Proteolysis - Induced Changes in Meat Collagen During Conditioning

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ABSTRACT

Changes in intramuscular connective tissue brought about by conditioning were investigated in bovine muscles of different quality. Perimysial and endomysial collagens were solubilized to a small extent during conditioning and residual insoluble collagens in both connective tissue domains were damaged by proteolytic processing.

Yields of soluble perimysial material from unconditioned muscles were significantly lower ($p = 0.096$) than from conditioned muscles. Solubilized perimysial collagen from unconditioned muscles was significantly lower ($p = 0.015$) than from conditioned muscles with 1 ± 0.8 % of original collagen solubilized for unconditioned muscles and 3.4 ± 3.3 % for conditioned muscles. 87.5 % of the muscles examined showed an increase in percentage solubilized collagen due to conditioning.

The main peptide components observed on analysis of insoluble perimysial fractions after CNBr digestion were derived from types I and III collagen. No changes were observed in the major peptide bands due to conditioning.

Yields of soluble endomysial fractions represented, on average, 94.5 % of total extracted endomysial material for unconditioned muscles compared with 97.5 % for conditioned muscles. Soluble endomysial fractions contained, on average, 0.13 % collagen from unconditioned muscles and 0.22 % collagen from conditioned muscles.

The main peptide components observed on analysis of insoluble endomysial fractions after CNBr-digestion were derived from types I and III collagen. Changes observed on the peptide maps, evident as the appearance of a number of new bands from conditioned samples, appeared to be muscle specific. % Type III collagen decreased on conditioning, indicating that endomysial type III collagen was preferentially destroyed during conditioning.

In model systems, insoluble perimysium treated with pepsin over 24 h resulted in little damage to the insoluble collagenous residue remaining. Insoluble perimysium treated with cathepsin resulted in changes to the major peptide bands on one-dimensional SDS-polyacrylamide gel electrophoresis which were evident after 24 h treatment.

Two-dimensional peptide maps obtained from conditioned insoluble perimysium and from insoluble perimysium treated with cathepsin for 24 h were altered relative to the unconditioned insoluble perimysium, indicating proteolytic damage to high molecular weight fractions. The *in vitro* case was extreme, but was comparable with conditioned

insoluble perimysium. In addition, new peptide material in conditioned perimysium and endomysium in the molecular weight range 40 000 to 50 000 was observed, while perimysial samples revealed loss of peptide material, due to conditioning.

Percentage solubilized collagen was higher ($p < 0.05$) from three muscles of varying quality when pre-injected with 0.1 M lactic acid and conditioned from 1 to 14 days than from untreated muscles. Analysis of the high molecular weight collagen peptides from lactic acid treated muscles by two-dimensional SDS-polyacrylamide gel electrophoresis revealed increased incidence of degradation in this region compared with untreated controls.

Sensory profiling using quality descriptive analysis (QDA) was carried out on three muscles of varying quality, pre-injected with 0.1 M lactic acid and results compared with untreated muscles. The results obtained failed to correlate the observed biochemical changes due to lactic acid treatment with perceived textural changes in these muscles. However, variability of the taste panel scores contributed significantly to the results obtained.

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The work outlined in this thesis was carried out to elucidate the effects of the conditioning (ageing) process on meat collagen, the major connective tissue protein, which has been implicated in contributing to the overall toughness of meat. Evidence in the literature to indicate that this protein is altered during conditioning is scarce, and the results available are conflicting in their conclusions. Therefore, the need for further research on this important subject was evident and it was hoped that such research would furnish greater insight into the events which take place in the connective tissue of muscle during its conversion to meat.

This first chapter deals with the background material relevant to the content of this thesis and includes a review of collagen chemistry, muscle chemistry and meat quality.

1.1.1 A Definition of Meat

Meat may be defined as muscle tissue which has undergone certain biochemical changes after death thereby rendering it suitable for use as food. In practice, the animals referred to in this definition are restricted to a few dozen of the 3 000 mammalian species. Also the definition is often widened to include, as well as the musculature, organs such as liver and kidney, brains and other edible tissues. However, these latter, often referred to as offal, will not be dealt with here and therefore, in this thesis, meat is taken to be derived only from skeletal muscle. The bulk of the meat consumed in the United Kingdom is derived from cattle, sheep, pigs and poultry. As the work reported here was carried out exclusively on bovine meat, a brief introduction of the bovine meat species is included.

The two main groups of domesticated cattle, *Bos taurus* (European) and *B. indicus* (India and Africa), are descended from *B. primigenius*, the original wild cattle or aurochs. Domestication of cattle followed the establishment of settled agriculture about 5 000 B.C. Domesticated hump-backed cattle (*B. indicus*, 'Zebu') existed in Mesopotamia by 4 500 B.C. and domesticated long-horned cattle in Egypt by about 4 000 B.C. Several breeds of domesticated cattle were known by 2 500 B.C. The fattening of cattle by forced feeding was practised in Egypt about 3 000 B.C.

The more immediate wild predecessor of most breeds of British cattle was *B. longifrons,* which was of relatively small frame, rather than *B. primigenius,* which is said to have been a massive animal. Indirectly, the development of many present British breeds was due to the early improvements initiated by Bakewell in the middle of the eighteenth century, who introduced in-breeding, the use of proven sires, selection and culling. In the United Kingdom prior to that time, cattle had been developed, primarily, for draught or dairy purposes. A deliberate attempt was now made to produce cattle, primarily for meat, which would fatten quickly when skeletal growth was complete. During the last 200 years the trend has been towards smaller, younger and leaner animals; there has been growing realization that breed potentialities will not be fully manifested without adequate food given at the right time in the growth pattern of the animal (Lawrie, 1985).

A beef animal should be compact and well covered with flesh, thus reducing the proportion of bone. Muscle development should be marked over the hind quarters, along the back and down the legs. The carcass, as prepared for the meat trade, represents those portions of the body remaining after removal of the blood, head, feet, hide, digestive tract, intestines, bladder, heart, trachea, lungs, kidney, spleen, liver, and adhering fatty tissue. On average, about 55 per cent of the live weight of cattle remains on the carcass (Gerrard, 1951). The carcass itself consists substantially of muscular and fatty tissues, of bone and of a residue which includes tendon, and other connective tissue, and large blood vessels. The joints into which beef carcasses are commonly split are shown in Fig. 1.1.

1.2 TYPES OF MUSCLE AND THEIR LOCATION

A universal characteristic of cells is their ability to respond to various kinds of stimuli. One form of this responsiveness is cell movement. Either a part of the cell, e.g. a cilium or mitochondrion, or the whole cell itself reacts by moving. Thus, cytoplasmic movement appears to be a fundamental feature of all cells. In multicellular animals, some cells become highly specialized during development with respect to cytoplasmic movement and are called muscle cells or fibres. There are three specialized kinds of muscle cell- smooth muscle and two varieties of striated muscle, skeletal and cardiac.

1.2.1 Skeletal Muscle

The number of studies contributing to an understanding of striated muscle, contractile protein biochemistry and the molecular basis of contraction is enormous (see for example Needham, 1971; Cassens, 1972; Bourne, 1973; Lawrie, 1985). The striated muscles constitute the muscles that ultimately provide meat. Skeletal muscle is both qualitatively

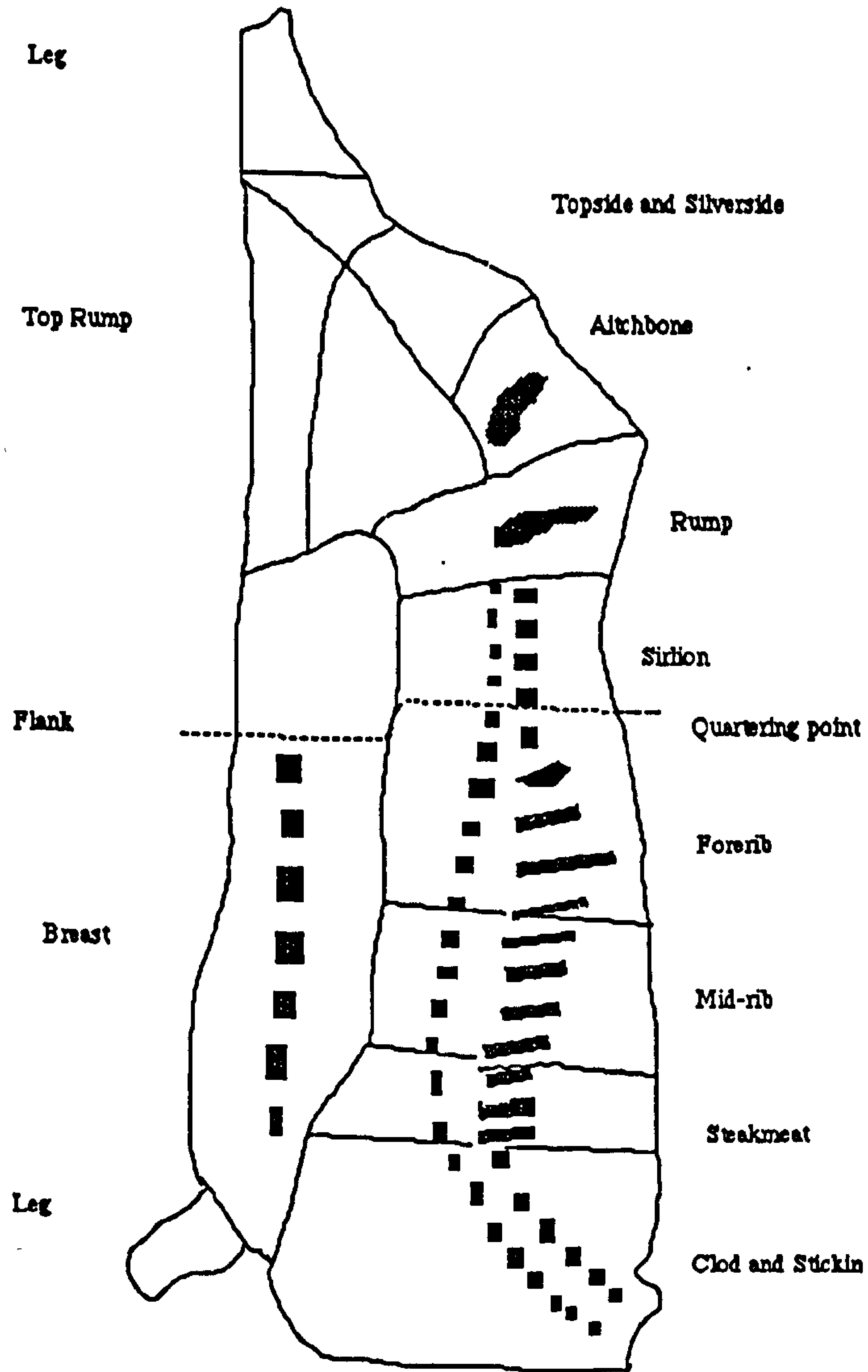


Fig. 1.1 Location of Wholesale Joints into which English Sides of Beef are Commonly Cut

and quantitatively the most important tissue of meat. There are more than 600 muscles in the animal body, varying widely in shape, size and function. Skeletal muscles are generally attached directly to bones, but some are attached to ligaments, fascia, cartilage and skin and therefore only indirectly to bone. The characteristic feature of the activity of striated muscle is that its fibres shorten rapidly and powerfully, producing movement. The specific characteristics of a given muscle are related to its function.

The essential unit of muscular tissue is the fibre, which consists of formed protein elements, the myofibrils, between which is a protein sol, the sarcoplasm (cytoplasm in non-muscle cells), and a fine network of tubules, the sarcoplasmic reticulum. The nuclei are generally situated at the periphery of the fibre. A muscle cell nucleus exhibits no unusual distinguishing structural features as compared to other types of cells, although each muscle cell or fibre contains many nuclei (Ashgar & Pearson, 1980). The fibre is surrounded by a very thin lipid membrane, the sarcolemma, the muscle analogue of the normal cell membrane to which connective tissue is attached on the outside. A summary of data on the chemical composition of a typical adult mammalian muscle, after rigor mortis, but before the occurrence of marked degradative changes is presented in Table 1.1. The principal free amino acids in fresh muscle are α-alanine, glycine, glutamic acid and histidine (Lawrie, 1985).

Muscles may be broadly classified as 'red' (slow-twitch) or 'white' (fast-twitch) (Needham, 1962), according to whether they carry out sustained action or operate in short bursts. However, differences between muscles are considerably more complex than their classification as 'red' or 'white' would signify, variability in function being influenced by a large number of factors. Intrinsic factors influencing muscle function include (1) species, (2) breed, (3) sex, (4) age, (5) anatomical location of muscle, (6) training or exercise, (7) plane of nutrition and (8) inter-animal variability. Extrinsic factors which may modify muscle behaviour in the immediate post-mortem period, during storage and processing include (1) food, (2) fatigue, (3) fear, (4) pre-slaughter manipulation and (5) environmental conditions at slaughter, in the immediate post-mortem period and during subsequent storage (Lawrie, 1985).

1.2.2 Smooth Muscle

Smooth muscle comprises only a minor proportion of meat. It is found in the body where continuous pulsation is required without any great strength, for example, in the walls of arteries, lymph vessels and in the gastrointestinal and reproductive tracts. Smooth muscle cells are spindle shaped, about 200 μm in length and 6 μm in width. However, they vary

Table 1.1 Chemical Composition of Typical Adult Mammalian Muscle after Rigor Mortis but Before Degradative Changes Post-Mortem (from Lawrie, 1985)

Components	Wet % Weight		
1. WATER			75.0
2. PROTEIN			19.0
(a) Myofibrillar		11.5	
myosin[1] (H- and L- meromyosins and several light chain components associated with them)	5.5 }		
actin[1]	2.5 }		
connectin	0.7 }		
tropomyosins	0.6 }		
troponins, C, I and T	0.6 }		
α, ß and γ actinins	0.5 }		
myomesin, N-line and C-proteins	0.7 }		
desmin, filamin, F- and I-proteins, etc.	0.4 }		
(b) Sarcoplasmic		5.5	
glyceraldehyde phosphate dehydrogenase	1.2 }		
aldolase	0.6		
creatine kinase	0.5 }		
other glycolytic enzymes	2.2 }		
myoglobin	0.2 }		
haemoglobin and other unspecified extracellular proteins	0.6 }		
(c) Connective tissue and organelle		2.0	
collagen	1.0 }		
elastin	0.05 }		
mitochondrial etc. (including cytochrome *c* and insoluble enzymes)	0.95 }		
3.LIPID			2.5
neutral lipid, phospholipids, fatty acids, fat-soluble substances		2.5	
4. CARBOHYDRATE			1.2
lactic acid	0.90 }		
glucose-6-phosphate	0.15 }		
glycogen	0.10 }		
glucose, traces of other glycolytic intermediates	0.05 }		
5. MISCELLANEOUS SOLUBLE NON-PROTEIN SUBSTANCES			2.3
(a) Nitrogenous		1.65	
creatinine	0.55 }		
inosine monophosphate	0.30 }		
di- and tri- phosphopyridine nucleotides	0.10 }		
amino acids	0.35 }		
carnosine, anserine	0.35 }		
(b) Inorganic		0.65	
total soluble phosphorous	0.20 }		
potassium	0.35 }		
sodium	0.05 }		
magnesium	0.02 }		
calcium, zinc, trace metals	0.03 }		
6. VITAMINS			trace
Various fat- and water-soluble vitamins, quantitatively minute.			

[1]Actin and myosin are combined as actomyosin in post-rigor muscle

slightly in size and shape in different organs, depending on their location. The cells are pointed at both ends and bulge in the centre, wherein the single nucleus is usually situated, although in large fibres it may be displaced slightly from the centre. The cells show feint longitudinal striations due to the long protein molecules in the sarcoplasm which run along the length of the cell. The cells are therefore birefringent but not cross-striated. There is no distinct sarcolemma but each cell is surrounded by a network of reticular fibres and in some cases, by collagen or elastic fibres. Elastic fibres are commonly associated with smooth muscle, where they provide strength in the walls of the larger arteries. The characteristic feature of the activity of smooth muscle is its capacity for sustained rhythmic contraction.

1.2.3 Cardiac Muscle

This tissue is the muscle of the heart and possesses the unique property of rhythmic contractility which continues ceaselessly from early embryonic life until death. Cardiac muscle has properties that resemble characteristic properties of both skeletal and smooth muscle. Like smooth muscle, its cells generally have a single centrally located nucleus and are innervated by the autonomic nervous system. Fibres of cardiac muscle branch and anastomose with others running parallel with them to form a network. The main fibre trunk of cardiac muscle is smaller in diameter and shorter than the fibres of skeletal muscle. The sarcoplasm contains numerous glycogen granules and the mitochondria are large and numerous. Cardiac muscle is endowed principally with a capacity for oxidative metabolism and consequently it has an extensive blood supply.

The myofilaments of cardiac muscle are not organized into discrete myofibrils as in skeletal muscle but instead, aggregates of myofilaments form fibrils of extremely variable size, and the dimensions vary along the longitudional axis of the fibrils. However, cardiac muscle has a striated appearance identical to that of skeletal muscle. Dense lines, known as intercalated discs transect the fibre at regular intervals along the longitudinal axis of cardiac muscle. The intercalated discs are continuous across the entire fibre and their paired membranes represent the cell membranes of adjoining muscle fibres. These discs provide a cohesive link between the fibres of the myocardium in addition to facilitating the transmission of the contractile force from one fibre to another. The myocardium is the contractile layer of the heart and contains the bulk of the cardiac muscle. The fibres of the myocardium are held in place by reticular and collagenous fibres, which are continuous with connective tissue sheaths that group them into fasicles (bundles) of fibres. Blood and lymph vessels and nerve fibres enter and exit the myocardium via the connective tissue between muscle bundles.

1.2.4 Structure of Skeletal Muscle Cells

Intact muscle is comprised of numerous fibres that generally lie parallel to the long axis of the muscle while in some muscles, the fibres are at an acute angle. Fibres are long, narrow, multinucleated cells which vary in length from a few millimeters to tens of centimeters and in diameter from 20 to 100 µm. In healthy animals, the diameters of muscle fibres vary from muscle to muscle and differ between species, breed and sex. They are increased by age, plane of nutrition, training, the degree of post-natal development in body weight rather than by actual body weight and by oestradiol administration (Lawrie, 1985).

Muscle fibres are composed of about 1000 smaller units, known as myofibrils, which are about 1 to 2 µm in diameter and which run the whole length of the muscle fibre. In the phase contrast light microscope the myofibrils reveal alternating light and dark bands. Different terms have been used to describe these bands. Under polarized light, the dark bands are birefringent and are therefore called A-bands or Q-bands, from the German word 'Querscheibe' meaning transverse disc. The light portion of the myofibrils, which are said to be nonbirefringent, are in fact, weakly birefringent, but the difference is substantially marked to merit their being called I-bands or isotropic bands. These are also called J-discs (the German equivalent) while other alternative names are clear discs, light bands, and hyaline substances. A narrow dark line in the middle of the I-band is called the Z-band (from 'Zwischenscheibe' meaning intervening disc) (Ashgar & Pearson, 1980). The dark bands (A-bands) contain a higher concentration of fibres than the light bands (I-bands) and the segment between two consecutive Z-lines, which bisect the light band, is known as a sarcomere, which is the functional unit of the myofibril. At higher magnification in the electron microscope, the fine details of the sarcomeres within the myofibrils can be seen more clearly and reveal the presence of two sets of filaments, the thick and thin filaments (Huxley, 1973). The thick filaments are about 15 nm in diameter and 1.6 µm in length. The structure is divided into zones as follows: the A-band consists of overlapped thick and thin filaments, the H-zone in the centre of the A-band consists of thick filaments only, the I-band consists of thin filaments only and the Z-line. At the centre of the A-band, the thick filaments are connected by a structure called the M-line. In transverse section, it is observed that the thick filaments are arranged in a regular hexagonal lattice. Where thick and thin filaments overlap in the A-band each thick filament is surrounded by six thin filaments. However, in the I-band the geometry of the thin filaments is not so regular as the lattice changes from hexagonal in the overlap region of the A-band to a square arrangement at the Z-line. The thick and thin filaments are now

known to consist of molecules of the contractile proteins myosin and actin, respectively (Hanson & Huxley, 1953; Hanson & Huxley, 1955; Huxley, 1960; Bailey, 1982).

1.2.4.1 Myosin

The name myosin was first given to a substance in muscle press juice which formed a gel on standing (Bailey, 1954). Myosin is the major protein of the thick filament. Each thick filament contains 200 to 400 molecules of myosin, with each molecule being 1.5 µm long and 130 Å wide (Bendall, 1973). Myosin is the most abundant of the myofibrillar proteins, constituting about half of the proteins of the myofibril (Table 1.2). It has a molecular weight of about 500 000, and can be visualized in the electron microscope as a long thin molecule (2 nm x 160 nm) with two globular heads (19 nm long) attached at one end of the long tail. The molecule, therefore, combines the features of fibrous and globular proteins. The heavy head is called heavy meromyosin (HMM) and the tail is called light meromyosin (LMM) (Szent-Gyorgi, 1953).

Treatment of HMM with papain yields two further subunits, HMM S-1, the anterior active globular portion and HMM S-2, the posterior helical region (Ashgar & Pearson, 1980). The cleavage of HMM S-1 under specified conditions gives rise to different molecular weight fractions, namely, a myosin heavy chain with a molecular weight of 200 000 and three light chains in each globular unit of HMM S-1. One of the light chains (DTNB-light chain, since it is separated by 5,5'-dithiobis (2-nitrobenzoic acid)) has a molecular weight of 25 000 and no ATP-ase activity, while the other two light chains are alkali-light chain 1, with a molecular weight of 18 000 (fragment A-1) and alkali-light chain 2 with a molecular weight of 16 000 (fragment A-2), so named because they are dissociated from myosin at high pH values. The latter two fragments of HMM are believed to possess both functionally active sites on the myosin molecule. One site contains the ATP-ase activity and the other site forms the cross-bridges that interact with the actin filaments to function in muscle contraction (Ashgar & Pearson, 1980). Myosin molecules are arranged in a bipolar fashion in the thick filament (Huxley, 1969). In the centre of the thick filament there is a zone free of cross-bridges whereas the remainder of the filament is covered with them. The bare zone contains only overlapped tails of the myosin molecule derived from both sides of the filament. The reversed polarity arrangement accounts for the fact that thick and thin filaments overlapping the two opposite ends of the thick filament must be propelled in opposite directions during movement.

Table 1.2 Muscle Myofibrillar Proteins (from Bailey, 1982)

Protein	Location	Fraction of Myofibril (%)
Myosin	Thick filament	55
C-protein	Thick filament	2
H-protein	Thick filament	0.3
Actin	Thin filament	23
Tropomyosin	Thin filament	6
Troponin	Thin filament	6
α-actinin	Z-line	1
M-protein	M-line	?
Desmin	Connection between myofibrils	?
Titin	Connection between Z-lines	?

1.2.4.2 Actin

The main protein of the thin filament, actin, comprises 23 % of the myofibril (Table 1.2) (Bailey, 1982) and exists in two forms. The monomer, G-actin, consists of relatively small globular units with a molecular weight of 41 700 and contains one bound Ca^{2+} ion and one non-covalently bound adenosine triphosphate (ATP). The fibrous nature of the actin filament occurs because of the longitudinal polymerization of G-actin monomers to form F-actin (fibrous), presumably linked by a Mg^{2+} or Ca^{2+} bridge through a nucleotide prosthetic group (Ashgar & Pearson, 1980). Two stands of F-actin are sprially coiled around one another to form a 'super helix' that is characteristic of the actin filament (Murray & Weber, 1974).

1.2.5. The Muscle-Tendon Junction

Skeletal muscles are attached to the bone of the skeleton via inelastic tendon. The precise manner in which the tendon is attached to the muscle has been a matter of controversy. Two main theories have been put forward; the first, or continuity theory, supposes that there is a direct union of the myofibrils and the tendon fibres, while the second, or sarcolemma theory proposes that there is no such continuity and that the sarcolemma forms a limiting membrane between the substance of the muscle fibre and the tendon elements. The manner in which tension generated by skeletal muscle fibres is transmitted from intracellular contractile proteins to extracellular connective tissue proteins is incompletely understood. It is generally believed that active force transmission occurs at specialized regions at the extreme ends of the fibres, designated the 'muscle-tendon junctions' or 'myotendinous junctions' (Trotter et al., 1981; Ovalle, 1987)

Ultrastructural studies have revealed that the terminations of skeletal muscle fibres are characterized by longitudinal foldings of the surface membrance that produce processes that take the form of either extensions or invaginations (Trotter & Baca, 1987). The sarcoplasm immediately adjacent to the processes contains actin filaments cross-linked into a dense matrix, designated the internal lamina (Trotter et al., 1983). These actin filaments are continuous with the actin filaments that associate with the myosin filaments of the terminal A-band. The internal lamina is linked to the plasma membrane by filaments that are oriented orthogonally to the actin filaments, traverse the plasma membrane and the lamina lucida and terminate in the lamina densa (Trotter et al., 1983). The lamina densa is then joined to the connective tissue matrix, and through it to the collagen fibrils of the 'microtendon', the oriented collagenous tissue associated with individual muscle fibres (Trotter & Baca, 1987). Three-dimensional images of the murine muscle-tendon junction

by scanning electron microscopy have shown that the finger-like processes at the ends of muscle fibres represent a network of cylindrical folds that anastomose with one another in an elaborate fashion (Ovalle, 1987). This geometric arrangement is consistent with the view that a marked increase in surface area at the muscle-tendon junction would reduce stress during force transmission between these two tissues. Surface amplification of the terminal sarcolemmal membrane enhanced mechanical strength at the site of force transmission, producing an interface with a high shear component (Ovalle, 1987).

1.2.6 Macroscopic Structure of Skeletal Muscle

Skeletal muscles consist of ordered arrays of muscle fibres arranged in bundles which are separated, yet linked by an integrated network of connective tissue.

At the muscle-tendon junction a spreading out of the tendon fibres can be observed with fibres extending both around and within the muscle (Fig. 1.2). The sheath of connective tissue arising from the tendon and surrounding the whole muscle is known as the **epimysium,** from the deep surface of which septa pass into the muscle at irregular intervals (Hamm, 1965). Those septa which constitute the **perimysium** open out into a supporting network which separates and invests bundles (fasciculi) of muscle fibres, the bundles being of somewhat angular outlines in section and of different sizes. Subdivision of the larger bundles into two or three successively smaller bundles is frequently observed. The name perimysium is retained for the connective tissue surrounding the lesser bundles. Finally, a third component of connective tissue within muscles constituting very delicate extensions of fine connective tissue is called the **endomysium** which surrounds the individual muscle fibres. Inside the endomysium is the sarcolemma, a thin membrane which encloses the soft sacroplasmic content of the muscle fibre. The sacrolemma appears as a homogeneous, apparently structureless, membrane, which is clearly distinguished from the finest divisions of the connective tissues.

1.3 MUSCLE CONNECTIVE TISSUE

The main biological function of connective tissue is to maintain the structure and appropriate spatial relationships within and between the organs in an organism. Connective tissue comprises the framework or scaffolding of organisms and maintains their characteristic shape and strength. Some connective tissues, such as tendons, must be resistant to extension, some, such as cartilage, must be resistant to compression, and others, such as bone, must be rigid and resistant to deformation.

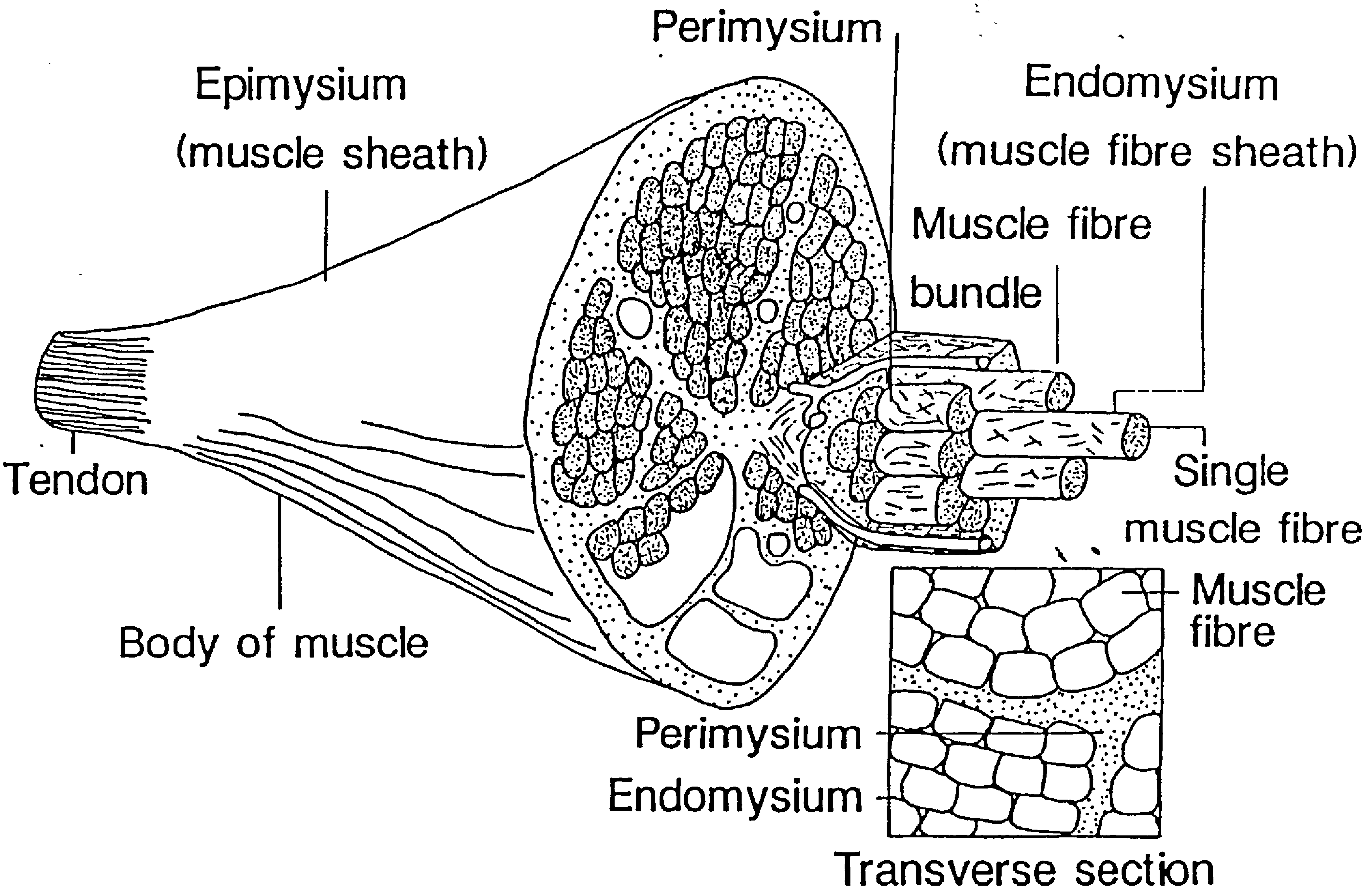


Fig. 1.2 Macroscopic Structure of Muscle

In mature beef animals, connective tissue comprises 2-8 % of muscle dry weight (Davey & Winger, 1979). Collagen usually makes up 95 % or more of the fibrous elements of muscle connective tissue; most of the remaining 5 % is elastin. Fibres of collagen are straight, inextensible and non-branching, while elastin fibres are smaller in diameter than collagen, elastic, branching and yellow in colour. Reticulin probably comprises predominantly collagen and the term is applied to the fine collagenous network of the perimysium and endomysium (Voyle, 1979). In fact, a great deal of doubt exists as to whether or not reticulin and collagen are identical. Some workers believe they are identical, while others believe that the basic fibre structure may be similar but an additional component exists in reticulin, which may represent a different mucopolysaccharide (Ashgar & Pearson, 1980). Reticulin also differs in the number and orientation of the fibres and in their relation to the protein-polysaccharide matrix which holds them together. Reticulin fibres are shorter and finer in structure than collagen, branch and stain black with ammonical silver stain.

Although predominantly acellular, connective tissue, in general, is also composed of several different cell types. Connective tissue fibres are normally embedded in and interact with an amorphous ground substance. The ground substance occupies the extracellular space of the connective tissues as a viscous fluid. It is comprised of globular mucoprotein, collagen and elastin. The predominant mucopolysaccharides include hyaluronic acid, chondroitin sulphates A, B and C, keratosulphate, heparitin sulphate and heparin and are derived from nucleoside diphosphate derivatives. The mucopolysaccharides also contain galactosamine or glucosamine. Galactosamine is found in chondroitin sulphates A, C and dermatin sulphate while glucosamine is found in hyaluronic acid and keratosulphate. The proportions of these mucopolysaccharides in ground substance varies in different tissues, with age and under the influence of corticosteroid hormones (Ashgar & Pearson, 1980).

Generally, two different types of cell populations have been recognized in the extracellular space, the fixed cells and wandering cells. Fixed cells include fibroblasts, mesenchyme cells and adipose cells. Wandering cells are comprised of mast cells, macrophages or histiocytes, lymph cells, eosinophils, and plasma cells and are concerned mainly with controlling infection. Connective tissue therefore, has both cellular and extracellular components. Fibroblasts, mast cells and macrophages are the predominant cell types which are surrounded by an extracellular matrix of amorphous mucopolysaccharides, chondroitin sulphates and hyaluronic acid set in a framework of elastin and collagen fibres (Ashgar & Pearson, 1980).

Collagen constitutes that part of meat connective tissue that has the major role in determining the texture of meat (Bailey, 1972; Light et al., 1985; Light, 1987) while elastin seems to be of little or no significance in determining meat texture due to its low content in the majority of muscles (Paul, 1963). Elastin represents 37 % of the connective tissue content of *semitendinosus*, however, (Bendall, 1967) and may have a role to play in determining toughness (Rowe, 1986). The amount of connective tissue relative to muscle fibres is much greater in some muscles than in others, a fact which to some extent explains why some cuts of meat are tougher than others although such a distinction is only a crude guide to the causative role of collagen in determining texture. The connective tissue content of muscle is greater in young compared with older animals and the concentration of both collagen and elastin diminish with increasing animal age (Lawrie, 1985) (Table 1.3).

As shall be shown later, connective tissue and especially collagen, has been strongly implicated in determining meat textural quality and this involvement changes with age, for example, the tenderness of veal and the toughness of meat from older animals. The connective tissue of young animals may contain more reticulin (Lawrie, 1985). There is a higher concentration of 'salt-soluble' collagen (a precursor of insoluble collagen) in young or actively growing muscle (Lawrie, 1985). The degree of intra- and intermolecular cross-links (see section 1.4.5) between the polypeptide chains in collagen and the nature of these cross-links change with increasing animal age. As collagen is the major connective tissue component in meat, therefore it is important to understand its structure and function. The following section deals with this subject.

1.4 COLLAGEN

1.4.1 Fundamental Characteristics of Collagen

Although the collagens of basement membrane and the fine filamentous component of articular cartilage can be readily distinguished from the interstitial fibrous collagens there are several biochemical properties common to all types of collagen. It can be defined (Ramachandran, 1963) as a protein with the following properties: (1) it contains three polypeptide chains, large proportions of which have a helical structure which possesses the repeating sequence Gly-X-Y, and which are wound into a stable triple helix; (2) one-third of the amino acids are glycine; (3) its wide angle X-ray diffraction pattern exhibits a 2.86 Å meridional arc and 12Å equatorial reflections; (4) the molecule possesses a high negative optical rotation and (5) it contains hydroxyproline and hydroxylysine, and no

Table 1.3 Collagen and Elastin Content of *L. Dorsi* Muscles in Cattle (from Lawrie,1985)

Cattle	Collagen (%)	Elastin (%)
Calves	0.67	0.23
Steers	0.42	0.12
Old Cows	0.41	0.10

tryptophan. Large proportions of the three polypeptide chains of collagen (each containing more than 1000 amino acids, the majority of which are arranged in regular repeating sequences of gly-pro-y and gly-x-hyp) are wrapped about a central axis to form a gradual right-handed superhelix, the individual chains being interconnected by hydrogen bonds. The collagen molecule can therefore be thought of as a stable triple helix, 1.5 nm in diameter and of variable length, linearly connected to variable proportions of non-helical globular domains.

1.4.2 The Biological Diversity of the Collagens

In recent years there have been considerable advances contributing to the wealth of knowledge of the chemistry, structure and function of collagen (Miller, 1976; Ramachandran & Ramachandran, 1976; Fessler & Fessler, 1978; Bailey & Etherington, 1980; Bornstein & Sage, 1980). The biological diversity of collagen had been difficult to account for, based as it had to be on a small number of side-chain modifications of a chemically very simple molecule. This diversity in structure and function is manifested in the forms in which collagen occurs: the strong ropelike fibres present in tendon, the flexible layers of interwoven fibres in skin, the transparent laminated sheets of fine fibres of cornea, the amorphous membrane structure of the lens capsule and glomeruli, the lubricated cartilage of the joints, the mineralized collagen of bone and dentine, and the fine filaments surrounding and supporting cells. Collagen is found in these various forms in the tissues of all types of multicellular organisms from the most primitive invertebrate, through sponges and coelenterates up to mammals. The basis of the structural organization of the whole animal kingdom is dependent on the properties of the extracellular collagen fibres.

The identification of a whole family of genetically distinct collagens over the past few years has illuminated the variations of the basic structure that can account for the biological diversity associated with collagenous tissues described above. At the present time twelve genetically distinct collagen molecules have been identified, which can be classified according to their macromolecular structure, as follows: Group I, interstitial collagen (striated fibre collagen) Group II, basement membrane collagen (nonfibrous collagen); and Group III, microfibrillar collagen (Table 1.4) (Bailey, 1987).

1.4.3 Location of Collagen Types

The best characterized collagen types are those found in group I; the fibrous types I, II, and III collagens and form the structural matrix of the tissues which contain them. These

Table 1.4 Classification of Collagen Types (from Bailey, 1987)

Group name	Types included
I-Interstitial collagen	I, II, III
II-Basement membrane collagen	IV
III-Microfibrillar collagen	
(a) Pericellular (cell-associated)	V, IX, X, (XI)
(b) Matrix	VI, VII,(V)

include skin, tendon and bone which contain predominately type I collagen and tissues such as aorta, placenta, lung, liver and kidney which contain both type I and III collagen (Piez, 1976). Type II collagen was first identified in cartilage (Miller & Matukas, 1969) where it is the major structural component, but has now been shown to be present in a variety of tissues including the intervertebral discs, notochord and the vitreous humour and neural retina of the eye (Bailey, 1987).

Collagens classified as group II aggregate to form nonfibrous sheets. The only known collagen in this group is type IV, which is specific to basement membranes, that is, the nonfibrous sheets underlying epithelial and endothelial cells, surrounding muscle and nerve cells, and providing the structure of lens capsule in the eye and the filtration properties of the glomeruli of the kidney.

The microfibrillar collagens present in group III can be subdivided into matrix collagen (types VI and VII) and pericellular (cell-associated) collagen (types V, IX and X) (Bailey, 1987). Type V collagen, first isolated from pepsin digests of placental chorionic and amniotic membranes (Burgeson et al., 1976), is widely distributed among collagenous tissues, and is associated with basement membranes but is distinct from type IV collagen. Type VI collagen was initially isolated as a pepsin-resistant fragment from human aortic intima and therefore called intima collagen (Chung et al., 1976). It has since been shown to have a wide distribution in the extracellular matrix, but has not been demonstrated in cartilage or basement membranes. Type VII was originally isolated from placenta by pepsin digestion and is believed to be confined to microfibrils underlying the dermal basement membrane and the chorioallantoic membrane (Burgeson et al., 1985). Type IX collagen was first isolated from pig articular cartilage, from intervertebral disc and from chick sternal cartilage, while type X collagen was initially identified in the medium of chondrocyte cultures and designated short-chain (SC) collagen, or G collagen. Type X collagen is concentrated around the chondrocytes, and can therefore be classified as a cell associated collagen (Bailey, 1987).

Several other collagens have been identified that have not, as yet been classified, including type VIII collagen, type XI collagen ($1\alpha,2\alpha,3\alpha$) (Bailey, 1987), type XII collagen (Dublet & van der Rest, 1987; Gordon et al., 1987) and invertebrate collagens (Bailey, 1987).

The collagens present in the connective tissue of muscle are types I, III, IV V and VI (Bailey & Sims, 1977) and the rest of this discussion will focus on these collagen types. Types I and III collagen has been located in the epimysium, types I, III and V in the

perimysium, types III, IV and V in the endomysium and type VI in the epimysium and perimysium (Bailey & Sims, 1977; Duance et al., 1977; Bailey et al., 1979; Hessle & Engvall, 1984; Light & Champion, 1984; Light et al., 1985).

1.4.4 Biosynthesis of Collagen

The collagen genes are very complex, containing about 50 exons and large intervening sequences. Following transcription, processing of the pre mRNA involves approximately 50 excisions and splicing steps. The collagens are first synthesized as pre-pro-collagens with remarkably large signal sequences (100 amino acids). Procollagen is the term reserved for the primary precursor form of the collagen molecule in the same sense as proenzymes such as procarboxypeptidase and prohormones such as proinsulin are precursors of carboxypeptidase and insulin, respectively. Procollagen molecules then undergo a series of enzymatically controlled post -translational modifications which take place intra- and extracellularly (Krieg et al., 1988). The procollagen molecule is extended at both the N- and C-termini by propeptides, which are extraordinarily large, both being of the order of a hundred amino acids in each chain (approximate Mr, 36 000) (Fessler & Fessler, 1978).

Newly synthesized procollagen α-chains undergo several postribosomal alterations and studies of the corresponding enzymes indicate a finely orchestrated series of post-transational modifications that facilitate successive assembly, secretion and cross-linking steps from individual polypeptides to fibres. A summary of the events is presented in Table 1.5.

Hydroxylation of proline residues, catalyzed by prolyl hydroxlyase, stabilizes the helical folding of a collagen chain (Bornstein, 1974; Prockop et al., 1976). However, hydroxylation of certain lysine residues (by lysyl hydroxylase), glycosylation of these and certain propeptide residues (by various monosaccharide transferases) and, possibly, catalysis of sulphydryl bond formation are all important steps in the post-translational modification of the collagen α-chain (Krieg et al., 1988). Prolyl hydroxylases, lysyl hydroxylases and the lysyl glycosidases require their substrates to be in unfolded, non-helical form, which suggests that proline hydroxylation and chain folding proceeds *pari passu* (Fessler & Fessler, 1978).

Time-labelling studies show that in the unperturbed system, proline hydroxylation closely follows peptide synthesis. Proline hydroxylase is located within the rough endoplasmic reticulum (RER), as shown by electron microscopy and by its presence within 'rough'

Table 1.5 Post-Translational Modifications of Collagen (from Krieg et al., 1988)

Intracellular	
	Hydroxylation of lysyl- and prolyl residues
	Glycosylation of hydroxylysyl residues
	Triple helix formation
	Translocation and secretion of procollagens
Extracellular	
	Cleavage of N- and C- terminal procollagen peptides
	Aggregration into fibrils
	Formation of intermolecular cross-links

microsomes prepared from it so it is postulated that there is a coordinated progression of peptide synthesis, entry into the RER, hydroxylation and concommitant helical folding. Disulphide linkage between the carboxyl propeptides is delayed until after synthesis of the completed pro α-chains (Fessler & Fessler, 1978).

The sequence of cleavage of the amino and carboxy propeptides varies; with type I collagen, the N- terminus is removed first, while for type III collagen, the C- terminus is cleaved first (Fessler et al., 1982). In chick embryo calvaria, the amino propeptides are removed at about the same time that proline-labelled protein leaves the cell (Morris et al., 1975), whereas the carboxyl propeptides are removed at a distinctly later time, suggesting that the amino propeptides are removed near the cell surface and the carboxyl propeptides extracellularly, however, all procollagen peptidase activity is extracellular (Nist et al., 1975). The peptidases are not serine proteases, are not inhibited by SH reagents, however, they are inhibited by ethylenediamine tetraacetic acid (EDTA) (Fessler & Fessler, 1978). Evidence for more than one endopeptidase in the processing of procollagen was obtained from a number of *in vitro cell* culture and cell incubation studies and from the action of tunicamycin on the processing of procollagen. The physiological sequence is predominantly that the amino procollagen peptidase first removes the amino propeptides and subsequently the carboxyl procollagen peptidase cleaves the carboxyl propeptides in a sequential manner yielding α-chains (Fessler & Fessler, 1978).

The molecules then arrange themselves side-by-side in a quarter-staggered manner, to facilitate fibril formation, which are stabilized by cross-links. These originate from an oxidation by lysyloxidase of distinct hydroxylysyl and lysyl residues localized in the telopeptide regions. The aldehydes, so-formed, react with a lysyl residue of a neighbouring molecule to form a Schiff's base, which can then undergo an Amadori rearrangement, resulting in formation of a stable cross-link. Regulation of fibril formation is still incompletely understood and sequential cleavage of procollagen peptides or mixtures of different collagen types are thought to be involved in the control of fibrillogenesis (Krieg et al., 1988).

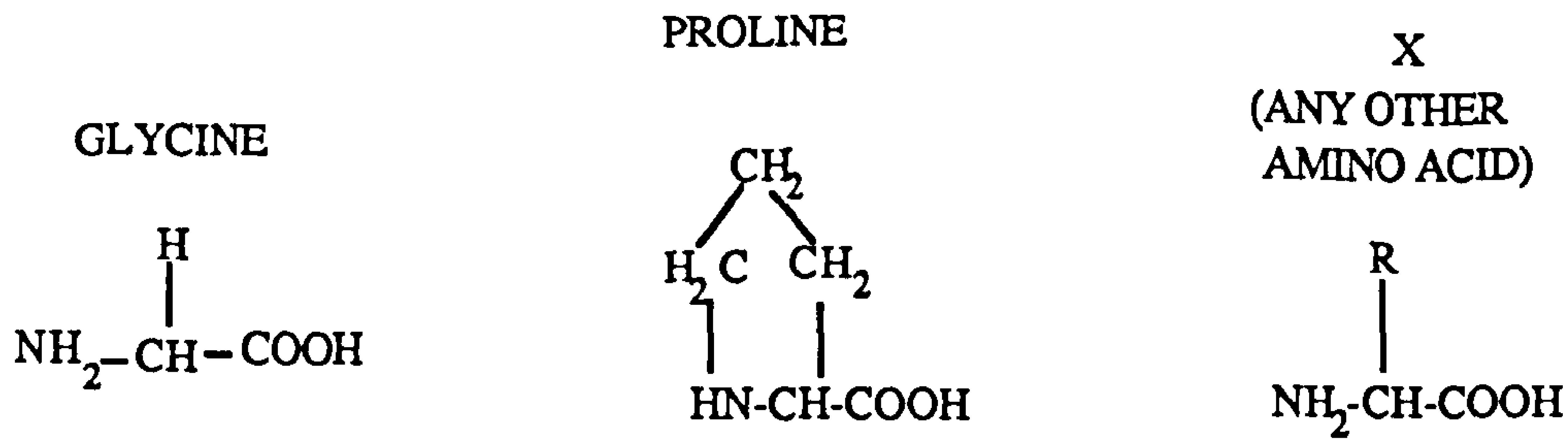
Degradation of mature collagen fibrils in the extracellular space is dependent on the activity of the mammalian collagenase, which cleaves the molecule between position 775 and 776 (glycine/isoleucine) within the triple helix (Harris & Krane, 1974; Werb, 1982). The resulting $\frac{3}{4}$ and $\frac{1}{4}$ fragments are unstable under physiological conditions and can be degraded by a variety of proteases. Collagenase is synthesized in an inactive form and its activity is regulated by several inhibitors, known to be present in the connective tissue and body fluids, ensuring complex control of the regulation of collagen turnover. The

mammalian collagenase synthesized by fibroblasts mainly cleaves the interstitial collagens I and III, but also has some activity against type II collagen, whereas for other collagens e.g. type IV, other collagenases have been shown to be highly specific (Krieg et al., 1988).

1.4.5 Structure and Stabilizing Cross-Links of Collagen

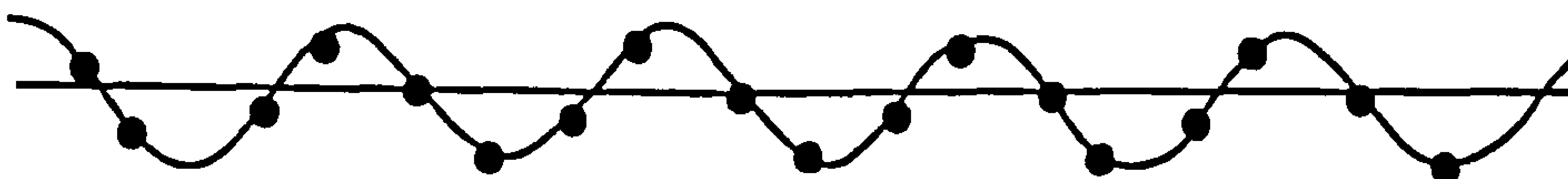
The structure of collagen, at various levels of organization is shown in Fig. 1.3. (Gross, 1961). The polypeptide α-chains which make up the collagen molecule vary in size and composition from collagen type to collagen type. However, there is a structural similarity and in many cases a high degree of sequence homology between all types. Collagens exhibit a characteristic amino acid analysis, the most notable feature being a high glycine and imino acid content, which together comprise approximately half the total amino acids. The presence of the amino acid hydroxyproline in collagen (about 14 % in fibrous collagens) is also a unique feature because this amino acid occurs in only a few other proteins, i.e. elastin (1.6 %), to a lesser extent in the serum complement protein Clq (Reid, 1979) and in certain plant proteins (Lamport & Miller, 1971). Its presence has also been reported in a 'collagen-like' sequence of acetyl cholinesterase (Lwebuga-Musaka et al., 1976). However, the hydroxyproline content of these proteins is remarkably less than that of collagen, and it has been used for many years as a means of determining the amount of collagen present in tissues. Collagen also contains hydroxylysine, very few aromatic or sulphur-containing amino acids and no tryptophan. The positioning in the sequence of the proline and hydroxyproline residues as mentioned above (section 1.4.1) confers a unique polyproline helical configuration on all three peptide chains which then bind together to form a compact triple helix. In its secondary structure, the greater part of each chain is arranged as a left-handed helix (Fig. 1.3 c); and the three of these intertwine to form a right-handed super-helix (Fig. 1.3 e, f), which is the helical portion of the collagen molecule.

The interstitial collagens (types I-III) all possess similar triple-helices, consisting of semi-rigid rods of about 300 nm long and 1.5 nm in diameter with a molecular weight of 300 000 (Gross, 1961; 1964). Each α-chain of the triple-helix contains approximately 1000 amino acid residues, existing as a left-handed polyproline triplet, with the final collagen chain being a right-handed structure. Each chain is comprised for over 95 % of its length by a repeating series of Gly-X(pro)-Y triplets, a structural feature which is an absolute requirement for assumption of the triple-chain helical conformation of the native molecule (Ramachandran, 1967). They are referred to as α–, β and γ-chains in the order of their increasing rate of sedimentation in the ultracentrifuge. The α- components are

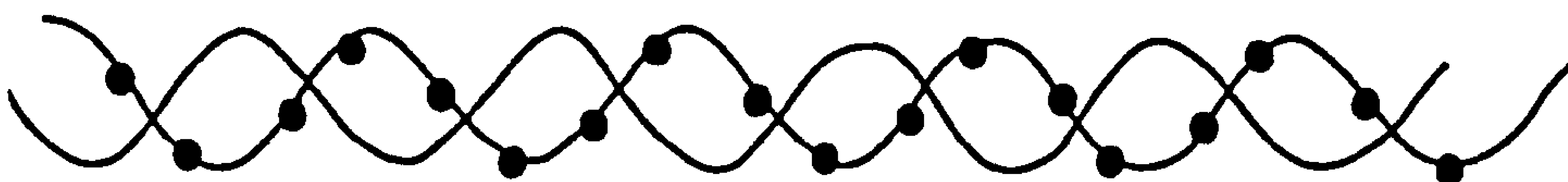


(a) Free Amino Acids

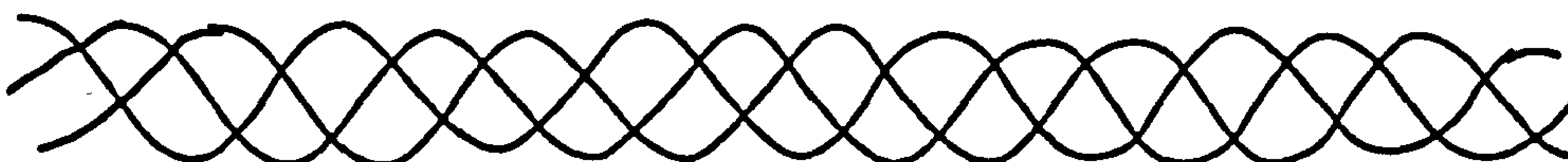
(b) Molecular Chain (Repeating Units)



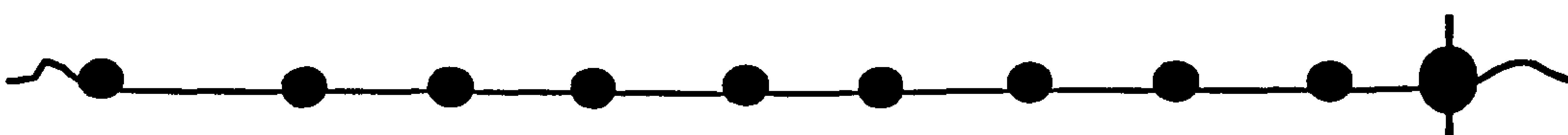
(c) Single Chain Molecular Helix



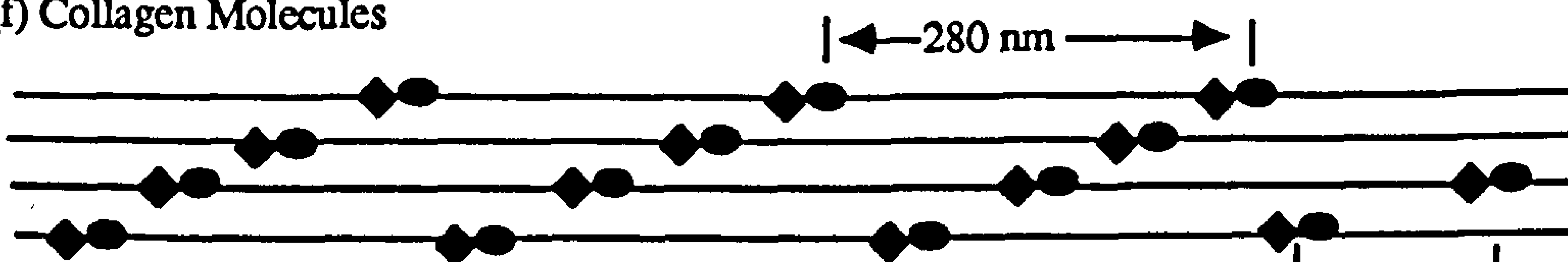
(d) Single Chain Coiled Helix



(e) Three-Chained Coiled Helix



(f) Collagen Molecules



(g) Collagen Fibril

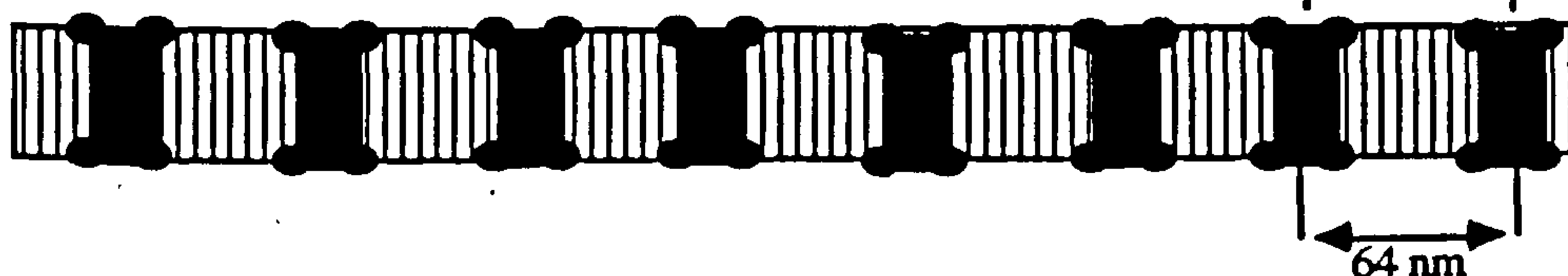


Fig 1.3 Schematic Illustrations of the Amino Acid Sequence and Molecular Structure for Collagen (from Gross, 1961).

single chains, while the β and γ- components represent dimers and trimers, respectively, of the α- chains, formed by covalent cross-links (Piez, 1967). The three α-chains pack tightly together due to hydrogen-bonding via the glycine at every third position. Internal hydrogen bonding, derived from the carbonyl of the peptide backbone and the hydrogen of the small glycine residue, stabilizes the triple helical structure. The ring structure of proline which prevents rotation of the N-C bond in the peptide chain is important in stabilizing the helix. Further stabilization occurs via the hydroxyl group of hydroxyproline and water. The importance of water in stabilizing the structure of the collagen molecule has been established (Yannas & Tobolsky, 1967; Traub & Piez, 1971; Privalov, 1982). The water molecule forms a hydrogen bonded water-bridge from one hydroxyl group of hydroxyproline to another and to the carbonyl oxygen of the peptide backbone. The X and Y positioned amino acids are located on the outer surface of the helix and have their side-chains orientated outwards. The pyrrolidine content of collagen is correlated with its thermal shrinkage temperature (Josse & Harrington, 1964) (Fig. 1.4). Underhydroxylated collagen is not sufficiently stable to maintain triple-helical conformation at physiological temperature (Berg & Prockop, 1973; Jimenez et al., 1973), indicating that the occurrence of hydroxyproline in collagen α-chains adds stability to the helix. The triple helical structure of collagen is extremely resistant to proteolytic attack. Varying susceptibility to proteolysis by collagenolytic enzymes results largely from differences in the tertiary and quaternary structure, determined not only by the sequence, but also by the presence of covalently bound carbohydrate and the type of inter- and intramolecular cross-links, which vary from tissue to tissue and with the stage of maturation (Woolley, 1984). As the collagen matures, coatings of mucopolysaccharides and glycoproteins are also thought to strengthen it (Gottschalk, 1966).

In addition to the larger triple-helical portion, all genetic forms of collagen contain short non-helical domains. These are generally globular in structure with an amino acid composition more closely resembling that of the majority of animal proteins. Non-helical domains can be sub-divided into those identifiable as procollagen extra peptides at the N and C termini of newly synthesized collagen, and those which remain as part of the molecule in between helical domains (as in type IV collagen). These regions are more susceptible to proteolytic attack than the triple helical region (Kühn et al., 1981; Timpl et al., 1981). In the case of those which remain attached to the N and C termini following processing of procollagen, non-helical domains are short compared to the triple-helical portions. The N-terminal amino acid of collagen chains is always pyrogyutamic acid, while all N-terminal non-helical sequences contain a lysine residue equivalent to position 9 in Type I collagen, in addition to relatively high proportions of hydrophobic amino acids including between one and two residues of tyrosine (Piez, 1976). The C-terminal non-

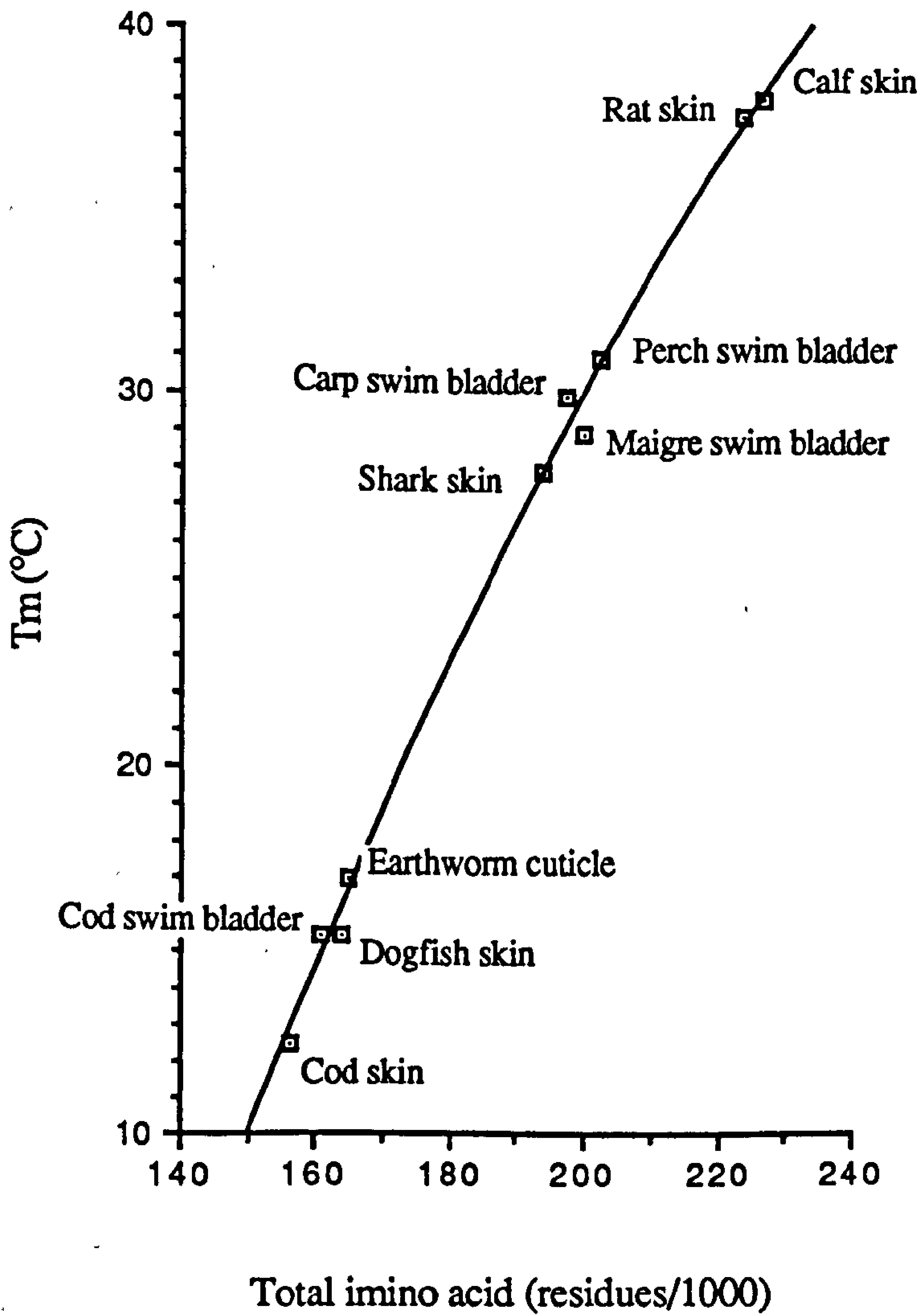


Fig 1.4 Thermal Stability of Collagen

helical region in α chains avoided detection for some time due to loss on isolation by proteolysis (Stark et al., 1971). However, with the use of proper precautions it has been isolated and its sequence determined (Rauterberg et al., 1972). The C-terminal non-helical domain also contains higher proportions of hydrophobic amino acids than the triple-helix, notably tyrosine. A lysine residue located close to the C-terminus, at residue 1044 in Type I collagen and that present in the N-terminal non-helical region at position 9 are substrates for enzymatic oxidative deamination and become involved in the formation of intra- and inter-molecular cross-links.

Two types of cross-link are known to exist in collagen - intramolecular bonds, occurring between polypeptides within the same molecule, and intermolecular bonds, which link one or more triple helices together. These cross-links originate from the N- and C- termini of the collagen molecule (Sims & Bailey, 1981) and are responsible for the high mechanical strength of the collagen fibre.

1.4.5.1 Intramolecular Cross-Links

The covalent bond which links α-chains together to produce dimers (ß) is known as α, ß-unsaturated aldol (Bornstein & Piez, 1966). Each non-helical sequence contains a single lysyl (Bornstein & Piez, 1966; Stark et al., 1971) or hydroxylysyl (Lane & Miller, 1969; Miller et al., 1969; Miller, 1971) residue, which becomes the cross-link precursor, as the result of oxidative deamination catalyzed by a specific enzyme designated lysyl oxidase (Siegel et al., 1970). Two of the resulting ∂-semialdehydes (allysine and hydroxyallysine) from adjacent α-chains then condense to form a very stable α, ß-unsaturated aldol condensation product. Lysyl oxidase inhibition, either by copper deficiency (Carnes, 1971) (the enzyme is a copper metalloenzyme) or by administration of ß-aminopropionitrile which inhibits the oxidizing reactions, causes various connective tissue disorders, due to a lack of this and intermolecular cross-links.

The location of the cross-link within the collagen molecule, makes it unlikely to contribute to the overall tensile strength of the fibre, since it cannot restrict movement of one molecule relative to another (Sims & Bailey, 1981). There is no evidence for the existence of this cross-link at the C-terminus of the collagen molecule.

1.4.5.2 Intermolecular Cross-Links

It is now well established that the structural integrity of the collagen fibril is determined to a large extent by the type and density of collagen intermolecular cross-links and in recent

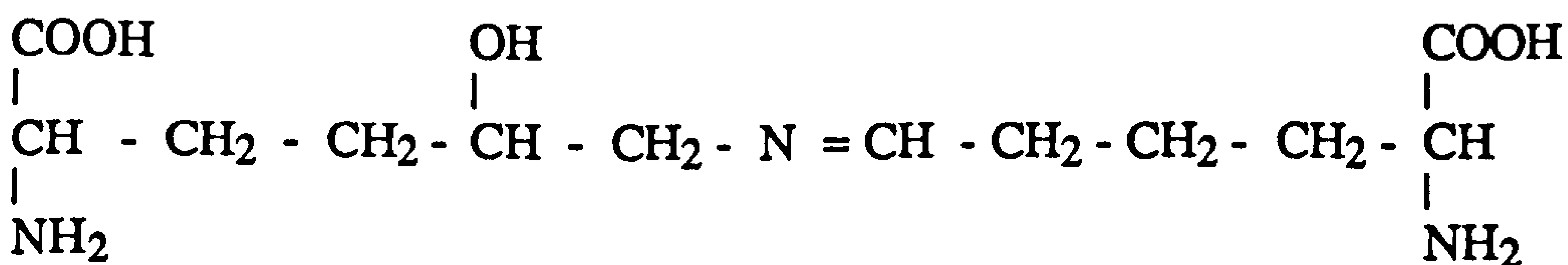
years their chemistry has been extensively investigated (Bailey et al., 1974; Light & Bailey, 1979; Light & Bailey, 1980a; Eyre et al., 1984). The two intermolecular cross-links formed in newly aggregated collagen fibres are aldimines, which are formed by the reaction of a lysine- derived aldehyde (allysine) residue in one collagen molecule with a hydroxylysine residue in another collagen molecule yielding dehydro-hydroxylysinonorleucine, and oxo-imines, which are formed by reaction of a hydroxylysine-derived aldehyde (hydroxyallysine) in one molecule with a hydroxylysine residue in another molecule yielding dehydro-dihydroxylysinonorleucine. Special features of these compounds are that they are both reducible. Dehydro-hydroxylysinonorleucine exists as a labile aldimine (Bailey & Peach, 1968) while dehydro-dihydroxylysinonorleucine exists as the stable oxo-imine, hydroxylysino-5-oxo-norleucine, formed by spontaneous *in vivo* Amadori re-arrangement of the initial aldimine (Robins & Bailey, 1973; Robins et al., 1973;).

The two structural requirements necessary for formation of the intermolecular cross-links are the presence of a lysine or hydroxylysine residue in the N and/or C non-triple helical regions of collagen which have been enzymatically converted to an aldehyde form and secondly, the presence of a lysine or hydroxylysine residue in the triple helical region of the molecule in a position such that it aligns with the above described aldehyde when the molecules are in a quarter-staggered arrangement as found in collagen fibrils (Glanville & Fietzek, 1976).

The cross-link is initiated by oxidative deamination of the N- and C-terminal lysine or hydroxylysine residues by lysyl oxidase, which then condense with lysine or hydroxylysine residues in the helical region of an adjacent molecule to form a Schiff base, which is considered to be an unstable intermediate in the pathway leading to the formation of mature, chemically stable intermolecular cross-links (Robins et al., 1973). Hydroxylysine residues at two sites within the α–1 chain are known to participate in intermolecular cross-linking in Type I collagen of several tissues. These are: residue 87 and residue 930 (Scott, 1982). The four residues are located precisely where head-to-tail intermolecular cross-linking can occur between molecules staggered by 4D periods (where D = 67 nm or about 234 amino acid residues). The α 2(I) chain and the α 2 (V) chain lack the cross-linking lysine in the C- telopeptide (Eyre, 1987). In the first case, the aldehyde partner for Schiff base formation is derived from hydroxylysine at position 17 in the α-1 chain carboxy-terminal extra-helical sequence. The importance of this region of the molecule in intermolecular cross-linking has been stressed (Bailey et al., 1980; Light & Bailey, 1980b, c). The aldehyde which condenses with residue 930 is derived from a lysine or hydroxylysine residue in the α-1 chain or α-2 chain amino-terminal region. In

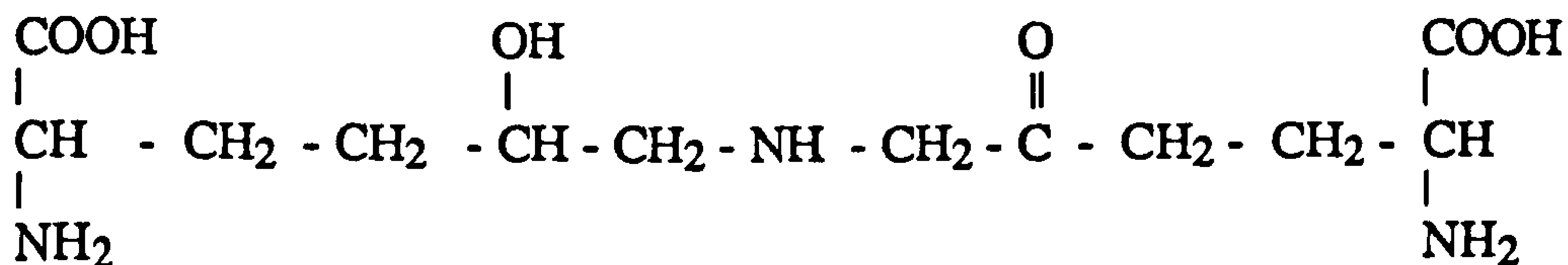
both cases, the cross-linked peptides, which may be isolated after CNBr digestion of the cross-linked collagen consist of α1CB6, the carboxy-terminal peptide of the α-1 chain, linked to the smaller peptides, α1CB5 or α1CB0,1 (or α2CB1) (Scott, 1982). The presence of the cross-links can be shown by their reduction with tritiated sodium borohydride followed by acid hydrolysis and ion-exchange chromotography of the tritiated components (Sims & Bailey, 1981). The following naturally occurring cross-links have been isolated in reduced form using this technique:

Dehydro-hydroxylysinonorleucine



This compound is formed by the condensation of lysine-aldehyde in the telopeptide of one α-chain with hydroxylysine in the helical body of an adjacent molecule. Although stable under physiological conditions, this aldimine cross-link is labile to dilute acids and heat. It is a major cross-link in skin and some other 'soft' tissues such as rat tail tendon (Bailey & Peach, 1968; Tanzer, 1976).

Hydroxylysino-5-oxo-norleucine



This cross-link is derived entirely from hydroxylysine by a condensation between hydroxylysine and hydroxylysine-aldehyde. Originally thought to have had an aldimine structure, it has subsequently been shown that it undergoes an Amadori rearrangement where a shift of the double bond occurs to form a more stable 'oxo' structure. It is the major reducible cross-link in bone, cartilage and embryonic skin and occurs in about equal proportions to dehydro-hydroxylysinonorleucine in tendon (Sims & Bailey, 1981). While hydroxylysino-5-oxo-norleucine is relatively stable to thermal denaturation (2 min at 70° C), to dilute acids, and to α-amino-β-thiols, dehydro-hydroxylysinonorleucine is extremely labile (Bailey et al., 1970).

The proportions of these two cross-links in a tissue is a reflection of the degree of hydroxylation of the lysine residues in the N- and C- terminal telopeptides. These

residues are almost 100 % hydroxylated in bone, which accounts for the predominance of hydroxylysino-5-oxo-norleucine in this tissue. In tendon collagen, where there is only 50 % hydroxylation, equal proportions of hydroxylysino-5-oxo-norleucine and dehydro-hydroxylysinonorleucine are found (Sims & Bailey, 1981)

1.4.5.3. Mature Cross-Links

The increased stability of mature collagen is due to the presence of complex multivalent intermolecular bonds, forming a system of lateral and transverse cross-links in the fibre (for review see Light & Bailey, 1979; Light & Bailey, 1980a) and which form spontaneously in the tissue during ageing (Robins et al., 1973; Robins & Bailey, 1975) derived from aldimine bonds (Bailey & Peach, 1968) and oxo-imine bonds (Mechanic et al., 1971; Robins & Bailey, 1973; 1975). Two chemical pathways of covalent cross-linking are apparent in collagen, based on the telopeptide lysine aldehydes and telopeptide hydroxylysine aldehydes (Eyre, 1987). The first reactions in both the lysine aldehyde and hydroxylysine aldehyde pathways produce borohydride-reducible aldimines and related compounds, which disappear as the collagen fibrils mature to form stable cross-links that are not reducible (Eyre, 1987) (Fig 1.5). The chemical structures of the nonreducible mature products of the lysine aldehyde pathway are still contentious but two compounds have been isolated and characterized which are thought to fulfil this role (Housley et al., 1975; Barnard et al., 1987). One possible product of this pathway is a non-reducible amino acid that dominates the cross-linking profile of adult bovine skin and cornea, and is the tri-functional cross-linking residue in a three-chained peptide from bovine skin collagen (Eyre, 1987). This compound was tentatively identified as hydroxyaldolhistidine by mass spectrometry, embodying two aldehydes (one allysine and one hydroxyallysine) (Housley et al., 1975). The second possible product, designated compound M (Barnard et al., 1987) is an amino acid of high molecular weight, derived from lysine, and is only present in high molecular weight cross-linked peptides derived from mature collagen. It is present in mature skin and bone (Barnard et al., 1987).

In most tissues that use the hydroxylysine aldehyde pathway, the mature cross-links are trifunctional 3-hydroxypyridinium residues, which have a characteristic natural fluorescence (Fujimoto et al., 1977). The mature cross-link is formed through the interaction of three hydroxylysine residues by a mechanism which may proceed either via the reaction of two oxo-imine cross-links or by the reaction of the oxo-imine with a hydroxylysine aldehyde (Eyre, 1987). It predominates in most fibrous connective tissues other than skin, cornea, and sclera, which are all surface tissues exposed to sunlight (Eyre et al., 1984). The structure of the two forms of this mature cross-link are shown in

LYSINE
Lysyl Oxidose
ALLYSINE
+Lys
+ Allysine
LN
Δ LN
$NaBH_4$
+Hyl
ALLYSINE ALDOL
+Hyl
Δ HMD
+His
Δ AH
+Hyl
+His
HLN
$NaBH_4$
Δ HLN
Δ HHMD
$NaBH_4$
HHMD

NH_2-CH-COOH
$(CH_2)_4$
NH
CH_2
CHOH
$(CH_2)_2$
NH_2-CH-COOH

?
MATURE PRODUCTS STILL UNKNOWN

?

NH_2-CH-COOH
CH_2
CH_2
CH_2
NH_2
CH-CH_2
COOH
N
N
CH
CH — CH_2 — NH — CH_2 — CHOH
CH
CH
NH_2-CH-COOH
CH_2
CH_2
NH_2-CH-COOH

Fig. 1.5a Chemical Pathway of Cross-Linking in the Fibrillar Collagens Based on Telopeptide Lysine Aldehydes (from Eyre, 1987)

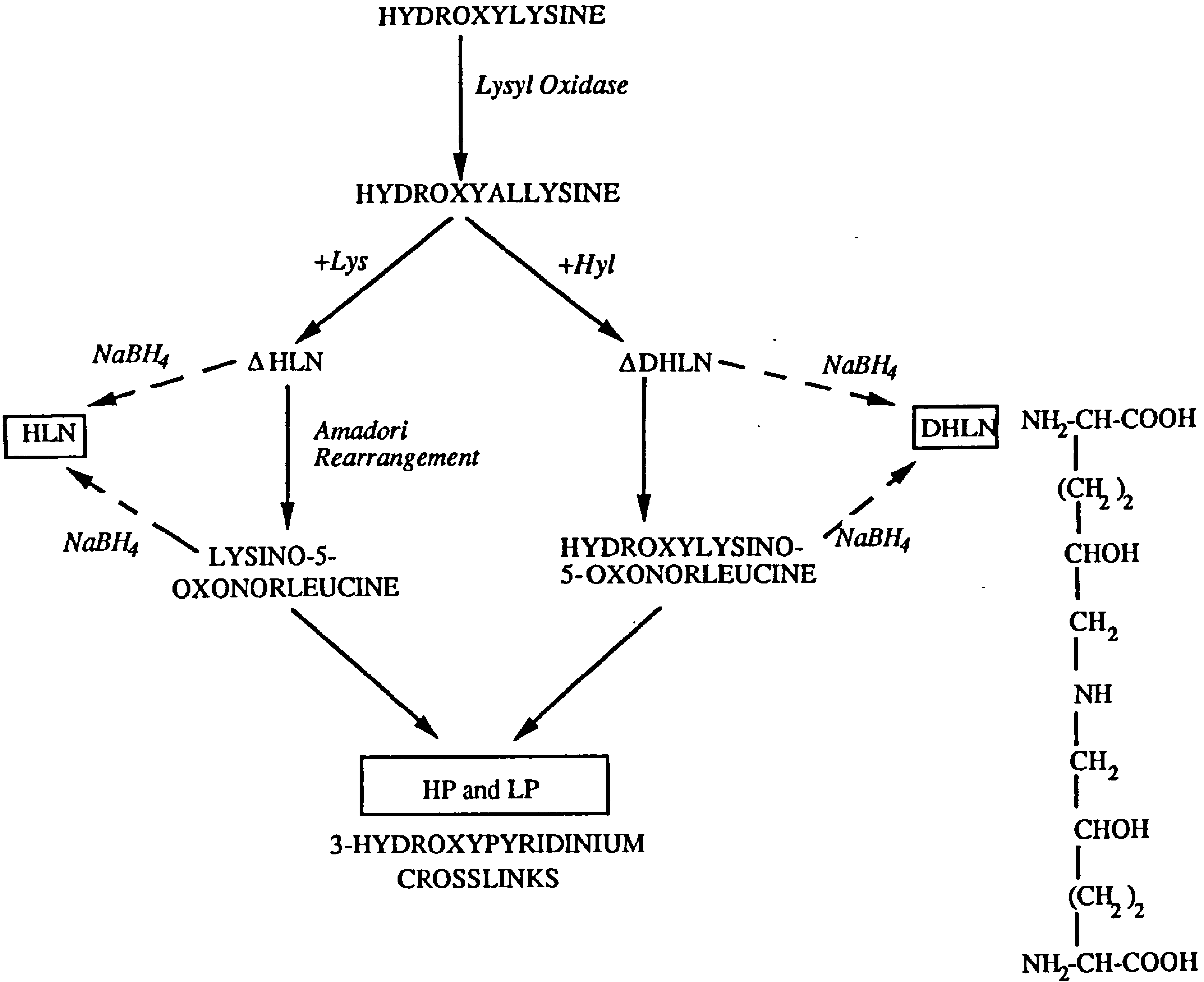


Fig. 1.5b Chemical Pathway of Cross-Linking in the Fibrillar Collagens Based on Telopeptide Hydroxylysine Aldehydes (from Eyre, 1987)

Fig. 1.6. Hydroxylysyl pyridinoline is the more abundant form. Bone and dentin, however certain significant levels of lysyl pyridinoline. The latter is probably formed when a lysine, rather than a hydroxylysine is present at a triple helical site due to incomplete post-translational modification (Eyre, 1987). However, Light & Bailey (1985) cast doubt on the role of pyridinoline as the stabilizing cross-link of mature collagen and suggested that pyridinoline may only link two collagen molecules, thus being an age-related intra-molecular bond. Therefore, other, as yet undiscovered mechanisms of collagen stabilization may exist.

1.4.6 Structure of the Most Abundant Collagen Types, Including Those Found in Muscle

By the late 1950's it became apparent that collagen fibres are formed by the aggregation and precise alignment of individual collagen molecules (Hodge & Schmitt, 1960). During the subsequent decade, a number of studies designed to chemically characterize the monomer molecule and its component polypeptide chains indicated that the triple-stranded collagen molecule was comprised of two identical chains (designated α–1) and a distinct but clearly homologous chain (designated α–2) (Piez, 1967). Since these studies were performed on collagen extracted from a variety of tissues such as skin, tendon, and bone, the results tended to reinforce the concept that all vertebrate collagens could be characterized as a single class or type of molecule consisting of two α–1 chains and one α–2 chain with only minor variations in composition of the component chains from species to species. This is now known to be incorrect and as briefly discussed in sections 1.4.2 and 1.4.3 at least twelve genetically distinct collagens have now been described.

The existence of more than one genetic type was realized at the end of the 1960's, following the demonstration that the cartilage collagen molecule is comprised of only a single type of α chain and that the latter chain exhibits several major compositional differences when compared to α–1 or α–2 chains derived from other tissues of the same species. The chain derived from cartilage collagen more closely resembled α–1 than α–2 in general compositional features and chromatographic properties. It was therefore designated α1(II) and the previously characterized α1 chain common to collagen in other tissues was termed α1(I). The more ubiquitously occurring collagen molecule with the chain composition, $(\alpha 1(I))_2\ \alpha 2$, is now referred to as Type I collagen. Similarly, the cartilage collagen molecule with the chain composition, $(\alpha 1(II))_3$, is designated Type II collagen (Miller, 1976). As it is not associated with meat connective tissue, this type will not be discussed further here. Types I, III-VI are, however, commonly found in meat connective tissues (Light & Champion, 1984) and so these will be considered in detail.

(a)

(b)

Fig 1.6 Structures of the Two Forms of Hydroxypyridinium Cross-Link, the Mature Structures in the Hydroxylysine Aldehyde Pathway (a) HP, Hydroxylysyl Pyridinoline; (b) LP, Lysyl Pyridinoline (from Eyre, 1987).

1.4.6.1 Interstitial Collagen - Type I Collagen

Type I collagen was the first collagen to be characterized and is by far the most abundant form, comprising over 90 % of the collagen found in bone and tendon and about 80 % of the collagen found in skin. It is readily extracted as native, largely monomeric molecules from the skin and other soft tissues of young animals by employing neutral salt or dilute acid solvents (Piez, 1967). The collagenous portion of both chains lacks cysteine.

As mentioned above, type I collagen contains two $\alpha 1(I)$ chains, hydrogen bonded to each other and to a third chain, designated $\alpha 2(I)$, $(\alpha 1(I)_2\ \alpha 2)$, arranged in a triple helix. Each chain has a molecular weight of approximately 95 000 and the $\alpha 1(I)$ and $\alpha 2(I)$ chains are products of separate structural genes and thus have distinct primary structures. The complete amino acid sequence of the $\alpha 1(I)$ chains has been determined (Fietzek & Kühn, 1976). The $\alpha 1(I)$ chain contains 1052 residues beginning with an N-terminal pyroglutamic acid and ending with a C-terminal tyrosine. The Gly-X-Y triplets start at residue 17 and extend without interruption for 337 triplets, ending at residue 1027. The N-terminal 16 residues and the C-terminal 25 residues are extra-helical extensions. Cleavage of the $\alpha 1(I)$ and $\alpha 2(I)$ chains with CNBr yields a limited number of peptides, since collagen α-chains generally contain only a moderate number of methionyl residues and resolution of the CNBr cleavage products can be readily achieved by employing standard techniques of polyacrylamide gel electrophoresis, ion-exchange and molecular sieve chromatography. All of the CNBr peptides derived from $\alpha 1(I)$ and $\alpha 2(I)$ chains of collagen from rat skin, chick bone, chick skin, human skin, baboon skin, bovine skin, guinea pig skin, pig skin, and bovine dentin, have been isolated and characterized with respect to molecular weight and amino acid composition (see Miller, 1976 for review). Comparable data are also available for some of the peptides from rat tendon $\alpha 1(I)$, rat tendon $\alpha 2(I)$, rat dentin $\alpha 1(I)$, rat bone a1(I),and rat bone $\alpha 2(I)$ (Miller, 1976).

In native fibrils of type I collagen, monomeric molecules of 300 nm in length and 1.45 nm in diameter have been shown by X-ray diffraction and electron microscopic investigations to be staggered by multiples of a distance D, equal to 67 nm or 234 residues, (Hodge & Petruska, 1963; Fraser & MacRae, 1981; Parry, 1988). The aggregation of collagen molecules results in the formation of precisley banded fibrils. Fibril assembly is directed by acidic and basic groups and is stabilized by hydrophobic groups, which are present in four repeat regions along the molecule to give a quarter-staggered alignment. The molecular length is equal to 4.4 times the length of these regions and the molecules are therefore overlapped by 0.4 D. It has been shown from X-ray data that the overlap regions of the fibrils are composed of highly ordered straight segments of tilted collagen

molecules linked to one another by relatively disordered molecular segments that constitute the gap filaments (Parry, 1988). The collagen molecule thus has a D-periodic molecular crimp.

1.4.6.2 Intersitial Collagen - Type III Collagen

Studies on the cyanogen bromide (CNBr) peptides released from insoluble collagen of infant dermis indicated the existence of yet another collagen α-chain. The CNBr cleavage products from the total insoluble collagen pool contained at least two peptides which could not have been derived from sequences in the α1(I) and α-2 chains of Type I collagen or the α1(II) chain of Type II collagen. The size and compositional features of the new peptides, however, suggested a considerable degree of homology with certain cyanogen bromide peptides from both α1(I) and α1(II) chains. The new chain was therefore designated α1(III). Collagen molecules comprised of α1(III) chains have been isolated and characterized after release in native form from several tissues such as skin, aorta and uterine wall during limited digestion with pepsin. The latter collagen molecule with the chain composition, $(\alpha1(III))_3$, is now referred to as Type III collagen (Miller, 1976).

Although small amounts of Type III collagen as well as a form of Type III procollagen can be extracted from young rat and bovine skin by neutral salt solvents, this collagen can be effectively extracted in good yield from skin and other tissues by limited proteolysis with an enzyme such as pepsin (Miller, 1976). Type III collagen was initially identified in fetal dermis (Epstein, 1974) and has since been shown to be present in small amounts in many tissues, particularly the vascular system. It accounts for about 60 % of the collagen in foetal skin but drops to about 10 % by birth and is maintained at this level throughout adult life (Epstein, 1974). Its most unusual feature is that the α-chains are intramolecularly linked via disulphide cystine bonds situated at the junction of the non-helical C-terminal domain with the triple helix (Glanville & Fietzek, 1976). Type III collagen is not found in cartilage or bone and only in very small amounts in tendon. However, a high proportion of Type III collagen is present in the perimysium of muscle, and it may play a role in determining the texture of meat (Light, 1987).

1.4.6.3 Basement Membrane Collagen - Type IV

Early structural studies on the characterization of basement membrane collagen indicated this collagen to be comprised of three identical α-chains which exhibit chromatographic characteristics very similar to other α1 chains, but which differ markedly from the other known collagen chains in general compositional features (Kefalides, 1971). Based on

these observations, the basement membrane collagen chain has been designated as $\alpha 1(IV)$, and the basement membrane collagen molecule with the apparent chain composition, $(\alpha 1(IV))_3$, has been referred to as Type IV collagen (Miller & Matukas, 1974). Subsequent investigations, however, suggested that the number of distinct α-chains present in basement membrane collagen may have been underestimated in the earlier studies (Miller, 1976). Structural studies on fragments from a variety of tissues, indicated that most collagen type IV protomers exist as heterotrimers $(\alpha 1(IV)_2)\alpha 2(IV)$.

It is now agreed that the collagenous proteins in basement membranes is mainly a single protein species with M_r of 550 000 to 600 000 and consisting of two different peptide chains, $\alpha 1(IV)$ and $\alpha 2(IV)$, assembled into a triple-stranded molecule. Since basement membrane collagen shows many unique structural features when compared to fibril-forming interstitial collagens (types I, II and III), it was named type IV collagen. The unique properties of type IV collagen are its multidomain structure and frequent interruptions of the triplet sequence (Gly-X-Y). These chemical features and specific antibodies are used for the identification of type IV collagen (Timpl & Dziadek, 1986).

A four-domain structure is generally accepted as a model for the overall structure of type IV collagen monomers (Fig. 1.7). About three-quarters of the molecular mass is made up by a major rodlike triple helix located in the centre of the molecule. It has a length of about 330 nm and an M_r of 145 000 was found for the constituent chain segments (Timpl & Dziadek, 1986). The N-termini of four type IV molecules are cross-linked together to form a tetrameric domain called "7S", 60 nm long with an M_r of about 25 000 (Mayne, 1986; Timpl & Dziadek, 1986). It was originally discovered as a tetrameric fragment which was named 7 S collagen according to its ultracentrifugal behaviour. Its relationship to collagen type IV was demonstrated by rotary shadowing of larger fragments. Both triple helical segments are connected by a short segment (NC2) which contains triplet and non-triplet sequences and presumably acts as a flexible hinge. A second, more prominent non-collagenous domain (NC1) has the shape of a globule and is located at the C-terminal end of the molecule. It is isolated from tissues as a cross-linked dimer (M_r of 170 000) consisting of six chain subunits. These type IV collagen monomers have the specific potential to form tetramers by interaction at the N terminus and/or dimers by interaction at the C terminus, thus forming larger aggregates (Timpl & Dziadek, 1986).

Evidence for the existence of two chains in type IV collagen is available from characterization of two different sets of pepsin fragments obtained from the major triple helix and from biosynthetic studies where a doublet of chains is usually found after

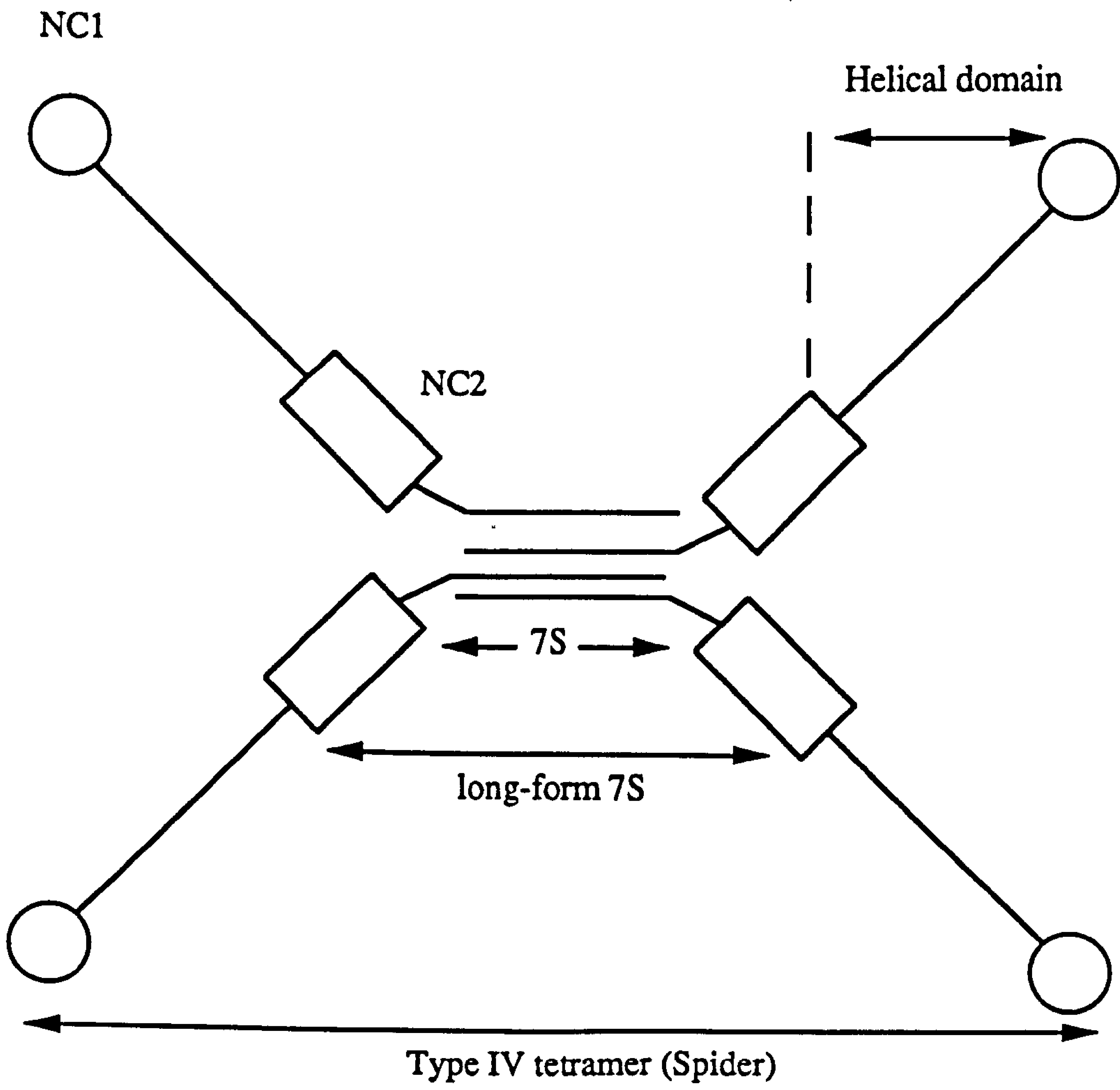


Fig. 1.7 Schematic Representation of the Structural Units of Type IV Collagen ('spiders') as Proposed by Kühn et al. (1981)

reduction and electrophoresis (Timpl & Dziadek, 1986). The two genetically distinct polypeptide chains, designated α1(IV) and α2(IV) are structurally distinct, as shown by peptide mapping (Crouch et al., 1980; Fessler & Fessler, 1980). These chains form helical protomers with a length of about 390 nm (Oberbaumer et al., 1982; Fessler et al., 1984) corresponding to the length of authentic type IV collagen.

Most of the collagen type IV in tissues is insoluble due to the formation of large networks which are stabilized by disulphide bridges and nonreducible cross-links. Brief digestion with pepsin or bacterial collagenase (Kühn et al., 1981; Timpl et al., 1981) solubilizes rather large fragments containing 90 % of the mass of collagen IV monomers. Some type IV collagen may also be extracted by acid (Timpl et al., 1978; Veis & Schwartz, 1981) but it has then lost the 7 S domain, presumably due to endogenous proteolysis (Timpl et al., 1981; Kleinman et al., 1982). Intact structures may be obtained by total (Dean et al., 1983) or partial reduction (Karakashian et al., 1982; Kleinman et al., 1982).

1.4.6.4 Type V Collagen

Type V collagen is cell associated, has first been isolated from amniotic membranes and is found in relatively high amounts in muscle (Burgeson et al., 1976; Chung et al., 1976). The molecular structure of type V collagen is similar to the interstitial collagens with a large triple helical domain of 300 nm. It is composed of three α chains that may be combined as homtrimers or heterotrimers (Krieg et al., 1988).

The macromolecular organization and location of type V collagen in tissues remains controversial and is poorly understood. It has been detected as fine non-banded fibres in certain tissues but is often found pericellular to chondrocytes and smooth muscle cells (Bailey, 1987). This collagen type could be considered as a microfibrillar matrix or pericellular collagen (Bailey, 1987). Type V collagen forms fibrils *in vitro* and segment-long-spacing (SLS) crystallites similar to those formed by the interstitial collagens (Mayne, 1986).

The procollagen form of type V collagen contains large noncollagenous domains at both ends of the molecule. The conversion of type V procollagen to the tissue form of the molecule involves a complex series of processing events (Mayne, 1986). Type V collagen extracted from tissues without pepsin digestion retains a globular domain at one end of the molecule which is believed to be responsible for the inability of type V collagen to form banded fibres *in vivo* which inhibits lateral association (Mayne, 1986; Bailey,

1987). An additional inhibiting factor may be the presence of sulphated tyrosine side-chains (Bailey, 1987).

Type V collagen may serve a binding or linking function to the larger collagen fibres while it has also been suggested that type V collagen forms small fibrils without involvement of other collagen types (Mayne, 1986). Type V collagen is closely associated with fibrils of type I or III collagen and may be present within the same fibrils. The presence of type V collagen appears to limit the diameter of the fibrils (Mayne, 1986).

1.4.6.5 Type VI Collagen

Biochemical analysis of the pepsin resistant fragment from Type VI (intima) collagen showed the presence of three different polypeptide chains (Jander et al., 1983), which were designated α1(VI), α2(VI) and α3(VI). Equimolar amounts of the three chains were isolated, suggesting a single molecule of chain organization α1(VI)α2(VI)α3(VI). Although type VI collagen is not yet fully characterized however, it seems to represent a unique collagen. The triple helix of type VI collagen is shorter than other collagen types and has a length of approximately 103 nm with large globular domains at both ends (Krieg et al., 1988). Type VI collagen is widely distributed in connective tissue (Bailey, 1987).

1.5 THE CONVERSION OF MUSCLE TO MEAT

The conversion of living muscle to tender meat results from a series of biochemical and biophysical changes which are initiated in muscle at the death of the animal.

1.5.1 Post-Mortem Glycolysis

Normally living muscles obtain their biochemical energy, ATP from respiring mitochondria, but aerobic respiration ceases when blood circulation stops after slaughter. For a time, however, the level of ATP (about 10 µg/g) is maintained by the breakdown of creatine phosphate (about 8 µmol/g) which serves to phosphorylate ADP to ATP. However, the major supply of biochemical energy post-mortem is from catabolism of glycogen (60 µmol/g) via the Embden-Meyerhof pathway (Pearson, 1971), which not only generates ATP, but also produces lactate (Bendall, 1973; Ashgar & Pearson, 1980; Hultin, 1984).

The sequence of chemical steps by which glycogen is converted to lactate is essentially the same post-mortem as *in vivo* when the oxygen supply may become temporarily inadequate

for the provision of energy in the muscle, but it proceeds further. Except under conditions where the glycogen reserves are appreciably diminished, the conversion of glycogen to lactic acid will continue until a pH is reached when the enzymes effecting the breakdown become inactivated. In muscle, a gradual decrease in pH from approximately pH 7.0 to a value of 5.6 to 5.7, within 8 h post-mortem is attained, while 24 h after slaughter the ultimate pH value is reached. In typical mammalian muscles this pH is about 5.4 to 5.5. Glycogen is generally considered to be absent at pH values above this level, but certain atypical muscles may have as much as 1 % residual glycogen when the ultimate pH is above 6 (Lawrie, 1955). The final pH attained, whether through lack of glycogen, inactivation of the glycolytic enzymes or because the glycogen is insensitive or inaccessible to attack, is referred to as the ultimate pH or pHu (Callow, 1937).

Ashgar & Yeates (1978) stated that the post-mortem pH drop is caused primarily from the accumulation of lactate from glycogen via the glycolytic pathway in the intracellular space, while Honikel & Hamm (1974) stated that despite the formation of two H^+ ions and two molecules of lactate from each glucose unit, the drop in pH is not due to lactate formation, since H^+ ions are released before the reduction of pyruvate to lactate. At about pH 7, H^+ is bound during phosphorylation of ADP to ATP and is liberated during hydrolysis of ATP to ADP. On the other hand, at pH 5.5 to 6, H^+ is released during glycolysis, but none is released during hydrolysis of ATP. The authors concluded that 90 % of the H^+ ions formed are due to glycolysis and 10 % are due to breakdown of ATP. Phosphofructokinase was found to be the rate-limiting factor in post-mortem glycolysis (Dalrymple & Hamm, 1975).

A number of factors determine the overall rate of glycolysis, post-mortem (Bendall, 1979; Ashgar & Pearson, 1980; Lawrie, 1985). These include antemortem treatments, the physiological and hormonal status of the animal, the temperature at which the carcass is held, subcutaneous fat cover and nutritional status of the animal. Animals that are well rested before slaughter will have adequate glycogen reserves which allow glycolysis to continue post-mortem until the pH has fallen to a value of about 5.5, after which no further breakdown of glycogen occurs. Exhausted and stressed animals will have depleted muscle glycogen reserves, resulting in cessation of glycolysis when this has been consumed. The intravenous administration of relaxing doses of magnesium sulphate before slaughter will slow the subsequent rate of post-mortem glycolysis (Lawrie, 1985), while injection of calcium salts and of adrenaline and noradrenaline accelerates the rate. Insulin shock, subcutaneous adrenaline injection, tuberculin and tremorine will produce a high ultimate pH, through depletion of glycogen reserves. The rate of post-mortem glycolysis increases with increasing external temperature above ambient, however the rate

also increases from about 5 to 0° C (Lawrie, 1985). At these lower temperatures there is a cold-induced contraction of the muscle (cold-shortening), which consumes more ATP because mechanical work is now done. The detailed mechanism of cold-contraction is not certainly known, but it appears that at low temperatures the ATP-driven Ca^{2+} pump is unable to maintain the normally very low Ca^{2+}-ion level in the sarcoplasm of the resting muscle. The passive outflow of ions from the sarcoplasmic reticulum then triggers a myofibrillar contraction, which consumes ATP and this stimulates a faster breakdown of glycogen. Increasing the temperature reverses the flow of Ca^{2+} ions and again relaxes the muscle until such time as rigor mortis develops, but beyond this point the muscle fibres will become locked in their shortened state. According to their location within the carcass, different muscles will have different rates of temperature decline, post-mortem. As a result, the rate of post-mortem glycolysis will tend to be higher in muscles which are slow to cool (Bendall, 1978).

Both the rate of pH decline and the pHu of post-mortem muscle affect the quality of meat (Ashgar & Pearson, 1980; Lawrie, 1985). A relatively slow rate of glycolysis and a moderately low ultimate pH (about 5.4) are characteristic of normal muscle, and results in tender meat, while high ultimate pH (above 5.8) results in dark coloured and close structured meat with poor keeping quality. Meat of normal ultimate pH has an open structure and is bright red. Rapid pH decline, from the physiological value (about 7.3) to 6.0 within 20 min, and a very low ultimate pH (5.3) tend to disrupt the ultrastructure of muscle, causing the meat to appear pale and watery (Briskey, 1963). Precipitation of the sarcoplasmic proteins on the myofilaments may also occur (Bendall et al., 1963). This is responsible for low solubility of the proteins, resulting in highly undesirable meat. However, evidence for this has been found only in some breeds of pigs (Ashgar & Pearson, 1980) and may not be generally applicable.

1.5.2 Rigor Mortis

Post-mortem biochemical changes are accompanied by ultrastructural changes in muscles which together are responsible for the complex phenomenon of rigor mortis. Rigor mortis is evidenced as dramatic stiffening and loss of extensibility of the musculature, which occurs in the carcass when muscle ATP is nearly depleted and glycolysis is slowing to a halt. With insufficient ATP present in the muscle cell, actin and myosin complex together to form rigid chains of actomyosin (Ashgar & Pearson, 1980). It is this cross-bridging of the thick and thin filaments of the myofibril that causes the muscle to become stiff and inextensible. The extent to which these filaments are overlapped in the myofibril determines to a large degree the ultimate quality of the meat.

The 'sliding-filament' theory of Huxley & Hanson (1954) has been generally accepted as the primary mode for muscle contraction. Contraction starts when free Ca^{2+} ions initiate conformational changes in the tropomyosin and troponin complex. Other associated chemical changes have also been reported (see Ashgar & Pearson, 1980 for review). Further evidence suggests that contraction occurs in a series of successive bursts in groups of adjacent sarcomerces (Pollack et al., 1977). In addition to the actin-myosin system, the regulatory proteins (tropomyosin, the troponin complex, α- and β- actinin, C- protein, M-protein components), the sacroplasmic reticulum, the T- system, Ca^{2+}/Mg^{2+} ions and ATP are all involved in the overall process of muscle contraction. Many similarities exist between the mode of physiological contraction of muscle *in vivo* and development of rigor-mortis in post-mortem muscle. The same series of events explain the mechanism of rigor mortis, the main difference being that the latter is an irreversible process (Ashgar & Pearson, 1980). The loss of extensibility of the musculature, post-mortem, proceeds slowly initially (the delay phase), then with great rapidity (the rapid phase) after which extensibility remains constant at a low level (Bendall, 1960).

In addition to losing its extensibility, unrestrained muscle shortens during rigor-mortis. Locker & Hagyard (1963) found that minimum shortening (about 10 % of the initial excised length) occurred in the temperature range 14 to 19° C. The extent of shortening was greater the further removed the pre-rigor holding temperature was from this temperature range in either direction. Muscles that are greatly shortened at the time of rigor development have more attached cross-bridges in the actomyosin complex, resulting in tough meat (Marsh & Leet, 1966). Abnormal types of rigor mortis, brought about by improper handling of pre-rigor muscle are discussed below.

1.5.2.1 Cold-Shortening

The phenomenon of cold-shortening, which is the shortening of pre-rigor muscle due to over-rapid cooling, results in a two- to three- fold increase in toughness (Ashgar & Pearson, 1980). The tendency for the muscle to shorten is greater the closer the temperature of the pre-rigor muscle is to freezing.

Originally, cold-shortening was believed to be induced by general release of Ca^{2+} ions from the sarcoplasmic reticulum into the myofibrillar regions at low temperatures. However, further evidence suggested that the mitochondria release Ca^{2+} ions at low temperatures and thus overload the sarcoplasmic reticulum, so that an excess of free Ca^{2+} ions initiate shortening. However, mitochondria play no role in Ca^{2+} re-accumulation and relaxation upon re-warming unlike the sarcoplasmic reticulum. Moreover, high pH values

and ATP levels are pre-requisites for cold-shortening while cold provides the direct stimulus for shortening, which is ultimately responsible for the increase in toughness (Ashgar & Pearson, 1980).

Although temperature is the main determinant affecting the extent of shortening (Locker & Hagyard, 1963), other factors including species, fibre-type and stage of physiological maturity of the animal also have significant bearing on the extent of shortening and consequently on meat toughness (Ashgar & Pearson, 1980).

Various methods have been used to minimize the incidence of cold-shortening in carcasses during chilling. Cold-shortening was more pronounced in excised pre-rigor muscles than in intact ones, while stretching of these resulted in quite tender meat (Ashgar & Pearson, 1980). Since the extent of shortening is believed to be largely dependent on the amount of tension exerted on a particular muscle in the carcass, an increase in tenderness of the major muscles has been attained by suspending the carcass from the oburator foramen or aitchbone (Harrison & Macfarlane, 1971). Similar effects have been obtained by suspension from the cervical vertabrae or by adding weights (Buege & Stouffer, 1974), rather than by using conventional vertical suspension from the Achilles tendon.

Post-mortem glycolysis is hastened by stimulating the muscles to contract very shortly after slaughter (Carse, 1972; Chrystall & Hagyard, 1973). The rapid depletion of glycogen by stimulation advances the onset of rigor mortis, thus permitting the carcass to be refrigerated much earlier and without the problem of cold-shortening.

1.5.2.2 Thaw - Rigor

If muscle is frozen in the pre-rigor state, before the ATP level has fallen appreciably, an exceedingly fast rate of ATP breakdown and of rigor mortis onset ensues on thawing. This phenomenon is known as thaw-rigor (Bendall, 1973; Locker et al., 1975). The greatly enhanced ATP-ase activity on thawing causes marked shortening, the muscle may contract to 50 % of its initial length and exudation of excessive quantities of drip which may amount to 30 to 40 % of the muscle weight occurs leading to highly undesirable meat.

The rate of contraction depends mainly on the rate of thawing. Ca^{2+} ions are released by the sarcoplasmic reticulum on thawing and stimulate ATP-ase activity (Bendall, 1973). Where thawing is instantaneous, Ca^{2+} is quickly recaptured by the sarcoplasmic reticulum, and stimulation of the ATP-ase system is brief.

1.5.3 Resolution of Rigor Mortis

Post-mortem changes in muscle greatly affect the tenderness of meat. Meat cooked in the pre-rigor state and 1 to 2 h post-mortem is quite tender. However, meat cooked while in the full rigor state is tough and 'flat' in taste (Ashgar & Pearson, 1980). The two major contributors to meat toughness are the contractile protein complex (Locker, 1960; Locker & Hagyard, 1963; Marsh & Leet, 1966) and the collagen fibres (Bailey, 1972). The terms 'conditioning', 'ageing', 'ripening', 'maturing' and the 'resolution of rigor' have all been applied to post-mortem storage of meat for periods beyond the normal time taken for cooling and setting. The best quality meat is obtained when the carcass is chilled slowly and allowed to hang, which for beef may be two weeks or more at the chill temperature. During this period of storage, toughness of the meat decreases. The reduction in toughness is rapid during the first few days of storage, but, thereafter the rate declines. However, considerable variation exists in the rates of conditioning between different muscles and between different species (Dransfield, 1986). Beef, lamb, veal and rabbit have similar rates, pig is faster while chicken is very fast, reaching low toughness values within 48 h of death (Dransfield, 1986).

Much confusion regarding rigor mortis and its resolution has developed in the literature. It has been suggested that the resolution of rigor may be caused by the slow dissociation of the actomyosin complex into actin and myosin, that is, a reversal of the events that brought about rigor mortis (Wierbicki et al., 1954). However, it is now generally agreed that there is no reversal, as such in the cross-bridging of the thick and thin filaments but there is a loss of strength in the myofibrils (Marsh, 1954).

1.5.4 Proteolysis during Conditioning

As already stated (section 1.5.3) conditioning of meat is the practice of storing meat (at 1 to 3° C) for some time after death of the animal, after which an improvement in quality is observed. Conditioning is an enzymic process, resulting in proteolytic changes to the two major contributors to meat toughness, the contractile protein complex (Locker, 1960; Locker & Hagyard, 1963; Marsh & Leet, 1966) and the connective tissue collagen fibres (Bailey, 1972).

It is now well accepted that during conditioning, proteolytic changes occur in the myofibrillar proteins, leading to their increased fragility (Penny, 1980; Etherington, 1984). Disruption occurred initially at the junction of the I-band with the Z-disc (Penny, 1980). As conditioning proceeds, degradation of the actual Z-discs occurs, while in fully

conditioned meat both the Z-disc and M-line structures are substantially degraded (Penny 1980; Etherington, 1981). Desmin, located circumferentially at the Z-disc, which has a role in the alignment and attachment of adjacent myofibrils (Lazarides, 1980) is degraded in post-mortem muscle, facilitating fibre splitting (Etherington, 1984). Other proteins found to be degraded were troponin T, troponin I and C-protein (Penny, 1980). In addition to these degradative changes, there was shown to be an accumulation of smaller polypeptides with one particularly strong band of 30 000 daltons, possibly derived from troponin T (Azanza et al., 1979; Penny, 1980).

Compared with the amount of evidence for degradation of the myofibrillar structure during conditioning (Penny, 1980; Etherington, 1984), little or no direct evidence exists to show that proteolysis of collagen takes place during the conditioning process, although the biochemical and mechanical status of the connective tissue component of meat has also been associated with tenderness of the cooked product (Mackintosh et al., 1936; Cover et al., 1962; Cross et al., 1973). Previous studies on the effects of conditioning on intramuscular collagen have produced conflicting results. Sharp (1963) found no change in soluble hydroxyproline containing peptides over long periods of conditioning. The yield of intramuscular connective tissue from beef was found to be less at 3 days post-mortem, than at death (McClain et al., 1970). In addition, the amounts of heat-labile collagen (Pfieffer et al., 1972), which would be expected to influence toughness after storage, and the amounts of collagen soluble in neutral or acid solutions from beef (Pierson & Fox, 1976) were found to be unchanged on conditioning.

On the other hand, evidence for a role for conditioning on collagen degradation is provided by Sharp (1964) who showed that aged beef muscle collagen had become much more readily solubilized into gelatin by heat, while Pfieffer et al. (1972) detected small changes in the covalent cross-linking of connective tissue after 21 days of storage of beef muscle. It was found that the heat-labile fraction of intramuscular collagen was significantly increased at 10 days but not at 5 days of conditioning (Herring et al., 1967). Conditioning was found to result in an increase in the yield of collagen α-components (Kruggel & Field, 1971; Wu et al., 1982). A decrease in the structural and thermal stability of collagen after conditioning was reported (Mills et al., 1984), suggesting that these changes occurred within a few hours post-mortem. The integrity of collagen fibres after conditioning was investigated by examination of isolated bovine intramuscular connective tissue using differential scanning calorimetry (Judge & Aberle, 1982) and it was found that a fall of 3 to 5° C in the thermal shrinkage temperature after 24 h of conditioning and of 7 to 8° C by 7 days post-slaughter resulted.

Ashgar & Yeates (1978) proposed that both pH - dependent and enzymatic changes take place during conditioning. The pH - dependent changes include alterations in the extracellular proteins (especially collagen) and in the protein - ion relationships, in the structure of the sarcolemma and in the conformation of the myofibrillar proteins. The enzymatic changes include the influence of conditioning on regulatory proteins, especially on α-actinin and the tropomyosin - troponin complex, and on the myosin filaments. Enzymatic changes during conditioning are brought about by the action of Ca^{2+} activated neutral proteinase (CANP) and the lysosomal acid proteases (cathepsins) (Etherington, 1981). However, at the post-mortem limit pH of 5.5, lysosomal cathepsins could function more effectively than CANP in weakening the myofibrils (Dutson, 1983; Etherington, 1984) although this has been disputed by Koohmaraie et al. (1986) and Koohmaraie et al. (1987) who claim that CANP may play a major role during post-mortem tenderization of meat.

Indirect evidence suggests that cathepsin action during conditioning brings about limited proteolytic cleavage of the collagen component of meat (Robbins & Cohen, 1976; Kopp & Valin, 1980; Etherington, 1987a). The cumulative effect of all these changes is an increase in the tenderness of meat during conditioning.

1.5.4.1 Technology of Conditioning

The normal slaughtering and marketing procedures in developed countries results in the consumer receiving the meat only after a period of conditioning. The carcasses are subjected to storage under controlled atmospheric conditions for specific periods of time to allow ripening of the meat (Etherington, 1981). Duration of the conditioning process is determined by the holding temperature and species (Dransfield, 1986). At chill temperatures, satisfactory tenderness was produced in young beef carcasses of different sexes, sizes and degrees of fatness, following 6 days of conditioning, while after 11 days of conditioning no further tenderization occurred (Ashgar & Pearson, 1980). The application of electrical stimulation may provide some benefits by accelerating the rigor process. As a result of this, the muscle proteinases appear to be activated earlier, and can thus begin to soften the muscle while it is still warm (Bendall, 1980). However, unless the carcasses are allowed to hang for several days, the full benefits of electrical stimulation will not be realized (George et al., 1980).

Since the rate of biophysical and biochemical reactions are temperature dependent, and temperature also affects the kinetic energy and metabolic activity of the cellular systems of muscle, conditioning periods can be reduced by subjecting the carcasses to higher

temperatures (14 to 44° C) (Davey & Gilbert, 1976), From 0 to 40° C, the rate increases about 2.5 fold for every 10° C rise in temperature (Davey & Gilbert, 1976; Dransfield, 1986). Above 60° C the rate drops rapidly due to enzyme denaturation (Davey & Gilbert, 1976). In beef, therefore, which achieves 80 % of the tenderizing in 10 days at 0° C, 4 days would be required at 10° C, and 1.5 days at 20° C. However, bacterial growth, bone taint, and excessive shrinkage are major problems associated with high-temperature ageing of meat (Ashgar & Pearson, 1980).

The use of bacteriostatic agents such as CO_2, antibiotics, and ultraviolet light aid in preventing microbial spoilage, while careful control of relative humidity in storage can prevent shrinkage. However, disadvantages are associated with some of these approaches. UV light induces homolytic reactions resulting in the formation of free radicals, which lead to photooxidation and to off-flavours in the adipose tissue while excess CO_2 results in metmyoglobin formation and discolouration of meat. In view of the limitations, improvements in tenderness observed during high temperature conditioning may not be large enough to warrant industrial adoption of such practices (Ashgar & Pearson, 1980).

1.6 Enzymes Involved During Conditioning

The number of known tissue proteases is extensive although it remains unclear how many are found in muscle (Goll et al., 1983). The proteinases that may participate in the conditioning of meat, together with the main pH range in which each is active, are listed in Table 1.6.

Until recently, almost all studies of muscle proteinases involved homogenization of whole muscle tissue followed by assay of proteolytic activity in the supernatant remaining after sedimentation of insoluble material or of activity in the sedimented lysosomal fraction. In addition to skeletal muscle cells, whole skeletal muscle contains many cells such as mast cells, fibroblasts, macrophages and the various cells found in blood. Many of the non-muscle cells found in skeletal muscle tissue contain very high concentrations of proteolytic enzymes (Goll et al., 1983). Several proteolytic activities thought to originate from muscle cells are in fact, located only in non-muscle cells found in muscle tissue (Goll et al., 1983). These include the alkaline, chymotrypsin-like serine proteinase which was shown immunologically to be specific to the mast cells (Etherington, 1985). Lysosomal proteases, including lysosomal carboxypeptidase A and B, cathepsin B, D and E, dipeptidyl aminopeptidase I and collagenenase, arising from leukocyte lysosomes are

Table 1.6 Proteinases of Muscle (from Etherington, 1987b)

Proteinase	Main pH range for proteolysis
Non-lysosomal	
Calcium activated neutral proteinases (CANP)	6.5-8.0
Trypsin-like (serine) proteinase	6.5-8.0
Neutral (thiol) proteinase	6.5-8.0
Alkaline (serine) proteinase	7.5-10.5
Lysosomal	
Cathepsin B	5.0-6.0
Cathepsin D	2.5-4.5
Cathepsin H	5.5-6.0
Cathepsin L	5.5-6.0
Cathepsin N	3.0-6.5

believed to contribute to the post-mortem tenderization of meat (Venugopal & Bailey, 1978a, b).

Use of assays of crude muscle homogenates or crude muscle lysosomal preparations for detection of muscle proteinases failed to detect any proteolytic enzyme that may be associated with an inhibitor. Present evidence indicates that specific inhibitors are one of the methods used by cells to regulate activity of intracellular proteolytic enzymes, which would destroy the interior of the cell if their activity were not controlled in some way. Specific protein inhibitors have been described for several of the proteolytic activities detected in muscle tissue; cathepsin B and the neutral serine protease from smooth muscle and the Ca^{2+}-dependent proteinase have all been shown to be associated with specific inhibitors *in vivo* (Goll et al., 1983). Several procedures, such as direct immunological localization or assays on cultures of pure muscle cells, now exist to indicate whether proteolytic activities detected in crude muscle homogenates originate from muscle cells, non-muscle cells or both.

The neutral thiol proteinase was present in the mononuclear cells, but in addition was located in a few muscle fibres, particularly in the slow-red, soleus muscle (Stauber et al., 1983). In addition to the mast cells there are other mononuclear cells such as leucocytes, fibroblasts and satellite cells and these would be comparatively rich in proteinase activity. However, the fraction of lysosomal hydrolase activity associated with these cells was found to account for only 2 to 4 % of total cathepsin activity and 15 % of glycosidase activity (Etherington & Wardale, 1982) of muscle. Therefore, by far the greater proportion of these enzymes was in multinuclear muscle fibres. The location of several lysosomal cathepsins has been studied by enzyme histochemical techniques. In general, the activities have been found usually close to the sarcolemma and not distributed randomly through the muscle fibre (Stauber & Ong, 1981; Whitaker et al., 1982).

1.6.1 Ca^{2+} Activated Neutral Proteinase (CANP)

Probably the most important of the non-lysosomal, neutral proteinases of muscle is the Ca^{2+} activated neutral proteinase (CANP). This enzyme is an intracellular thiol protease and has been purified to homogeneity from several species. The enzyme is present in many tissues but appears to be more abundant in muscle (Murachi, 1983). Immuno-histochemical studies have shown that it is located inside striated muscle cells at the level of the Z-disc (Ishiura et al., 1980) and adjacent to the cytoplasmic face of the plasma membrane (Goll et al., 1983). Although CANP is also found in non-muscle cells such as platelets, its location in striated muscle cells indicates that it could have a role in post-

mortem tenderization of muscle proteins (Etherington, 1981; Goll et al., 1983). From *in vitro* studies, the enzyme promotes weakening of the myofibril consistent with that observed in intact muscle. The Z-line protein α-actinin was found to be released and troponins T and I and C-protein degraded. A new protein fragment of 30 000 daltons was also generated. After more extensive hydrolysis the Z-line disappeared completely from the myofibrils (Penny, 1980). A summary of the effects of CANP on contractile proteins from skeletal and smooth muscle is presented in Table 1.7.

CANP, isolated from pig and beef muscle, consists of two dissimilar subunits of MW 80 000 and 30 000 daltons (Dayton et al., 1975). However, rabbit CANP exists as a single polypeptide chain of 73 000 daltons (Azanza et al., 1979) The original CANP activity isolated was maximally active between pH 6.5 and 8.0, required 1 to 5 mM Ca^{2+} for maximal activity and had very little activity below 0.1 mM Ca^{2+}, was not activated by most other divalent cations tested, and required a reduced sulphydryl group for activity (Etherington, 1981; Goll et al., 1983).

The form of CANP originally purified required millimolar levels of Ca^{2+} for maximum activity and is designated mCANP, whereas intracellular free Ca^{2+} concentrations rarely rise above 10 μM (Goll et al., 1983). A second form of the enzyme was discovered, that was maximally activated at lower concentrations (50 to 100 μM) of Ca^{2+} and that had detectable activity at 10 μM Ca^{2+} from bovine cardiac and skeletal muscle (Goll et al., 1983). This enzyme has been designated μCANP and like mCANP has polypeptides of 80 000 and 30 000 daltons, but possesses a lower net negative charge at pH 7.5 than mCANP. The low negative charge of μCANP allows it to be separated from mCANP because it elutes from DEAE-cellulose ion exchange columns between 110 and 140 mM KCl (average, 122 mM KCl), whereas mCANP elutes between 215 and 270 mM KCl (average, 235 mM) from the same columns. μCANP, however, elutes from Ultrogel 34 gel permeation and phenyl-sepharose hydrophobic colums identically with mCANP (Goll et al., 1983). Both μCANP and mCANP are completely inhibited by CANP inhibitor (Szpacenko et al., 1981). Consequently, μCANP seems to be the same enzyme as mCANP but modified in a way that reduces its net negative charge at pH 7.5 and its Ca^{2+} requirement for maximum activity (Goll et al., 1983).

Clearly, then, only μCANP would be fully functional in the muscle cell at the Ca^{2+} level that can be reached post-mortem. However, there is now evidence that mCANP may be converted autolytically into a more sensitive form. For the rabbit enzyme it has been suggested that this increase in Ca^{2+} sensitivity results from the degradation of an associated protein of 30 000 daltons, which may function as an enzyme regulator *in vivo*

Table 1.7 Summary of the Effects of CANP, the Ca^{2+} -Dependent Proteinase, on Contractile Proteins from Skeletal and Smooth Muscle (from Goll et al., 1983)

Contractile Protein	Location in Muscle Cell	Effect of CANP
Myosin	Thick filaments	No effect; may degrade heavy chain of myosin to 190 000 daltons if LC^2 light chain is unphosphorylated
Actin	Thin filaments	No effect
α-actinin	Z-disc	No effect
Troponin C	38.5 nm intervals along the thin filaments	No effect
Tropomyosin	All along the thin filament; possibly in the Z-disc	Degrades to peptides of approximately 17 000 and 15 000 daltons
Troponin T	38.5 nm intervals along the thin filament	Degrades rapidly to several different peptides of approximately 30 000 daltons that are then degraded to 14 000 daltons
Troponin I	38.5 nm intervals along the thin filaments	Degrades fairly rapidly to peptides of approximately 14 000 daltons
C-protein	43 nm intervals along the thick filament	Degrades from a 130 000 dalton peptide to approximately 120 000 dalton peptide
Filamin	Z-discs	Degrades to two fragments 240 000 and 9 500 daltons
Desmin	Z-discs	Degrades to peptides of 32 000 and 18 000 daltons
Vinculin	Attachment plaques in smooth muscle	Degrades to major peptides of 96 000 and 28 000 daltons and then later to two minor peptides of 83 000 and 70 000 daltons
Gelsolin	I-band	Degrades to peptides of 43 000 and 38 000 daltons; these are then degraded slowly to peptides of 42 000 and 36 000 daltons

(Cottin, 1983). The enzyme itself showed no apparent loss in molecular weight. This high sensitivity form of mCANP, however, did not appear to be the same enzyme as the μCANP prepared directly from muscle. In their studies on the pig enzyme, Dayton et al. (1981) demonstrated using immunodiffusion techniques, that mCANP and μCANP cross-react. Their antibodies were raised against denatured CANP purified by SDS-polyacrylamide-gel electrophoresis. However, recent work using specific antibodies to each of the native rabbit enzymes, failed to show any cross-reactivity either by immunodiffusion or immunoprecipitation, although there was some cross-reaction between the SDS-denatured forms, which suggests that there are significant structural differences between the two proteins (Etherington, 1984).

Both forms of CANP were found to release α-actinin from the Z-line and to degrade troponin T, troponin I and C-protein while desmin was also degraded very rapidly. In addition, CANP can release Z-Nin protein from the Z-line (Penny, 1980; Suzuki et al., 1981).

In the living cell, the activity of CANP would be regulated by the free Ca^{2+} concentration. In addition, a protein inhibitor of CANP was discovered which can completely inhibit both mCANP and μCANP at either millimolar or micromolar Ca^{2+} concentrations. CANP inhibitor seems to be present in striated muscle in excess over CANP because no CANP activity can be detected in crude homogenates of either skeletal or cardiac muscle until CANP inhibitor is removed (Goll et al., 1983). After death, the Ca^{2+} ion pumps fail and μCANP would be activated by the influx of Ca^{2+}. It would seem probable that this enzyme could be directly responsible for the loss of rigor stiffness. More recently, it has been shown that the attachment site of the thin filaments in the Z-line involves a different form of actin and this protein appears to be especially sensitive to the action of CANP (Nagainis & Wolfe, 1982).

Lactic acid formation after death of the animal results in pH decline to about 5.5, 6 to 24 h after slaughter. The optimum pH of CANP (6.5 to 8.0) (Etherington, 1981; Goll et al., 1983) suggests that it is maximally active during the early stages following death of the animal, however, it would not be expected to have a significant contribution during the main conditioning period, once the carcass pH has fallen to 5.5.

1.6.2 Non-Lysosomal Enzymes Active at Neutral and Alkaline pH Values

Of the neutral proteinases of muscle, the trypsin-like proteinase has been found in smooth muscle and then only during muscle wasting. However, the enzyme is extremely active in

the breakdown of α-actinin, actin, myosin, and the troponin complex (Kay et al., 1982). A neutral thiol proteinase has been identified in normal and dystrophic human muscle. It has not been studied in the muscle tissue of meat animals and its action on myofibrillar proteins is not known (Kar, 1982).

1.6.3 Lysosomal Cathepsins

At the point of death, the muscle pH is near neutrality. As discussed earlier, post-mortem glycolysis can continue only anaerobically with the concomitant production and accumulation of lactic acid, which lowers the pH, in normal muscle, to pH 5.5, which is reached 6 to 24 hours after death. Any further lowering of the pH is precluded by accelerating side-reactions (Scopes, 1974; Bendall, 1979). Such conditions in the cell obviously favour the activity of acid hydrolases. During anaerobic glycolysis the redox state of the muscle would also fall such that those proteinases with a cysteine residue in the active centre would become fully active. Two other variables that are important in determining the activity of muscle proteinases are temperature and the free Ca^{2+} concentration in the muscle cells. The rate at which the temperature of the carcass falls depends on the size of the carcass and the holding environment. With forced chilling, the enzyme activities would decline rapidly, whereas for a slowly cooling carcass they would maintain a higher activity and the meat would age sooner. The Ca^{2+} ion concentration of the living muscle is normally maintained at about 10^{-8} M, with a transient rise to 10^{-5} M during contraction. After death, the Ca^{2+} ion pump fails, permitting the free ion concentration to equilibrate through the tissue, and thereby activating the Ca^{2+} requiring proteinases in the muscle cell (Etherington, 1981).

Above pH 6.0, CANP was the most effective enzyme in studies on the effect of pH on myofibrillar proteolysis. Proteolysis at pH 5.5 was greatly enhanced in the presence of EDTA and cysteine, which indicates that the cysteine cathepsins of the muscle lysosome are the more effective enzymes during the main conditioning period (Etherington, 1981). In the carcass, these enzymes act secondarily to the neutral proteinase activity and the myofibrillar structures are already partially damaged, however, in most studies intact myofibrils or myofibrillar proteins have been employed as test substrates. Therefore, it remains uncertain which of the lysosomal proteinases are the more important in the conditioning process when the muscle pH has reached a limit value of about 5.5. The two enzyme systems, CANP and lysosomal enzymes may act synergistically, since lysosomal enzymes may degrade the CANP inhibitor, and therefore promote CANP activity (Goll et al., 1983). However, lysosomal proteases may also accelerate deactivation of CANP by degrading it. In contrast to our knowledge of CANP, there is less information available

on the action of the lysosomal enzymes during meat conditioning. Lysosomal enzymes include proteases, glycosidases and phosphatases (White et al., 1978).

The sudden fall in muscle pH during electrical stimulation while the carcass is still hot will promote an earlier disruption of the muscle lysosomes. It has been suggested that the combination of low pH and high temperature promotes the rupture of lysosomal membranes and releases those enzymes which act on muscle components (Dutson, 1983). It is presumed that after their release from the lysosomes the enzymes can diffuse readily into the myofibrillar structures. Lysosomes lose their characteristic latency in the post-rigor muscle tissue due to the low pH (Dutson & Lawrie, 1974). Evidence shows that for electrically stimulated carcasses, more than half of the total measurable lysosomal activity is released within the first hour following stimulation, much sooner than for non-stimulated carcasses. These enzymes therefore are not only released earlier but they will exhibit greater proteolytic activity than in non-stimulated carcasses as the pH is reduced while the temperature of the muscles is still elevated (Dutson et al., 1980; Etherington, 1981).

At the limit pH of 5.5, the muscle enzymes promote a further improvement in the quality of the meat while it is held in refrigerated (but not frozen) storage. Since enzyme action is temperature-dependent, proteolytic degradation can be accelerated if the carcasses are held for a while above or near ambient temperatures. Furthermore, there is an earlier release of enzymes from the lysosomes under these conditions (Etherington, 1981).

The lysosomal proteases known to possess collagenolytic activity are cathepsins B, L, N and S (Bailey & Etherington, 1980; Maciewicz et al., 1987), with cathepsins L and N exhibiting the highest specific activity (Maciewicz & Etherington, 1985). To date, however, only cathepsins B and L have been located in muscle (Etherington et al., 1987), but cathepsin N may also have some role to play in proteolysis during conditioning, therefore these three enzymes are discussed below.

Cathepsin B (MW 27 000), cathepsin L (MW 22 000) and cathepsin N (MW 20 000) are all cysteine proteinases (Etherington, 1981).

Cathepsin B is an endopeptidase that hydrolyzes protein substrates in the pH range 5 to 6. It cleaves myosin, at pH 5.2, into a major fragment of 150 000 daltons that seems resistant to further degradation and into a heterogeneous group of fragments of 10 000 and 50 000 daltons (Goll et al., 1983). Using extracted myosin as substrate at pH 5.5, cathepsin B exhibited 60 to 70 % of maximum activity and it was also found to degrade actin and

collagen (Etherington, 1981).

Cathepsin L, an endopeptidase, first described from rat liver lysosomes is widely distributed (Kirschke et al., 1977; Etherington, 1981). Cathepsin L is a potent proteolytic enzyme (Kirschke et al., 1980), exhibiting maximal activity in the pH 3 to 6.5 region, depending on the substrate. This enzyme has been shown to degrade collagen (Kirschke et al., 1982; Mason et al., 1982) and myofibrillar proteins including myosin, α - actinin, actin, troponin T, troponin I, titin and nebulin (Matsukura et al., 1981; Matsukura et al., 1984; Penny et al., 1984). Cathepsin L is estimated to have ten times greater specific activity against myosin than cathepsin B. It is capable of cleaving myosin heavy and light chains completely after 22 h but its activity against actin is slower (Goll et al., 1983). Cathepsin L also cleaves the fluorogenic end group from the peptide benzyloxycarbonyl-L-phenylalanyl-L-arginine-7-amino-(4)-methyl-coumaryl-amide (Z-Phe-Arg-NHMec), which is a sensitive synthetic substrate for routine assay. However, cathepsin B is also capable of cleaving this substrate (Barrett, 1980). Cathepsin B activity can be distinguished from cathepsin L by its ability to cleave benzyloxycarbonyl-L-arginyl-arginine-7-amino-(4)-methyl-coumaryl-amide (Z-Arg-Arg-NHMec), while a specific substrate for cathepsin L has not yet been found.

Although not detected in skeletal muscle, cathepsin N is capable of cleaving collagen (Evans & Etherington, 1978). In its action against collagen, cathepsin N was almost indistinguishable from cathepsin B (Etherington, 1980). The enzyme exhibits maximal activity at pH 3.0 to 4.5.

The lysosomal cysteine endopeptidases are probably the most active tissue proteinases responsible for intracellular degradation (Barrett & Kirschke, 1981). Various measurements have shown that intra-lysosomal pH is approximately 4.5 (Poole et al., 1978) so the acidic pH optimum of most lysosomal peptide hydrolases means that these hydrolases are at or near their optimum pH for activity inside the lysosome.

At the post-mortem limit pH of 5.5, lysosomal cathepsins, released into the extracellular space continue to degrade connective tissue during the conditioning period (Etherington, 1981).

1.7 Artifical Methods of Tenderizing Meat

Attempts to artificially tenderize meat have included beating the meat, cutting it into small portions so that the strands of connective tissue were severed, marinating it with vinegar,

wine or salt and enzymic tenderizing - employed at least 500 years ago by the Mexican Indians when they wrapped meat in pawpaw leaves during cooking. In more recent times such attempts have become more systematic.

Attempts to improve the tenderness of meat by the application of certain physical forces include the use of high pressure and mechanical tenderization using a blade device (see Ashgar & Pearson, 1980 for review). The exertion of high pressure on pre-rigor muscle increased tenderness of meat by disrupting the myofilaments, possibly by transformation of F-actin to G-actin with minimal changes in the sarcolemma, but this operation significantly increased cooking losses. Similarly, blade tenderization improved tenderness to a varying extent as applied to beef, to different muscles from the same carcass, to different muscles from different grades to beef, to different muscles from cows and bulls, to top loins from animals produced on different nutritional regimens and to ovine carcasses (Ashgar & Pearson, 1980). Mechanically tenderized meat has been associated with a consistent decrease in juiciness on using blade tenderization, and an increase in mealiness (Ashgar & Pearson, 1980). The connective tissue content determines the extent of tenderization treatment required. For example, the *biceps femoris* and *semitendinosus* muscles require two passes through the blade tenderizer in order to attain a similar degree of tenderness as the *psoas major, gluteus medius, l. dorsi* and *semimembranous* muscles derived from a single pass through the blade tenderizer. Blade tenderization has been associated with the length of the conditioning period (Ashgar & Pearson, 1980). One pass through the blade tenderizer was necessary for beef rounds conditioned for less than 10 days while tenderization was not required if the conditioning period was longer than 10 days. However, the use of blade tenderization has been recommended even after conditioning in order to minimize moisture losses. Conflicting results regarding the extent of cooking losses from mechanically tenderized meat have been presented. Jeremiah (1978) stated that mechanical tenderization offers considerable merit when applied to cuts with inherent toughness or on carcasses of advanced maturity, but excessive shrinkage and microbial contamination can be serious problems resulting from use of mechanical tenderizers.

Chemical tenderization techniques employed include the use of weak acids (vinegar, lemon juice) and salts to increase the water-holding-capacity (WHC) of meat and hence tenderness. Sodium chloride and other salts have a tenderizing action on meat and post-mortem perfusion of joints with salt solutions has been of some success in this context (Bouton & Howard, 1960). Hamm (1958) has extensively studied the effect of different ions on WHC and found that addition of 0.5 to 3.0 % NaCl was required to cause an increase in WHC, whereas low concentrations of NaH_2PO_4 and $Na_2H_2PO_7$ were effective

in increasing WHC. A number of other studies have also indicated that addition of NaCl to meat improved WHC (see Ashgar & Pearson, 1980 for review). Hamm (1974) stated that NaCl increases WHC by affecting the electrical charges of the protein, which prevents the combination of actin and myosin. Na^+ and Mg^{2+} cations are equally effective in increasing meat tenderness (Palladino & Ball, 1979), and have a greater effect that K^+ and Ca^{2+} ions. These workers also observed that the Cl^- anion had more effect upon tenderness than the other halogen anions. However, Khan & Kim (1975) reported that the addition of $CaCl_2$ to pre-rigor muscle accelerated glycolysis and the onset of rigor mortis, which resulted in tough meat, but its addition to post-rigor muscle increased tenderness. Injection of phosphate and metaphosphate or hexametaphosphate into pre-rigor muscle has been shown to produce tender meat. The tenderization effect was ascribed to the function of phosphate (PO_4^{3-}), hexametaphosphate $(PO_3^{3-})_6$ and pyrophosphate $(P_2O_7)^{4-}$ ions, which either chelate Ca^{2+} and/or Mg^{2+} ions or dissociate the actomyosin complex (Ashgar & Pearson, 1980).

Addition of enzymes to meat to accelerate ripening and upgrade poor quality cuts was introduced in 1949 and a variety of enzymes are now widely used (Whitaker, 1959; Solov'ev, 1966). Commercially used enzymes fall into 3 groups: those of bacterial or fungal origin, those of plant origin and those of animal origin. Enzymes in the first group include Protease 15, Rhozyme, subtilisin, pronase and hydrolase D, while the second group include papain, bromelain and ficin and the third group includes trypsin. Three methods of enzyme application are used. The first involves sprinkling a powdered preparation on the meat surface or immersing the meat in an enzyme solution. These methods are applicable only to small cuts and often result in uneven tenderization. The second method of application involves pre-slaughter intraveneous injection of oxidized papain. In the Proten process, developed by Swift & Company, Limited, oxidized papain at a level of 1.5 mg/lb live weight is injected about 30 min before slaughter, and the animals are slaughtered, dressed and chilled conventionally. The papain is reduced to the active forms by the reducing environment in the muscles, post-mortem. The conversion of the oxidized to the reduced form is slow in chilled meat but is completed rapidly on warming. An increase in tenderness in all carcass cuts was observed, but organs with a good blood supply such as heart, liver and tongue tend to disintegrate on cooking due to their very high papain content (Pritchard, 1971). The third method of enzyme application to meat involves post-mortem pre-rigor pumping of dressed carcasses, which is praticed in the USSR (Solov'ev, 1966). Post-mortem pre-rigor pumping is done either by intraveneous injection or by intramuscular injection using a series of needles. This method allows more rigorous control of enzyme distribution between tough and tender joints.

Papain, the major enzyme from papaya latex, is now widely used in the food industry (Caygill, 1979). The enzyme is a cysteinyl-proteinase which degrades both myofibrillar and connective tissue proteins. It has little action at ambient temperatures and its main action occurs during cooking of meat. The enzyme has a very high temperature optimum and considerable thermostability with proteolysis being most rapid near 50° C. Undenatured collagen is resistant to attack but above 50° C, the collagen structure begins to loosen and is then solubilized by papain, with maximum breakdown occurring in the range 60 to 65° C (Dransfield & Etherington, 1981). Other plant proteinases widely used as tenderizers include bromelain from pineapples and ficin from figs. These enzymes are very similar to papain but the availability and lower cost of papain makes this the preferred enzyme. Bromelain and ficin are thermostable and exhibit little action against undenatured collagen (Dransfield & Etherington, 1981).

1.8 MEAT QUALITY

The quality of a food may be defined as the composite of those characteristics which differentiate individual units of a product and have significance in determining the degree of acceptability of those characteristics by the consumer (Kramer & Twigg, 1962). Subjective quality evaluation involves the physiological, psychological, social and economic status of the consumer (Pilgrim, 1957) which is why quality and its components mean different things to different individuals. Evaluation of meat quality encompasses two main considerations. Firstly, the requirements of the meat trade must be met, and secondly, consumer preferences must be satisfied. The meat trade is concerned with carcass quality which is assessed on the basis of conformation, finish (fat status) and a combination of characteristics including colour of muscle and fat, marbling, texture of muscle and appearance of bone and cartilage as related to the species, sex and age of the animal (Ashgar & Pearson, 1980). Consumer preferences are concerned with meat quality, taking into account such factors as tenderness, juiciness, taste and aroma.

Of the attributes of eating quality, colour, water-holding capacity and some of the odour of meat are detected both before and after cooking, while juiciness, texture, tenderness, taste and most of the odour of meat are detected on mastication (Lawrie, 1985). Attempts to identify common standards of meat quality have been made between the member countries of the European Economic Community (Dransfield et al., 1984). The study involved the assessment of beef from the same animals by eight member countries. The results indicated that Irish and English participants tended to value flavour more highly than tenderness and juiciness while the latter attributes were more highly favoured by Italian

panellists. French and Belgian participants favoured the flavour of aged beef from older animals. A standard scale or cooking procedure could not be recommended for the eight participating countries, however, the assessment of texture was consistent and comparable. Texture describes the tissue structure of a substance. Szczesniak (1963) and Sherman (1970) have proposed the following definition:

> *'Texture is the composite of those properties which arise from the physical structural elements and the manner in which it registers with the physiological senses'.*

The term, texture, therefore, encompasses all properties of foods which are perceived by the kinesthetic and tactile senses of the mouth, including tenderness, density, granular structure, fragility and humidity. Tenderness is registered by the ease with which the teeth and jaw muscles perform their task of mastication. Simultaneously, an impression of juiciness is also experienced, which depends on the amount of liquid released from the meat together with the amount of saliva added to it, the latter being adjusted to make up for any deficiency of juice in the meat (Ashgar & Pearson, 1980). During the mastication process, the gustatory papillae, stimulated by the juice, receive the impulse of taste. The perception of flavour originates from the combined perceptions of taste and odour assessed by the gustatory papillae and nasal mucous membrane, respectively (Ashgar & Pearson, 1980). These stimuli are transmitted by nerves to the higher brain centres (Amerine et al., 1965; Amoore, 1967; Ashgar & Pearson, 1980) at which level the quality criteria for tenderness, juiciness and flavour are registered. Tenderness is the first and foremost quality sought in meat. It is the main criterion for judging meat quality and can improve the remaining quality characteristics. Flavour and juiciness are less variable qualities and can be compensated for, by use of gravies, seasonings and vegetables (Ashgar & Pearson, 1980).

Meat quality is often subjectively evaluated by a panel of judges, who may range from a very highly trained and competent group to a household type without previous training (Amerine et al., 1965). Frequently, a score card utilizing the hedonic scale, where each quality criterion is described on an arbitrary numerical scale, is used by the panel. The ability of the judges to differentiate correctly for various attributes of quality can be ascertained by several statistical methods (Weir, 1959; Amerine et al., 1965).

1.8.1 Factors Affecting Meat Quality

The three main components of meat which may be expected to affect the textural quality of

meat are water content (and water-binding), the contractile apparatus and the connective tissue.

1.8.1.1 Water Content

The WHC of meat affects the appearance of meat before cooking, its behaviour during cooking and juiciness on mastication. Diminution of the *in vivo* WHC is manifested by exudation of fluid known as 'weep' in uncooked meat which has not been frozen, as 'drip' in thawed uncooked meat and as 'shrink' in cooked meats, where it is derived from both aqueous and fatty sources (Lawrie, 1985).

Striated muscle from meat animals contains about 75 % water, which varies inversely with the fat content (Hamm, 1975). Water has a significant impact on certain quality characteristics of meat as a result of water-protein interactions (Fennema, 1973; Kinsella, 1976; Ashgar & Pearson, 1980).

The relationships between WHC and tenderness and juiciness of meat are matters of controversy (see Ashgar & Pearson, 1980 for review). Correlation coefficients for free water and tenderness were negative for the *longissimus dorsi* and positive for the *biceps femoris* muscle (Ritchey & Hostetler, 1964) who concluded that the influence of water, either free or bound, may be different in various muscle. Both in beef and pork the *longissimus dorsi* had a lower WHC than the *psoas* muscle (Hamm, 1960). This was so, even when the rates and extent of pH decline were identical, suggesting the presence of different types of proteins (Lawrie, 1985).

1.8.1.2 The Contractile Apparatus

Ample evidence is available in the literature showing that changes in the myofibrillar component pre-rigor can markedly influence the tenderness of the resulting meat.

Meat cooked soon after slaughter, before the onset of rigor mortis, is usually tender, but toughness increases as rigor mortis develops, resulting from interlocking of the myofibrillar proteins actin and myosin together in the actomyosin complex (Ashgar & Pearson, 1980). Pre-rigor muscle is plastic and highly extensible, and becomes firm and relatively inextensible in the full rigor state. In addition to loss of extensibility, unrestrained muscle shortens during the onset of rigor mortis. Locker (1960) concluded that there was a relationship between post-mortem shortening and tenderness. The increase in toughness due to shortening is caused by changes in the myofibrillar structure

(Marsh & Leet, 1966), while the contribution of the connective tissue to the increase in toughness observed in cold-shortened muscles is likely to be small compared to that of the myofibrillar porteins but can be minimized by careful handling (Sims & Bailey, 1981).

Methods of preventing myofibrillar toughness caused by muscle shortening, resulting from excessive cross-bridging of the actomyosin complex include allowing rigor mortis to develop at a temperature at which post-mortem shortening is minimal and by physically restraining the muscle from shortening (section 1.5.2.1).

1.8.1.3 Connective Tissue

A considerable amount of research work on the contribution of the connective tissue component to meat toughness has been carried out (see for example, Bailey, 1972; Light et al., 1985; Light, 1987). Stretching muscle and allowing to to go into rigor mortis in this condition has little effect on tenderness (Herring et al., 1967). The toughness of unshortened muscle has been referred to as 'background' toughness (Bailey, 1972) and is believed to represent the situation where the contribution of the connective tissue to toughness is predominant.

1.8.1.3.1 Total Collagen Content

Evidence in the literature for a correlation between total collagen content and meat toughness is contradictory. Many studies including Rea et al. (1970), Smith et al. (1970) and Cross et al. (1973) support the role of connective tissue collagen in meat toughness while other groups suggested that the collagen content of meat does not correlate with textural quality (McClain et al., 1965; Herring et al., 1967; Field, 1968; Hunsley et al., 1971).

More recent studies (Dransfield, 1977) indicated a good correlation between collagen content and the mechanical toughness of a variety of beef muscles, cooked at different temperatures. Bailey et al. (1982) showed that muscles, and especially double-muscles from Charolais bulls had less collagen and were more tender than controls from other breeds. Similarly, steaks obtained from intact male Angus and Simmental cattle had less collagen and were more tender than similar steaks from castrates, while meat obtained from animals fed on a low-energy diet contained less total collagen and was more tender than that of controls (Crouse et al., 1985). A variety of beef muscles of known textural quality indicated a correlation between total collagen content and eating quality (Light et al., 1984; 1985; Light 1987). In general, high quality muscles yielded low collagen

contents while intermediate and poor quality muscles yielded higher collagen contents.

1.8.1.3.2 Collagen Solubility

Collagen solubility in meat, as assessed by extraction with salt, acid and alkali, has been positively correlated with textural quality (Light, 1987). Collagen solubility in meat decreased with physiological age (Hill, 1966), whilst the meat steadily became tougher. Cross et al. (1973) reported that both collagen and elastin content and solubility within bovine muscles were the major factors determining tenderness. The correlation between collagen solubility and textural quality has been refuted by other workers, including Rea et al. (1970), Smith et al. (1970), Stewart et al. (1974) and Jeremiah & Murray, (1984).

1.8.1.3.3 Collagen Cross-Links

Collagen solubility is directly related to its cross-linking. Goll et al. (1964) suggested that differences in the structure of the collagen molecule, rather than total amount of collagen accounts for much of the variation in tenderness. This was substantiated by a number of other studies including Kruggel et al. (1970) and Kruggel & Field; (1971). Pfeiffer et al. (1972) found a closer relationship between tenderness and the number of cross-links within and between collagen fibres than between tenderness and total collagen content or collagen solubility. Immature aldimine and oxo-imine cross-links are converted to stable 'mature' cross -links with increasing age (Shimokomaki et al. 1972; Sims & Bailey, 1981), which render the collagen extremely insoluble (Light, 1987 and section 1.4.5). Therefore, it is the type of cross-link present in the collagen matrix which determines collagen solubility and has a significant influence on the tenderness of meat.

Intramuscular collagen contains both aldimine and oxo-imine cross-links in proportions similar to those present in tendon (Shimokomaki et al. 1972). However, the content of the aldimine cross-link is unlikely to influence the strength of the collagen matrix as this unsaturated bond is heat-labile and would be expected to be cleaved at cooking temperatures (Light, 1987). Consequently, the heat-stable oxo-imine cross-link is considered more likely to influence collagen insolubility and texture of meat.

Tension developed in the collagen matrix on shrinkage was due to the presence of the heat-stable oxo-imine cross-links (Bailey & Sims, 1977). Good quality muscles were found to contain the lowest number of oxo-imine cross-links, in a study of six beef muscles of varying quality, in both perimysium and endomysium (Light, 1987). In addition, a good correlation was found between heat-stable oxo-imine cross-link content and compressive

force developed after cooking (Dransfield, 1977; Light et al., 1985).

However, all beef animals are slaughtered at greater than one year of age and the oxo-imine and aldimine cross-links are being actively replaced by the stable 'mature' cross-links, at this stage (Light, 1987). These 'mature' cross-links are heat-stable and account for the polymeric nature of collagen in older and mature tissues (Shimokomaki et al., 1982; Light, 1987), and the impact of this cross-link on meat texture must be expected to be great.

1.8.1.3.4 Collagen Types

It has been suggested that a correlation exists between type III collagen content and meat toughness (Bailey et al. 1979). The work of Deethardt & Tuma (1971) who showed that reticulin fibres, which may be type III collagen (Nowack et al., 1976) showed greater resistance to cooking than other fibres and that of Wu et al. (1982) who showed that type III collagen was more resistant to proteolytic attack during conditioning than type I collagen, indicates a role for type III collagen in meat toughness. However, subsequent studies failed to confirm a relationship between the ratio of type I and type III collagen in meat and toughness (Light et al., 1984; 1985), although the total amount of type III collagen was correlated to some extent with quality aspects (Light, 1987). It was found that high quality muscles contained significantly lower quantities of type III collagen than intermediate and poor quality muscles (Light, 1987).

1.8.1.3.5 Collagen Fibre Size

Collagen fibre diameter increased with increasing physiological age (Parry & Craig, 1979), whilst collagen strength and resistance to attack by proteases similarly increased. Differences in fibre diameter were found to exist between perimysial collagen fibres isolated from bovine muscles of varying quality (Light, 1987). Collagen fibres isolated from high quality muscles were found to have smaller diameters than some poor quality muscles investigated. It is known that type III collagen fibres are often of a smaller diameter than type I fibres, as are type II fibres (Bailey & Etherington, 1980). However, the issue of a correlation between small fibre diameter and toughness of meat is not resolved at this time.

In addition to the factors mentioned above ageing and glycosylation may have effects on the contribution of collagen to meat toughness. Collagen content does not increase in muscle during physiological ageing, however, the formation of 'mature' stable collagen

cross-links, with age (Robins et al., 1973) and the increase in overall collagen fibre size with age (Parry & Craig, 1979) indicate that collagen must be a determinant in increased meat toughness from older animals (Light, 1987). During normal ageing many low-turnover proteins become randomly glycosylated through Schiff-base reaction of glucose with ε-amino groups resulting in formation of glucose-mediated intermolecular co-valent cross-links in the collagen matrix. Random glycosylation occurs in collagen, resulting in increased tensile strength of the collagen matrix during heating (Light, 1987).

1.9 AIMS AND OBJECTIVES

The work carried out in this thesis was aimed at investigating the effects of conditioning on the major meat connective tissue protein collagen.

Investigation of the effects of conditioning on the solubility of isolated intramuscular connective tissue fractions and connective tissue collagen has been carried out. The insoluble perimysial and endomysial residues, remaining after conditioning, have been analyzed for evidence of proteolytic 'clips' resulting from conditioning and in a model conditioning system by electrophoretic methods.

Finally, the potential of a procedure for accelerating the conditioning process was investigated for its ability to degrade the connective tissue fraction of meat and for its potential as a meat tenderizing method.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

All organic solvents used for HPLC were HPLC grade from Rathburn Chemicals, Walkerburn, Scotland. Helium used for solvent purging was obtained from Gas and Equipment, Shropshire, U.K.

The chemicals used for sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis were Analar grade or specially purified for electrophoresis (SDS), obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and included acrylamide, NN'-methylene bisacrylamide, ammoniuum persulfate, NNN'N'-tetramethyl ethylene-diamine (TEMED), tris (hydroxymethyl) methylamine (tris), glycine, acetic acid and methanol. Coomassie Brilliant Blue R250 was obtained from Sigma Chemicals, Poole, Dorset, U.K. All other chemicals were Analytical grade reagents and distilled water was used throughout. pH was measured using a pH-meter CG 822 (Camlab Ltd., Cambridge, U.K.), fitted with glass electrodes, and standardized with pH 7.00 ± 0.02 and 4.00 ± 0.02 buffer solutions at 20° C (BDH Chemicals Ltd., Poole, U.K.)

2.2 EQUIPMENT

HPLC was carried out on a Gilson System (Gilson Medical Electronics, Inc. WI, USA). The system consisted of (1) two model 302 Pumps, (2) a model 121 fluorometer; excitation filter 310 nm to 410 nm, emission filter 480 nm to 520 nm, (3) a uv variable wavelength spectrophotometer (4) a model 802 C manometric module (5) a temperature controlled column compartment (6) an Apple Computer (7) a system controller (8) a TRIO chromatography integrator (TriVector Systems International Ltd. Bedfordshire, U.K.) and (9) an Epson LX-80 printer. An auto-sampling injector (Gilson Model 231-401) was fitted to the system and HPLC System Manager Model 704 Version 2.0 (Gilson) Software was used.

SDS polyacrylamide gel electrophoresis was carried out on the LKB 2001 Vertical Slab Gel Electrophoresis Unit, connected to the LKB 2301 Macrodrive 1 Power Supply (LKB, South Croyden, U.K.)

Other equipment used in the preparation and analysis of samples is described in the relevant sections.

Methods which were used routinely throughout the work described in this thesis, or which occur in more than one chapter are described below.

2.3 PREPARATION OF BOVINE MUSCLES

Slaughter and handling of carcasses was carried out by staff of the Carcass Section at AFRC Institute of Food Research (Bristol Laboratory). Bovine carcasses were split immediately after slaughter and both sides were hot de-boned. Chosen muscles listed below (representing high, medium and low quality meat) were dissected out and vacuum packed. Muscles from one side of the carcass were blast frozen immediately; these were termed unconditioned muscles, while the corresponding muscles from the other side of the carcass were held at chill temperature (1° C) for a prescribed time (14 days to 21 days) and subsequently blast frozen and stored at -20° C; these were termed conditioned muscles. Storage at -20° C did not exceed 3 weeks for unconditioned samples or 1 week for conditioned samples. The muscles selected at the outset of this research were; *psoas major, pectoralis profundus, gluteus medius, gastrocnemius, sternomandibularis, serratus ventralis, supraspinatus* and *extensor capri radialus.*

2.4 PREPARATION OF CONNECTIVE TISSUE FRACTIONS

2.4.1 Preparation and Homogenization of Muscle Samples

Bovine connective tissue fractions (perimysium and endomysium) were prepared by a modification of the method of Light & Champion (1984) as described by Stanton & Light (1988).

After dissection of the epimysium, meat samples (25 to 50 g) were cut into 1 cm cubes and homogenized in 100 ml of ice-cold 0.05 M $CaCl_2$ for 10 sec at full speed in a Waring Blendor. The homogenate was filtered through a graded copper grid with 1 mm square holes. The material not passing through the filter was re-homogenized in a further 100 ml ice-cold 0.05 M $CaCl_2$ and re-filtered. This process was repeated a further two times, at which point all the filtrates were combined and the gross insoluble connective tissue was retained. The filtrate was centrifuged (25 000 g x 30 min) and the supernatant discarded. The pellet was referred to as the crude endomysial fraction, while the gross insoluble connective tissue was referred to as the crude perimysial fraction.

2.4.2 Purification of Perimysium

Various solvents were assessed in terms of their usefulness in purifying muscle connective tissue as described in Chapter 3. The following was the method of choice. The crude perimysial fraction (from Section 2.4.1) was stirred in 50 ml 6 M urea, 0.05 M tris-Cl, pH 7.4 for 30 min at room temperature on a magnetic stirrer, and subsequently centrifuged at 2 500 g x 10 min. The supernatant was saved and a further 50 ml of buffered urea solution was added to the aggregated insoluble fraction and the extraction procedure repeated twice, with the insoluble material being collected by centrifugation between each wash. The supernatants were combined (denoted the soluble perimysial fraction), and the insoluble material remaining following extraction in 6 M urea, 0.05 M tris-Cl, pH 7.4 was denoted the insoluble perimysial fraction. Soluble and insoluble perimysial fractions were dialyzed for 48 h against running tap water and against two changes of distilled water for a further 48 h. All fractions were immediately frozen following dialysis and subsequently freeze-dried, if necessary, e.g. for total dry weight estimations.

2.5 PURIFICATION OF TYPE I COLLAGEN

Rat tail tendons were finely chopped and dispersed and stirred in 0.5 M CH_3COOH. The dispersion was homogenized at 4° C, and centrifuged at 15 000 g x 20 mins. Precipitation of collagen was effected by making the supernatant 0.7 M in NaCl and precipitated collagen collected by centrifugation. The pellet was water-washed, dialyzed against distilled water and freeze-dried.

2.6 LYOPHILIZING

Frozen samples were lyophilized using a Christ Alpha lyophilizer fitted with an RD4 vacUbrand pump for 2 to 3 days, or until the material was completely reduced to a dry powder.

2.7 CNBr DIGESTION

CNBr cleaves proteins selectively at methionine residues thus, with its limited number of methionines, collagen gives a simple set of peptides readily resolved by SDS-polyacrylamide gel electrophoresis. CNBr digestion of samples was carried out as previously described (Light & Bailey, 1979). Samples were dissolved to a final concentration of 10 mg/ml in 70 % (v/v) formic acid and an equal weight of CNBr dissolved in acetonitrile (2 g/ml) was added. Incubation was carried out at 30° C for 4 h,

at which time samples were rotary-evaporated in a vacuum rotary evaporator (type 349/2, Bibby) after 10-fold dilution and lyophilized.

2.8 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Native collagens were separated using 7.5 % (w/v) polyacrylamide linear resolving gels and CNBr-cleavage peptides were separated on 10 % (w/v) polyacrylamide linear resolving gels as previously described (Light, 1982) by the method of Laemmli (1970). These methods are described in detail below.

2.8.1 Assembly of Electrophoresis Unit

Glass plates (16 x 19 cm) were washed thoroughly with distilled water followed by ethanol and then dried. The plates were separated from each other by placing a pair of spacers (1 mm thick) along either side. These were held in place by a set of clamps which also acted to waterproof the sides. The whole unit was placed onto a casting stand whereby the bottom of the glass plates were sealed. The acrylamide gel mixture could then be poured from the top without any leakage occuring from the other three sides.

2.8.2 Sample Preparation

Lyophilized samples for SDS-polyacrylamide gel electrophoresis were dissolved in a protein disaggregating buffer (gel sample buffer) of the following composition: 2 % (w/v) SDS, 0.125 M tris-HCl, pH 6.8, 10 % (v/v) glycerol and 0.05 % (w/v) bromophenol blue marker dye. This was stored as a stock solution at room temperature. Samples were dissolved in gel sample buffer to a final concentration of 5 mg/ml. Dissolution was generally aided by heating the sample in a boiling water bath for 2 min. Samples treated in this way were either used immediately in the electrophoretic procedures described below or were stored prior to use at -20° C. On all gels used for analyzing collagen a sample of CNBr digested rat tail tendon collagen was used as the standard marker.

2.8.3 Preparation of Resolving Gels

Polyacrylamide gels were formed by polymerizing monomeric acrylamide with a cross-linking co-monomer, N N'-methylene-bis-acrylamide in the presence of free radicals provided by the chemical initiator ammonium persulphate. The proportion of the two monomers, acrylamide and N,N'-methylene-bis-acrylamide, and not their total concentration is the major factor in determining gel pore size, the latter having more effect

on the elasticity and transparency of the gel. Finally, the polymerization reaction is controlled through the addition of, usually, an equimolar amount of TEMED which provided a source of tertiary amines.

The procedure of gel preparation was as follows: stock acrylamide (29.2 % acrylamide/ 0.8 % N N'-methylenebis-acrylamide) was diluted to the required concentration, usually 10 % (w/v), prior to polymerization in 0.375 M tris-Cl , pH 8.8, containing 0.1 % (w/v) SDS. 50 µl/100 ml stock acrylamide of TEMED was added. The solution was stirred gently taking care not to introduce air into the solution as oxygen inhibits the polymerization process. Polymerization of the acrylamide was initiated by the addition of 50 mg/100 ml of stock acrylamide of ammonium persulphate. As soon as the ammonium persulphate had dissolved, the gel mixture was quickly but carefully applied between the prepared glass plates to a height of about 2 cm from the top using a long Pasteur pipette ensuring that no air bubbles were formed. Water was gently placed on the top of the gel and it was left to set (usually about 1 h). As soon as polymerization was complete, the water overlay was poured off. Excess water was removed by gently applying a sheet of filter paper to the surface of the gel and absorbing any remaining liquid.

Stacking gel was prepared as follows: stock acrylamide (29.2 % acrylamide/0.8 % N N'-methylenebis-acrylamide) was diluted to 5 % (w/v) prior to polymerization in 0.2 M tris-Cl, pH 6.8 containing 0.1 % (w/v) SDS. 25 µl/100 ml stock acrylamide of TEMED was added. The solution was stirred gently and polymerization of the acrylamide was initiated by the addition of 50 mg/100 ml of acrylamide of ammonium persulphate. The stacking gel was carefully poured onto the top of the resolving gel using a long Pasteur pipette. A 10 or 20 well comb was immediately inserted and the gel allowed to set for at least 30 min. At this stage, the gels were ready for use or could be stored by keeping them moist wrapped in cling film at 4° C for 2 to 3 days. The comb was carefully removed from the polymerized stacking gel and overlaid with distilled water. 30 - 50 µl of the prepared sample (5 mg/ml) was placed in the appropriate well, and electrode buffer (see below) was carefully overlaid on to the samples.

Analytical gels were electrophoresed in an apparatus which could hold 2 slab gels. The upper (anodal) buffer chamber was attached to the top of the glass plates and the entire assembly, together with the applied samples, was introduced into the electrophoretic unit. Both the upper and lower (cathodal) chambers were filled with electrode buffer (0.05 M tris-glycine (6 g tris, 28.8 g glycine per litre), pH 6.8, containing 0.1 % (w/v) SDS) and connected to a power supply. Electrophoresis was carried out at 100 to 200 V. The course of the electrophoresis could be followed by means of the tracking dye,

Bromophenol Blue, and was stopped when the tracking dye was observed to be approximately 0.5 cm from the bottom of the gel (2.5 to 3 h). The electrode buffer was cooled by passing cold running tap water through the heat exchanger during the course of the electrophoresis run. At the end of electrophoresis the glass plates were removed from the electrophoresis unit, and the resolving gel carefully separated from them. Gels were then stained as described below.

2.8.4 Staining and Destaining Procedure

Gels were placed in a solution of Coomassie Brilliant Blue R250 dissolved to 0.1 % (w/v) in 5:1:5 (v/v) methanol: acetic acid: water, at room temperature. After 1 h the gels were removed from the stain, rinsed in water and re-immersed in destain solution (7.5 % (v/v) acetic acid containing 5 % (v/v) methanol). Gels were left to destain in several changes of this solution at room temperature however, the destaining process could be accelerated at 37° C.

2.9 DETERMINATION OF COLLAGEN

Collagen has an unusual amino acid composition which includes hydroxyproline. Methods for quantification of collagen in tissues depend upon the determination of the protein-bound hydroxyproline. A factor is then applied to transform this figure to yield the collagen content. Since there is considerable variation in the hydroxyproline content of collagen from different species (Gross, 1963), each requires a different conversion factor, the accuracy of which depends on the purity of the collagen on which the hydroxyproline content was based. A value of 14.4 % hydroxyproline has been assigned to bovine collagen (Jackson & Cleary 1967). The method used for collagen quantitation was based on determination of hydroxyproline by HPLC analysis of the dansyl-derivatized amino acid from collagen hydrolyzates (Light & Champion, 1984).

2.9.1 Acid Hydrolysis

10 mg lyophilized material was added to 4 ml 6 M HCl and incubated at 105-110° C in a Dry-Block DB-3 (Techne, Cambridge, U. K.) for 16 to 24 h (Light, 1985)) in sealed glass tubes. The hydrochloric acid concentration was reduced in the hydrolyzate by rotary evaporation at 55° C three times from 25 ml HPLC grade water. The residue was then dissolved in 1 ml of the HPLC Buffer B (see below) and transferred to microfuge tubes.

2.9.2 Dansylation

50 µl of the hydrolyzate in HPLC Buffer B (see below) was mixed with 200 µl 0.5 M lithium borate buffer, pH 9.6 and 50 µl dansyl chloride in acetonitrile (6 mg/ml) in a 1.5 ml microfuge tube wrapped in foil. The mixture was incubated for 1 h at 40° C. The dansylated material (300 µl) was transferred to a 5 ml volumetric flask, mixed with 3 ml of HPLC Buffer A (see below) and adjusted to pH 6.2 by careful addition of 0.1 M HCl. The final volume was adjusted to 5 ml with HPLC Buffer A (see below). Samples were centrifuged at 9 000 g for 1 min in an Anderman Eppendorf Centrifuge 541 S. Sample volumes of 20 to 100 µl were commonly used.

Stock Amino Acid Standard Solution for collagen hydrolyzates (Sigma Chemicals, St. Louis, U. S. A.) was diluted to 1 mg/ ml. 25 µl aliquots of this solution were lyophilized. The lyophilized material was dissolved thoroughly in 150 µl of 0.5 M lithium borate buffer, pH 9.6 at 25° C and 50 µl of dansyl chloride in acetonitrile (10 mg/ ml) was added to the solution in a 1.5 ml microfuge tube wrapped in foil. The mixture was incubated for 2 h at 40° C. The dansylated material (200 µl) was lyophilized and was used immediately or stored at -20° C. Standard samples were readily dissolved by addition of 100 µl Buffer A (see below) and 100 µl acetonitrile and vortexed thoroughly. The solution was centrifuged at 9,000 g for 1 min before use. 3.5 nmoles hydroxyproline in a sample volume of 100 µl or less was injected into the HPLC system.

2.9.3 High Performance Liquid Chromatography (HPLC)

Hydroxyproline was analyzed using a reverse phase high performance liquid chromatographic procedure modified from that of Light & Champion (1984). The terms 'starting solvent' and 'eluting solvent' refer to the practice in gradient chromatography of using two solvents. The starting solvent (denoted solvent A in this thesis) refers to the weaker solvent which in reverse phase chromatography is the aqueous based solvent. Solvent strength in reverse phase chromatography increases with decreasing polarity therefore the 'eluting solvent' (denoted solvent B in this thesis) in this system is the organic solvent. The gradient is developed by increasing the amount of 'eluting solvent' flowing through the column.

Solvents were prepared using HPLC grade water. Aqueous based solvents were filtered prior to use through 0.45 µm pore size aqueous Millipore filters, and organic based solvents were filtered using 0.45 µm pore size Millipore filters for organic solvents. The solvents were degassed by helium purging before use.

The columns were maintained in a temperature controlled column compartment, the temperature of which could be controlled from 0 to 100° C.

The composition of solvent A for hydroxyproline analysis was as follows :- 0.02 M sodium phosphate buffer, pH 6.2 containing acetonitrile (5 %, v/v). Solvent B was 95 % (v/v) acetonitrile containing 5 % 0.02 M sodium phosphate buffer, pH 6.2. The dansylated amino acids were eluted on a 15 x 0.46 cm column of Rainin 5 μ C18 reverse phase material at 20° C, pH 6.2 with a flow rate of 1.5 ml/min. A gradient of solvent B was used to elute the dansylated amino acids as follows: 15 % solvent B for 5 min, 15 to 40 % solvent B for 8 min, 40 to 50 % solvent B for 7 min, 50 to 70 % solvent B for 5 min, 70 to 100 % solvent B for 5 min. The column was regenerated by washing with 100 % solvent B for 2 min and 100 to 15 % solvent B for 5 min. Detection was by optical density at 254 nm.

2.9.4 Calculation of Hydroxyproline

The areas under the peaks on the chromatograms recorded during the analyses were measured and integrated using a TRIO Chromatography Integrator. Hydroxyproline in the collagen hydrolyzate was calculated from the area of the hydroxyproline peak, using as reference the area of the hydroxyproline peak in a amino acid standard solution. (Sigma Chemicals, Poole, Dorset, U.K.) Hydroxyproline was calculated using the following equation:-

$$(HYP)\ Sample = \frac{Area\ of\ HYP(Sample)}{Area\ of\ HYP(Reference)} \times (HYP)\ Reference$$

A conversion factor of 7.14 from hydroxyproline to collagen was used (assuming an average hydroxyproline content of 14 % in the meat collagens).

CHAPTER 3

CHANGES IN GROSS SOLUBILIZED MATERIAL AND PERIMYSIAL COLLAGEN AFTER CONDITIONING

3.1 INTRODUCTION

The work outlined in Chapter 3 was concerned with investigation of the effects of conditioning on the solubility and biochemical status of gross connective tissue and perimysial connective tissue collagen, derived from bovine muscle. Purification of connective tissue is a pre-requisite to further biochemical analysis.

Initially, SDS, an extractant which was used successfully by Laurent et al. (1981) and Barnard et al. (1987) for purification of collagen, was employed for bulk extraction of bovine connective tissue collagen. This method was designed to isolate combined perimysial and endomysial connective tissue from the bulk of muscle protein. Although clean insoluble collagenous preparations were obtained, which were suitable for analysis, the method proved unsuitable for assessment of the effects of conditioning on solubility of intramuscular collagen. The fact that SDS binds to proteins and that it is an extremely powerful extractant, meant that it succeeded in producing such high yields of solubilized material that the quantification of the collagenous component, which was proportionately relatively low, was impossible.

Perimysium comprises the bulk of intramuscular connective tissue, therefore, the effects of the conditioning process would be expected to be evident in the isolated perimysial collagenous fraction. In this regard, an effort was made to 'dissect' perimysial connective tissue from the remainder of intramuscular connective tissue. Initially, a study was conducted to investigate the effectiveness of a series of extracting agents for their usefulness in isolating perimysial material from bovine muscle. The use of SDS in this regard again highlighted the problem of accurate quantification of connective tissue yields, due to the fact that it binds to proteins. However, this reagent was the most successful employed for extraction of solubilized perimysial collagen. Other reagents employed were successful in extracting solubilized perimysial material and solubilized perimysial collagen. Acidic reagents were unsuitable for the purpose of this study, due to the presence of weak acid-labile peptide bonds and labile aldimine cross-links (Light & Bailey, 1979), which may be cleaved by such solvents. The possibility of random cleavages was avoided by employing a neutral pH solution of urea which proved effective for yielding clean

collagenous preparations and for investigation of conditioning effects on solubilization of perimysial collagen.

3.2 MATERIALS AND METHODS

3.2.1 Collagen Content of Soluble and Insoluble Fractions obtained from Unconditioned and Conditioned Muscles by SDS Extraction.

Unconditioned and conditioned muscles were prepared as outlined in Chapter 2. The muscles selected for this study were: *serratus ventralis, gastrocnemius, psoas major, pectoralis profundus, sternomandibularis, gluteus medius, extensor capri radialus and supraspinatus.*

The epimysium was dissected from each muscle and intramuscular connective tissue was extracted as outlined below and summarized in Fig. 3.1 The muscle was sampled such that 2 x 25 g samples obtained from different locations within the muscle were taken. These were cut into 1 cm cubes and each 25 g sample was homogenized in 100 ml 0.05 M tris-Cl, pH 7.4 for 20 sec at full speed in a Waring Blendor. SDS was added to the slurry to a final concentration of 1 % (w/v) (Barnard et al., 1987) and the mixture was stirred for 1 h at 20° C, after which it was centrifuged at 25 000 g x 20 min. The supernatant was saved. The pellet was re-homogenized in 0.05 M tris-Cl, pH 7.4 for 20 sec at full speed in a Waring Blendor. SDS was added to a final concentration of 1 % (w/v) and the mixture was stirred for 1 h after which it was centrifuged as described above. The supernatants were pooled and denoted the soluble fraction and the pellet was denoted the insoluble fraction. All fractions were dialyzed for 48 h against running water and against two changes of distilled water for a further 48 h. Soluble and insoluble fractions were immediately frozen following dialysis and subsequently freeze-dried if necessary, e.g. for total dry weight estimation.

3.2.2 Extraction and Purification of Perimysial Meat Connective Tissue

Seven reagents were used to extract perimysial collagen from a sample of conditioned meat. These were, 1 M NaCl, 0.02 M Na_3PO_4 , pH 7.4; 0.5 M CH_3COOH; 0.5 M CH_3COOH containing 4 M urea; 6 M urea, 0.05 M tris-Cl, pH 7.4; 1 M $CaCl_2$, 0.05 M tris-C1, pH 7.4; 1 % (w/v) SDS; 1 % (v/v) Triton X-100. These reagents were compared in their ability to extract collagen from muscle connective tissue and for obtaining a clean collagen preparation, i.e. for washing.

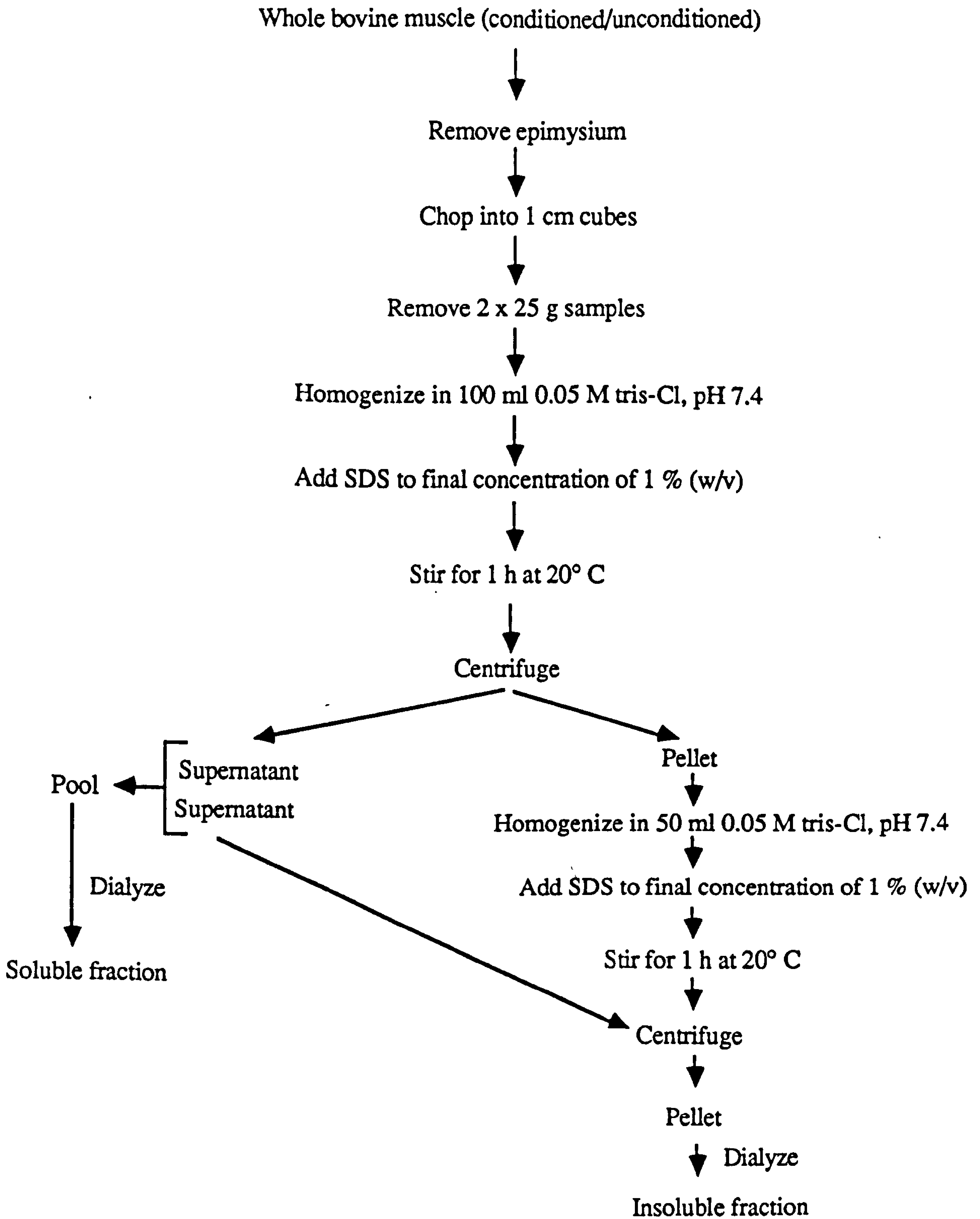


Fig. 3.1 Schematic Representation of Extraction of Gross Solublized and Insoluble Collagen Using SDS

Crude perimysium was prepared from uncooked, conditioned *gastrocnemius* muscle by the methods described below. 150 g of meat was homogenized in 100 ml ice-cooled 0.05 M $CaCl_2$ for 10 sec at full speed in a Waring Blendor. The homogenate was filtered through a graded copper grid with 1 mm square holes. The insoluble material not passing through the filter was re-homogenized in the same solvent and re-filtered. This process was repeated a further two times. All filtrates were combined, and the gross insoluble connective tissue was retained. This latter fraction was referred to as the perimysial fraction and the filtered material was denoted the endomysial fraction, which was further purified and analyzed (see Chapter 4).

Press-dried perimysial fractions were divided into seven sub-fractions. Each was extracted at 20° C with the reagents described before separating soluble from insoluble fractions by centrifugation. The soluble and insoluble material was dialyzed for 48 h against running water and against two changes of distilled water for a further 48 h. All fractions were immediately frozen following dialysis and subsequently freeze-dried if necessary, e.g. for total dry weight estimation.

3.2.3 Collagen Content of Soluble and Insoluble Perimysium obtained from Unconditioned and Conditioned Muscles by Urea Extraction

Unconditioned and conditioned muscles were prepared as outlined in Chapter 2. The muscles selected were: *serratus ventralis*; *gastrocnemius; psoas major; pectoralis profundis; sternomandibularis; gluteus medius;extensor capri radialus;* and *supraspinatus*.

The epimysium was dissected from each muscle and the intra-muscular connective tissue of each muscle was then extracted as follows (see Fig. 3.2). The muscle was sampled such that 3 x 25 g samples representing the general aspects of the whole muscle were taken. These were cut into 1 cm cubes, and each 25 g sample was homogenized and filtered as described above. The perimysial fraction was stirred in 50 ml 6 M urea buffered at pH 7.4 with 0.05 M tris-Cl for 30 min on a magnetic stirrer, then centrifuged at 2 500 g x 10 min. The supernatant was saved and a further 50 ml of buffered urea was added to the aggregated insoluble fraction and the extraction procedure repeated twice. All supernatants were combined, representing the soluble perimysial fraction, the insoluble material remaining following extraction in buffered 6 M urea represented the insoluble perimysial fraction.

Soluble and insoluble perimysial fractions were dialyzed as described above. The insoluble perimysial fraction was blot dried on Whatman 3 mm chromatography paper and

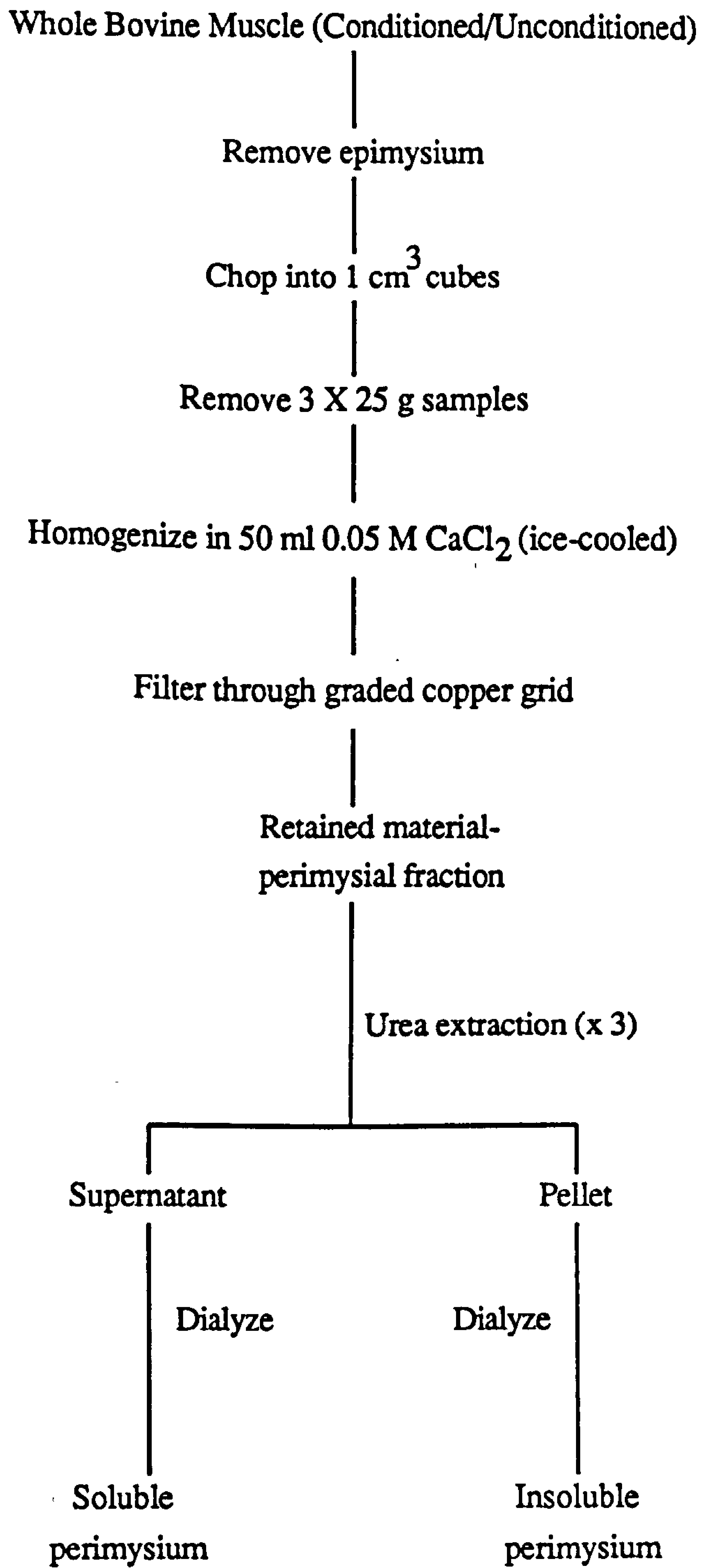


Fig. 3.2 Schematic Representation of Extraction of Perimysial Connective Tissue Using 6 M urea, 0.05 M tris-Cl, pH 7.4.

weighed. This represented the wet weight of the insoluble perimysium.

Samples of soluble and insoluble connective tissue components from conditioned and unconditioned muscles were compared for total collagen content and by SDS-polyacrylamide gel electrophoresis.

3.2.4 CNBr Digestion and SDS-Polyacrylamide Gel Electrophoresis

CNBr digestion of urea-washed perimysial and endomysial fractions was carried out in 70 % (v/v) formic acid at 30° C as outlined in section 2.7. SDS-polyacrylamide gel electrophoresis was carried out on whole collagen chains in soluble fractions and CNBr peptides of extracted insoluble perimysium and endomysium as outlined in section 2.8.

3.2.5 Determination of Hydroxyproline Content

Hydroxyproline content of insoluble fractions and solubilized material extracted by the various reagents investigated was measured in the soluble fractions by HPLC, as outlined in section 2.9.

3.3 RESULTS

3.3.1 Validation of Hydroxyproline Assay

A chromatogram of the elution profile of a standard amino acid mixture for collagen hydrolyzates is shown in Fig 3.3. The response of the hydroxyproline assay method employing HPLC to varying levels of hydroxyproline standard was linear from 0.1 nmole to 6 nmole (Fig. 3.4) and all subsequent hydroxproline analyses were designed to fall within this range. The reproducibility of hydroxyproline detection yielded a within-assay coefficient of variation (CV) of 3.9 % performed on five replicates of 1.9 nmole hydroxyproline injection.

3.3.2 Yields and Collagen Content of Gross Solubilized and Insoluble Connective Tissue Extracts using SDS

Yields of SDS extracted soluble and insoluble connective tissue fractions obtained from the set of eight unconditioned bovine muscles were compared with the yields from conditioned muscles (Table 3.1). Statistical analysis using two-way analysis of variance (ANOVA) indicated that the yield of soluble material obtained from the unconditioned muscles was

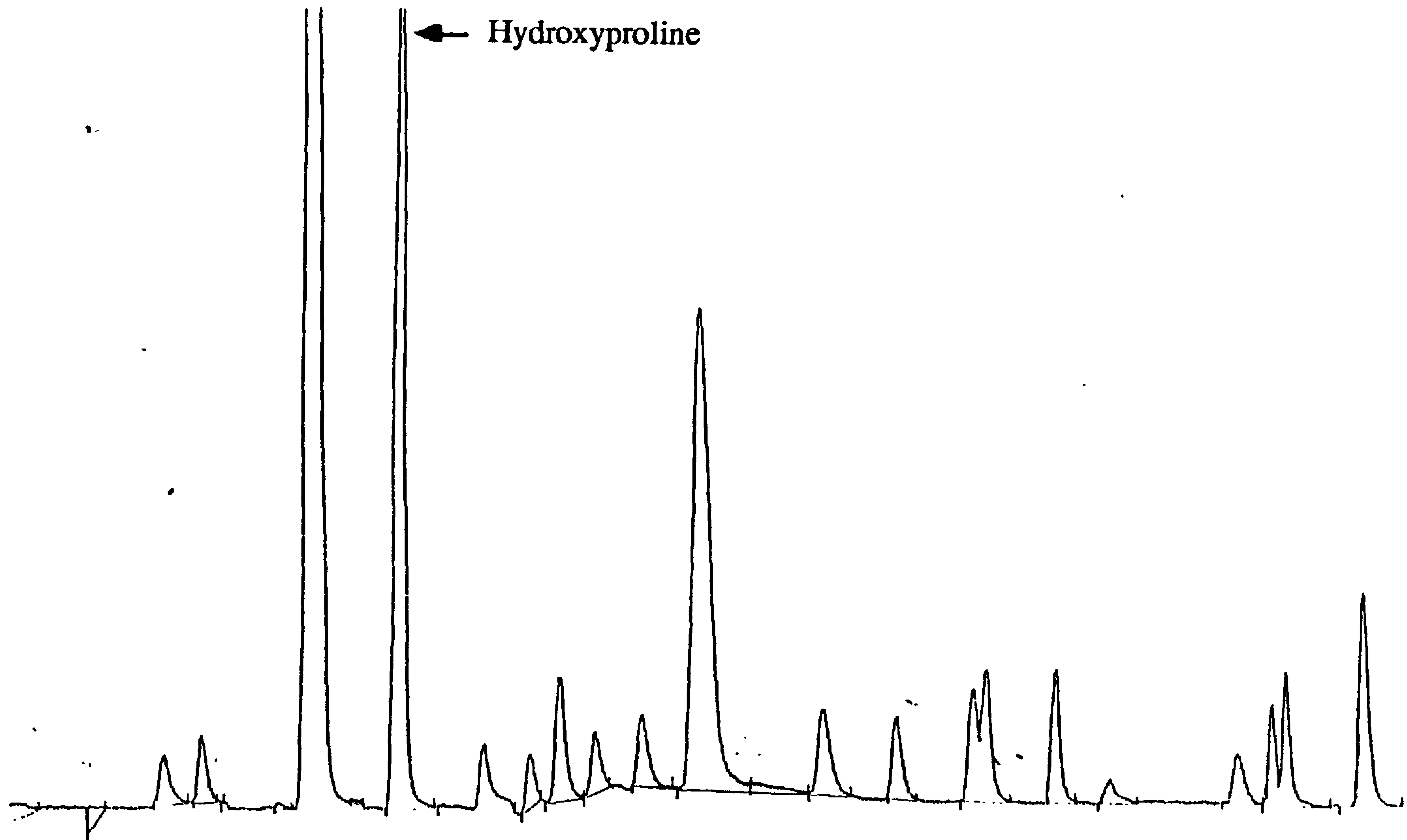


Fig. 3.3 Chromatogram of the Elution Profile of a Standard Amino Acid Mixture for Collagen Hydrolyzates

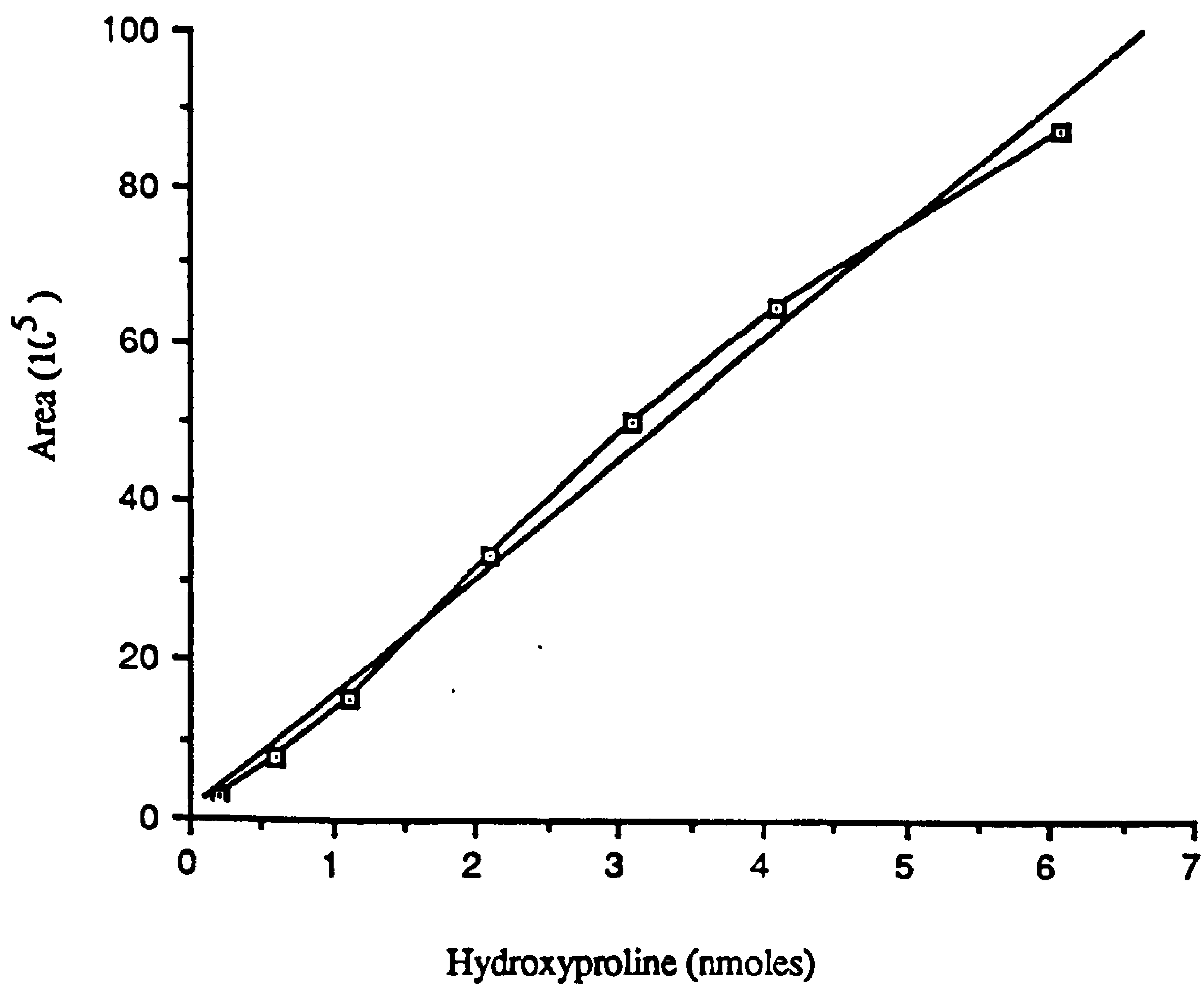


Fig. 3.4 HPLC Detection of Hydroxyproline

Volumes of 100 µl of standard amino acid mixtures, containing from 0.1 nmoles to 6 nmoles of dansylated hydroxyproline were eluted on a 15 x 0.46 cm column of Rainin 5 µl C18 reverse phase material, at 20 ° C, using conditions as described in Chapter 2. Detection was by optical density at 254 nm.

Table 3.1 Yields of Gross Solubilized and Insoluble Connective Tissue from Eight Unconditioned and Conditioned Bovine Muscle (Dry Weight) using 1 % (w/v) SDS

Muscle	Soluble Extract (g) ($\bar{x} \pm$ SD)		Insoluble Residue (g) ($\bar{x} \pm$ SD)		Soluble material as % of total dry weight	
	Unconditioned	Conditioned	Unconditioned	Conditioned	Unconditioned	Conditioned
Serratus Ventralis	2.73 ± 0.33	4.09 ± 0.99	1.08 ± 1.02	0.94 ± 0.05	72	83
Gastrocnemius	2.05 ± 0.50	3.76 ± 0.28	0.39 ± 0.18	0.71 ± 0.05	84	84
Psoas Major	4.66 ± 0.64	3.37 ± 0.007	1.07 ± 0.86	0.16 ± 0.05	81	95
Pectoratis Profundus	4.32 ± 0.53	4.81 ± 1.85	0.36 ± 0.01	0.58 ± 0.18	92	89
Sternomandibularis	3.08 ± 0.50	5.34 ± 0.18	0.97 ± 0.31	1.11 ± 0.10	76	83
Gluteus Medius	7.04 ± 0.04	7.28 ± 0.09	0.32 ± 0.08	0.47 ± 0.23	96	94
Extensor capri radialus	4.74 ± 0.27	3.00 ± 0.37	1.37 ± 0.47	0.45 ± 0.04	78	87
Supraspinatus	2.95 ± 0.32	3.57 ± 0.03	0.70 ± 0.08	0.59 ± 0.15	81	86

significantly lower ($p = 0.047$) than the yield of soluble material obtained from the same set of conditioned muscles. The yields of insoluble material obtained from the set of eight unconditioned and eight conditioned muscles were not significantly different. The soluble fraction represented, on average, 82.5 % of total extracted material in the unconditioned muscles and 88 % in conditioned muscles. Five out of the eight muscles examined indicated an increase in percentage of soluble material on conditioning (Table 3.1).

The collagen content of the soluble fractions obtained from the eight unconditioned and conditioned muscles was found to be negligible and could not be accurately quantified.

3.3.3 Analysis of Insoluble Material Extracted using SDS

Insoluble materials obtained after extraction of unconditioned and conditioned muscles were analyzed after CNBr digestion by SDS - polyacrylamide gel electrophoresis. The main peptide components were derived from types I and III collagen. Although clean gels were obtained no major differences were evident in the major peptide bands obtained from these extracts (Fig 3.5).

3.3.4 Effectiveness of Extractants in Solubilizing Collagen

All the reagents investigated were relatively efficient in extracting non-collagenous material from the crude perimysial preparations (Fig. 3.6). Unwashed insoluble perimysium (ie. before extraction) yielded an average collagen content of 46±2.5 % whilst the average collagen content of washed perimysia was over 60 %. 1 % (w/v) SDS and 4 M urea/0.5M CH_3COOH were the most successful extractants in that each solubilized 52 % of the perimysial fraction. 6 M urea at neutral pH was almost as effective in extracting soluble material from perimysium (45 %) whilst acetic acid yielded 43 % and other methods substantially less. However, some, including the SDS had drawbacks in terms of the needs of this study (see Discussion).

Using SDS for extraction, the total amount of extracted perimysial material was greater than 4 times the dry weight of perimysial material extracted using any of the other reagents (Table 3.2). This made accurate estimation of percentage collagen content of dry weights difficult. It also proved difficult to freeze-dry perimysial material solubilized using 1% (v/v) Triton X-100 even after exhaustive dialysis. A brown coloured sticky substance was observed which was not quantifiable and was subsequently discarded. This reagent were therefore not used further.

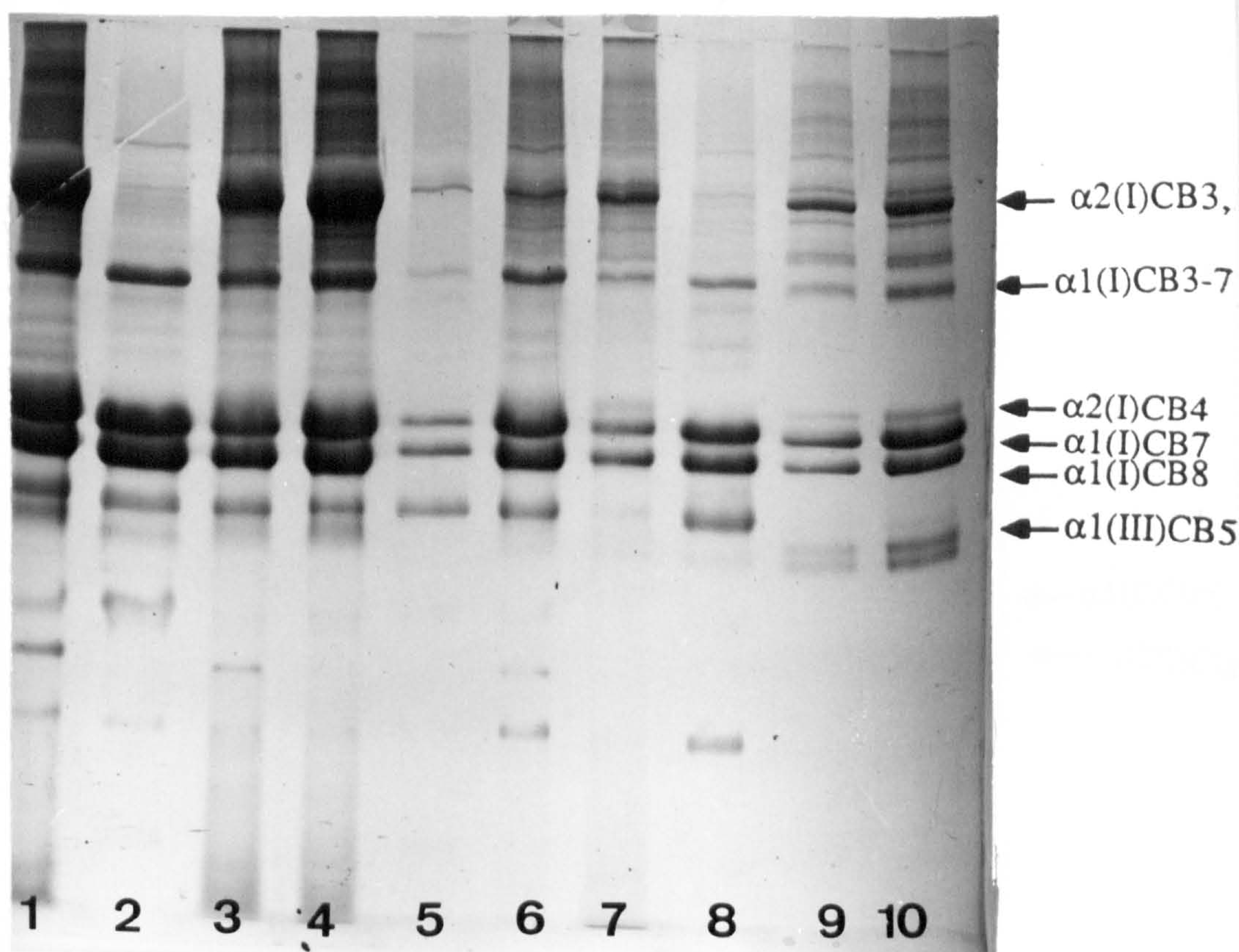


Fig. 3.5 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr Peptides of SDS Washed Insoluble Gross Connective Tissue

1 % (w/v) SDS washed insoluble connective tissue from unconditioned and conditioned *extensor capri radialus* and *supraspinatus* bovine muscle was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Tracks (1) and (2) are CNBr peptide maps of insoluble materials from unconditioned *extensor capri radialus* muscle. Tracks (3) and (4) are CNBr peptide maps of insoluble materials from conditioned *extensor capri radialus* muscle. Tracks (5) and (6) are CNBr peptide maps of insoluble materials from unconditioned *supraspinatus*. Tracks (7) and (8) are CNBr peptide maps of insoluble materials from conditioned *supraspinatus*. Tracks (9) and (10) are rat tail tendon collagen type I digest standards.

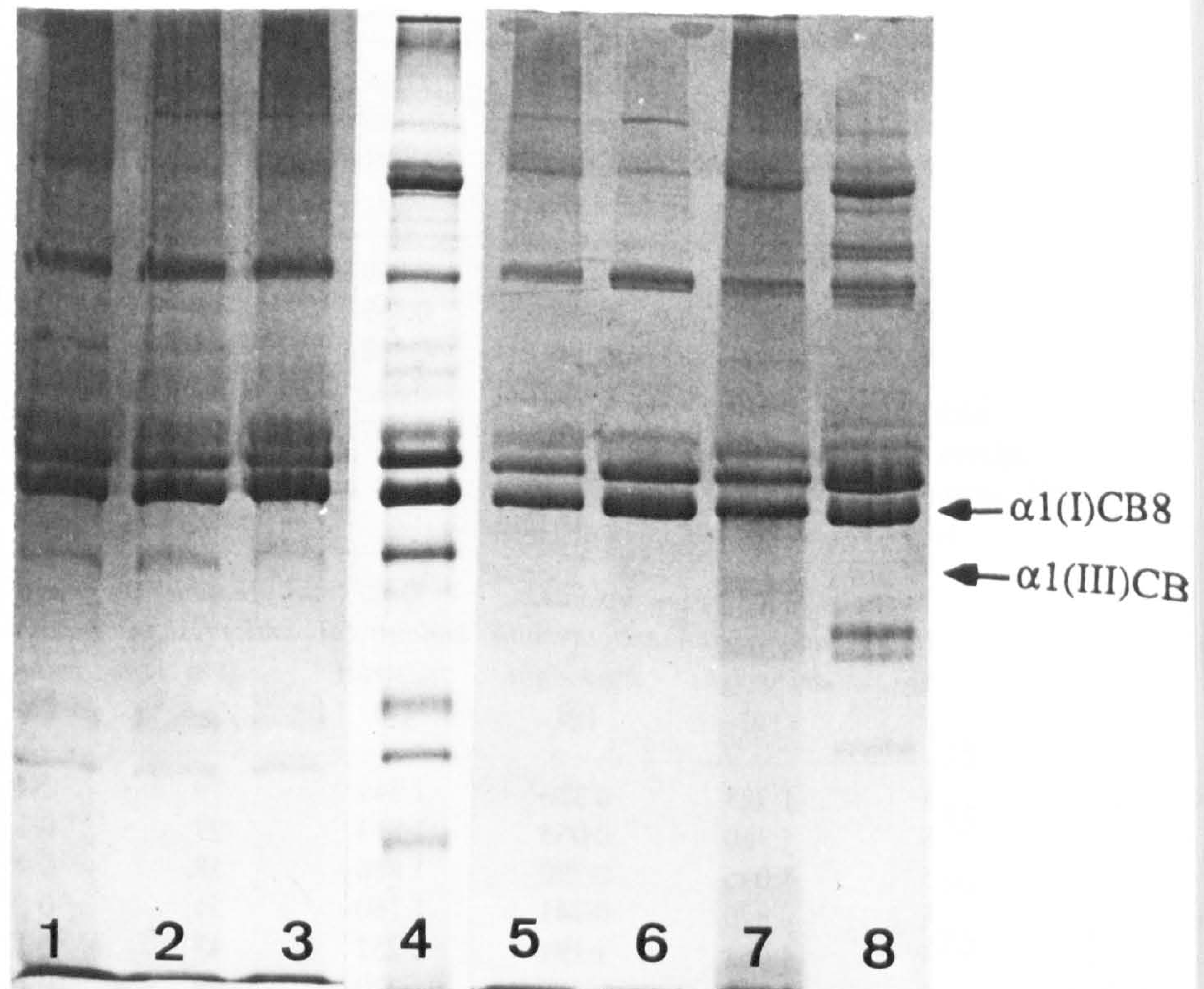


Fig. 3.6. SDS-Polyacrylamide Gel Electrophoretic Analysis of Washed Muscle Perimysium.

Washed perimysial fractions from conditioned *gastrocnemius* muscle were digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Track (1), 1 M NaCl, 0.02 M Na_3PO_4 ,pH 7.4 washed insoluble perimysium; track (2), 0.5 M CH_3COOH washed insoluble perimysium; track (3), 0.5 M CH_3COOH/4M urea washed insoluble perimysium; track (4), 6 M urea, 0.05 M tris-Cl, pH 7.4 washed insoluble perimysium; track (5), 1 M $CaCl_2$/0.05 M tris-Cl, pH 7.4 washed insoluble perimysium; track (6),1 % (w/v) SDS washed insoluble perimysium; track (7), 1 % (v/v) Triton X-100 washed insoluble perimysium; track (8), rat tail tendon collagen type I CNBr digest standard.

Table 3.2 Comparison of the Effectiveness of Methods of Extraction of Solubilized Perimysial Collagen

Extracting Agent	Perimysium wet weight (g)	Soluble Perimysium dry weight (g)	Insoluble Perimysium dry weight (g)	Total Perimysial material (g)	% Soluble Perimysium	Soluble Perimysial collagen (%)
1	1.5	0.182	0.717	0.899	20	0.34
2	1.5	0.377	0.492	0.869	43	1.07
3	1.5	0.431	0.397	0.828	45	2.35
4	1.5	0.374	0.456	0.830	45	2.35
5	1.5	0.256	0.661	0.917	28	2.30
6	1.8	1.970	1.82	3.8	52	3.25
7	1.5	...*	2.31	...	...	...

* Soluble perimysium extracted using Triton X-100 could not be freeze-dried.

1) 1 M NaCl, 0.02 M Na_3PO_4, pH 7.4
2) 0.4 M CH_3COOH
3) 0.5 M CH_3COOH, 4 M urea
4) 6 M urea, 0.05 M tris-Cl, pH 7.4
5) 1 M $CaCl_2$, 0.05 M tris-Cl, pH 7.4
6) SDS (1 % w/v)
7) Triton X-100 (1 % v/v)

3.3.5 Yields and Collagen Content of the Perimysia of Eight Unconditioned and Conditioned Bovine Muscles Extracted with Urea

Urea was selected for extraction purposes with separated perimysial fractions and was buffered with 0.05 M tris-Cl at pH 7.4 to avoid the possibility of randomly induced acid-catalyzed peptide bond cleavages (see Discussion). Yields of urea extracted soluble and insoluble perimysium obtained from a set of eight unconditioned bovine muscles were compared with the same eight conditioned muscles (Table 3.3).

Statistical analysis using two-way ANOVA indicated that the yield of soluble perimysium obtained from the unconditioned muscles was significantly lower ($p= 0.096$) than the yield of soluble perimysium obtained from the same set of conditioned muscles. The yields of urea extracted insoluble perimysium from a set of eight unconditioned and eight conditioned muscles were not significantly different. The soluble perimysial fraction represented, on average, 15 ± 7 % of total extracted perimysium in the unconditioned muscles, while this fraction represented 24.6 ± 10 % of total perimysial material in the conditioned muscles. Seven out of the eight muscles examined indicated an increase in percentage of soluble perimysium on conditioning, the exception being *sternomandibularis.*

Table 3.4 shows the collagen content of soluble and insoluble perimysium obtained from the eight unconditioned and conditioned bovine muscles. Statistical analysis using two-way ANOVA indicated that the collagen content of soluble perimysium obtained from the set of eight unconditioned muscles was significantly lower ($p = 0.015$) than the collagen content of soluble perimysium obtained from conditioned muscles.

The average percentage of collagen solubilized by the extractant was 1 ± 0.8 % for unconditioned muscles, while for conditioned muscles it was 3.4 ± 3.3 %. Seven out of the eight muscles examined indicated an increase in the proportion of solubilized perimysial collagen, the exception was *sternomandibularis*. The increase in solubilized perimysial collagen in conditioned muscles ranged from 2 to 10 times that seen in unconditioned muscles with an average increase of 3.4 times.

No significant difference was observed between the collagen contents of insoluble perimysium obtained from unconditioned and conditioned muscles.

Table 3.3 Yields of Urea Extracted Perimysial Fractions from Eight Unconditioned and Conditioned Bovine Muscles (Dry Weight)

Muscle	Unconditioned	Conditioned	Unconditioned	Conditioned	Unconditioned	Conditioned
	Soluble Perimysium (g)		Insoluble Perimysium (g)		(Soluble Perimysium as % of Total Dry-Weight)	
	($\bar{X} \pm SD$)	($\bar{X} \pm SD$)	($\bar{X} \pm SD$)	($\bar{X} \pm SD$)		
Serratus Ventralis	0.051±0.023	0.076±0.006	0.332±0.086	0.285±0.088	13	21
Gastrocnemius	0.073±0.004	0.048±0.022	0.485±0.339	0.248±0.155	13	16
Psoas Major	0.014±0.013	0.281±0.218	0.088±0.085	0.411±0.240	14	41
Pectoralis Profundus	0.028±0.026	0.060±0.012	0.380±0.142	0.309±0.098	7	16
Sternomandibularis	0.153±0.105	0.101±0.033	0.664±0.090	0.469±0.127	19	18
Gluteus Medius	0.030±0.017	0.042±0.009	0.298±0.208	0.181±0.116	9	19
Exterior Capri Radialus	0.301±0.255	0.505±0.314	0.706±0.460	0.780±0.635	30	39
Supraspinatus	0.105±0.043	0.166±0.202	0.585±0.573	0.453±0.306	15	27

Table 3.4 Collagen Content of Urea Extracted Perimysial Fractions from Eight Bovine Muscles

Muscle	Unconditioned Soluble (mg) $\bar{X} \pm SD$	Conditioned Soluble (mg) $\bar{X} \pm SD$	Unconditioned Insoluble (mg) $\bar{X} \pm SD$	Conditioned Insoluble (mg) $\bar{X} \pm SD$	% Solubilized Collagen Unconditioned	% Solubilized Collagen Conditioned
Serratus Ventralis	0.8±0.4	1.4±1.0	170±5.0	207±13.0	0.4	0.7
Gastrocnemius	2.2±2.7	2.7±2.2	283±7.3	163±16.4	0.8	1.7
Psoas Major	3.1±4.4	27.0±15.0	37±3.5	234±8.2	0.8	10.5
Pectoralis Profundus	1.0±0.1	2.7±2.7	259±65.0	198±12.4	0.4	1.3
Sternomandibularis	9.6±3.6	2.6±2.4	445±26.6	291±28.2	2.1	0.9
Gluteus Medius	1.2±0.1	2.6±1.4	192±26.9	109±2.7	0.6	2.3
Exterior Capri Radialus	9.6±8.8	28.0±21.1	412±28.9	511±11.7	2.3	5.2
Supraspinatus	1.6±1.0	15.0±12.3	379±70.5	308±67.9	0.4	4.6

3.3.6 Analysis of Urea Extracted Insoluble Perimysial Preparations

Insoluble materials obtained after urea extraction of unconditioned and conditioned muscles were analyzed after CNBr digestion by SDS-polyacrylamide gel electrophoresis. The main peptide components observed on analysis of perimysial fractions were derived from types I and III collagen as expected. Although very clean gels were obtained no significant differences were seen in the major peptide bands obtained from these preparations (Fig. 3.7).

3.4 DISCUSSION

Several groups of workers have attempted to devise simple methods of purifying the connective tissue component of the muscle mass including McCollester (1962), Fujii & Murota (1982) and Light & Champion (1984). SDS was used successfully for purification of collagen from lung (Laurent et al., 1981) and rabbit skin (Barnard et al., 1987). This extractant was employed in the present study for bulk preparation of connective tissue collagen from bovine muscle. The method of Light & Champion (1984) employed careful filtration and SDS in the washing procedures after the homogenization process described by McCollester (1962), resulting in the gross separation of the epimysium, perimysium and endomysium from the bulk of the muscle protein. This method was modified for investigation of conditioning effects on solubilization of the perimysial fraction and analysis of the insoluble material remaining after conditioning.

Yields of gross SDS-solubilized connective tissue fractions from conditioned muscles were significantly higher ($p = 0.047$) than those obtained from unconditioned muscles while the solubilized fraction represented, on average, 82.5 % of total extracted material in unconditioned muscles compared to 88 % in the conditioned muscles. However, the collagen content of all solubilized connective tissue fractions was found to be negligible. Analysis of the CNBr derived peptides from the insoluble material revealed that the major peptide bands present were derived from types I and III collagen. However, no damage to the conditioned samples could be detected by SDS - polyacrylamide gel electrophoresis. This study suggested that conditioning had an effect on solubilizing connective tissue material. However, because of the ability of SDS to extract large amounts of non-collagenous protein, in addition to connective tissue protein and because SDS forms a complex with proteins, such complexes containing a constant ratio of SDS to protein (about 1.4:1 by weight) the high yields of solubilized material relative to the low collagen content obtained caused the method of extraction employed to be inadequate for quantification of damage to the collagen component of meat connective tissue. The results

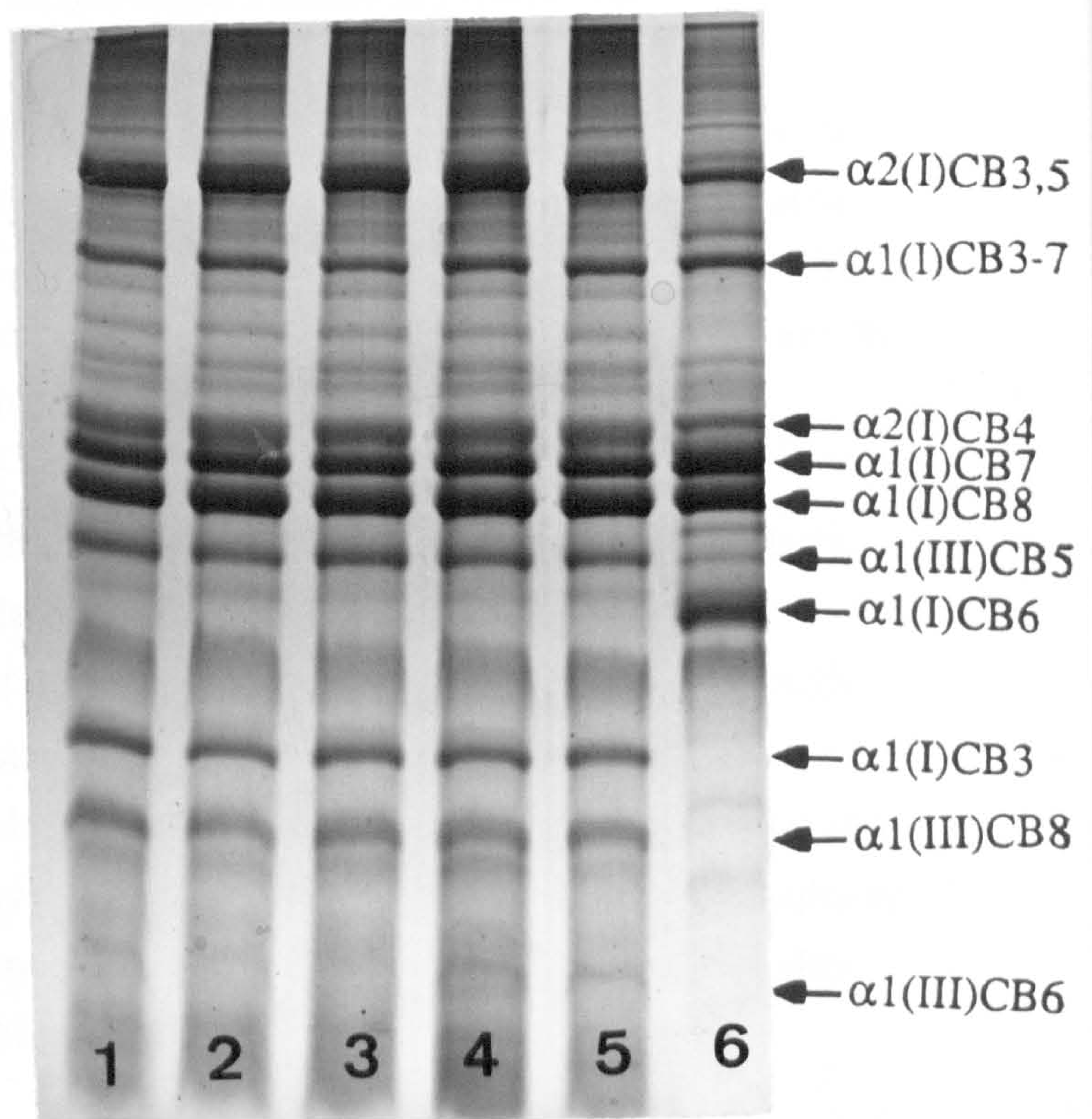


Fig. 3.7 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr-Peptides of Urea Washed Bovine Perimysium.

6 M urea, 0.05 M tris-Cl, pH 7.4 washed insoluble perimysium from unconditioned and conditioned bovine muscles was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in the Materials and Methods section. Tracks (1) and (2) are CNBr peptide maps of insoluble perimysium from unconditioned *gluteus medius* muscle. Tracks (3) (4) and (5) are CNBr peptide maps of insoluble perimysium from conditioned *gluteus medius* muscle. Track (6) is rat tail tendon collagen type I CNBr digest standard.

from this study suggested the need for a more sensitive connective tissue extraction procedure which would isolate the connective tissue fraction from the bulk of muscle protein, therefore allowing accurate quantification of solubilized collagen. In addition, dissection of perimysium from endomysium was required to quantify the effects of conditioning on the collagen component of this major connective tissue fraction.

Seven extracting agents were investigated in the study, using conditioned muscle as the source of connective tissue. The collagen content of fractions extracted with 4 M urea/ 0.5 M CH_3COOH, 6 M urea and 1 M $CaCl_2$, both buffered at neutral pH with 0.05 M tris-Cl, pH 7.4 and 1 % (w/v) SDS extracted fractions were similar while all other agents examined extracted less collagen from muscle. However, as expected, yields of perimysial fractions extracted employing SDS were significantly higher than those extracted using the other methods examined, even after exhaustive dialysis. Similarly, this study intended to examine the postulated effects of peptide bond cleavage by endogenous proteases. The method employing 4 M urea/0.5 M CH_3COOH, although efficient in washing meat connective tissue, was abandoned because it is known that some peptide bonds in collagen are weak acid-labile (see Light & Bailey, 1979 for review) and labile aldimine cross-links might be cleaved by such a solvent. Therefore, the possibility of such random cleavages was avoided by employing a neutral pH solution which also eliminated the possibility of activating acid proteases present in the meat samples.

SDS was used to extract gross muscle connective tissue from conditioned and unconditioned muscle in an attempt to characterize whole muscle changes whilst 6 M urea, 0.05 M tris-Cl, pH 7.4 was chosen as extracting agent for the extraction of separated perimysium. Both reagents were shown to yield clean insoluble perimysial preparations as evidenced by SDS-polyacrylamide gel electrophoresis.

While the soluble perimysial fraction only represented 15 % and 24.6 % of total perimysial material from unconditioned and conditioned muscles respectively, extracted with urea, these fractions represented 82.5 % and 88 %, as extracted by SDS. SDS was more efficient at solubilizing non-collagenous material, thereby diluting the collagenous fraction to such an extent that it was unmeasurable. On the other hand, urea proved efficient at solubilizing collagen, but did not extract such large quantities of non-collagenous material by the method employed. Therefore, accurate quantification of collagen in urea extracted fractions was possible.

Perimysial collagen accounts for 3.19 % of total perimysial material in unconditioned muscles compared with 5.64 % for conditioned muscles. It should be noted here that

these values only represent non-dialyzable protein and so the actual amount of collagen solubilized in these studies may be considerably higher.

The results of the urea extraction of separated perimysium study revealed that conditioned meat yielded significantly greater quantities ($p = 0.096$) of solubilized perimysial material (Table 3.3) and a significantly higher collagen content ($p = 0.015$) in the solubilized perimysial fractions than unconditioned meat. This is strong, direct evidence that proteolytic damage is inflicted upon collagens in the perimysium during the conditioning process. The fact that there were variations in the extent of this process confirm that it is random and uncontrolled. This might be expected as the source of the enzymes is almost certainly cellular (probably cathepsins) and so the enzymes have to breach the sarcolemma and endomysium before being able to penetrate the perimysium. Also, there is some variation in collagen fibre size amongst different muscles (Light et al., 1985), and perhaps in fibre bundle size, which would lead to variations in the effectiveness of enzymes in digesting collagen fibres.

Interestingly, *psoas major*, the most tender muscle investigated (Dransfield, 1977), showed the highest yield of proteolyzed soluble collagen after conditioning. *Gluteus medius*, another good quality muscle, also showed a high increase in collagen solubility after conditioning but other poor quality muscles showed similar increases, e.g. *extensor capri radialus* and *supraspinatus*. It seems, therefore, that there is little correlation between known muscle quality and solubility of perimysial collagen on conditioning. However, the extent of solubilized collagen may not reflect the residual damage in the insoluble matrix that remains after conditioning. It is probably this latter factor which most profoundly affects the way in which collagen contributes to textural changes on conditioning. Thus the apparent lack of correlation between known muscle quality and collagen solubility due to conditioning may be expected to be misleading.

It was concluded that the method employed for extraction and purification of perimysium using 6 M urea buffered at pH 7.4 with 0.05 M tris-Cl was adequate to produce a clean perimysial preparation.

No significant changes were observed in the insoluble perimysial fraction in terms of yields, collagen content or CNBr peptide spectrum. In respect of the first two parameters this is not surprising as the bulk of collagen remains insoluble after conditioning and so the small variations would not be expected to be seen by hydroxyproline analysis. However, it might be expected that there would have been some visible damage to insoluble collagen due to 'clips' or incomplete cleavages by hydrolases. This was not the case.

It is known that the C-terminal peptide of type I collagen is involved in the production of polymers which stabilize the fibre and can be visualized as high molecular weight components on gel analysis of CNBr digests of insoluble material (Light and Bailey, 1980a, b). This material is particularly sensitive to low temperature treatment with proteases like pepsin. It is reduced, through such treatment, to a peptide component of molecular weight 19 000 (Light,1979) readily visible on gel analysis. However, no significant change in the amount of this peptide (α 1CB6) could be observed in the insoluble collagen from conditioned muscles. This suggests that if any damage is incurred to this insoluble material it must be very minor.

However, such damage may still have a significant effect on fracture behaviour after cooking. Minor damage to insoluble collagen may result in changes in behaviour of the collagen during cooking, e.g. more extensive denaturation and solubilization, and may provide a weakened mechanical structure for fracture during eating (Purslow, 1985).

The collagen component of meat connective tissue has been implicated for many years as contributing to the tenderness of meat. There is strong evidence that lysosomal proteases are released during conditioning, once the muscle pH has fallen to pH 5.5 (Dutson & Lawrie, 1974; Wu et al., 1981). The lysosomal proteases capable of cleaving native insoluble collagen to soluble fragments are cathepsins B, L and N (Etherington, 1980; Locnikar et al., 1980).

As discussed earlier (Chapter 1) and by Stanton & Light (1987), little or no direct evidence existed prior to these studies to show that proteolysis of collagen takes place during conditioning even though the biochemical and mechanical status of the connective tissue component of meat has been associated with tenderness of the cooked product (Mackintosh et al.,1936; Cover et al.,1962; Cross et al.,1973) Using direct scanning calorimetry Judge & Aberle (1982) provided limited indirect evidence for the weakening of connective tissue during conditioning. Studies by Sharp (1963) revealed no change in soluble collagen after conditioning although aged beef muscle collagen had become much more readily solubilized into gelatin by heat (Sharp, 1964). Asghar & Yeates (1978) proposed that endogenous lactic acid could cause increased collagen solubility through cleavage of acid-labile cross-links and an increase in α-chains of collagen during conditioning was observed (Kruggle & Field, 1971; Wu et al.,1982) indicating limited proteolytic cleavage.

Results of this study on conditioned perimysium suggest that post-mortem effects on the connective tissue were small with only subtle modifications occurring, leading to equally

small but significant differences in terms of solubilized perimysial material and solubilized collagen. Results from more detailed investigations of the damaged insoluble perimysium after conditioning are presented in Chapter 6.

CHAPTER 4

CHANGES IN ENDOMYSIAL COLLAGEN AFTER CONDITIONING

4.1 INTRODUCTION

The endomysium is that fraction of muscle connective tissue in direct contact with the sarcolemma and as such represents the first collagenous barrier and line of attack to muscle proteinases, liberated during the conditioning process. Therefore, the extent of damage to this meat connective tissue fraction may be expected to have a significant contribution to the overall textural properties of meat.

Chapter 4 deals with the effects of conditioning on the endomysial fraction of bovine meat. Solubility studies on endomysial connective tissue and analysis of insoluble endomysial fractions remaining after conditioning were carried out. Initially, the extracting reagents described in Chapter 3 for perimysial fractions were assessed for their ability to purify the endomysial connective tissue fraction and endomysial collagen. Although a number of the reagents investigated proved efficient in extraction of endomysial connective tissue and soluble endomysial collagen, urea was the reagent chosen, for reasons similar to those outlined in Chapter 3 for extraction of perimysial connective tissue. The endomysial preparations, thus prepared, contained a mixture of endomysium, attached perimysial fibres and muscle protein, resulting in extracts with such low collagen contents that accurate quantification was not possible. Consequently, this method was abandoned for more effective methods of endomysial extraction. Subsequently, a combination of two of the reagents investigated in the initial study (6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v)) were employed in sequential extraction of endomysial fractions obtained from unconditioned and conditioned muscles. Although this method succeeded in extracting soluble endomysial material, a problem similar to that found in Chapter 3 for quantification of solubilized perimysial collagen arose. The total yields of solubilized endomysial material, which consisted of protein-bound SDS, in addition to non-collagenous material, resulted in dilution of the collagen content to such an extent, that it was again immeasurable. In addition, the method proved inadequate for purification of insoluble endomysial collagen. Finally, a documented method (Light & Champion, 1984) for endomysial extraction, which yielded clean insoluble endomysial preparations, was investigated in this study for its ability to highlight conditioning effects on the endomysial fractions of meat. This method combined a procedure described by McCollester (1962) for the preparation of endomysial 'ghosts', extraction solvents of Hasselbach & Schneider (1951) and Laurent et al. (1981), and SDS, for purification of endomysial fractions. The

method proved very effective in solubilizing endomysial material and endomysial collagen and for purification of insoluble endomysial collagen. This method of endomysial extraction proved effective in examination of conditioning effects on endomysial connective tissue fractions obtained from bovine muscle.

4.2 MATERIALS AND METHODS

Three methods of endomysial extraction were employed and examined for their effectiveness in investigating the effects of conditioning on the endomysial fraction of bovine meat. Simple urea extraction, sequential urea/SDS extraction (the sequential Extraction Method) and simple SDS extraction (the SDS Extraction Method) were employed for this purpose.

4.2.1 Preparation of Unconditioned and Conditioned Muscles

The preparation of unconditioned and conditioned muscles is outlined in Chapter 2. The muscles selected for the first study employing the sequential extraction method were *serratus ventralis, psoas major, sternomandibularis, extensor capri radialus* and *supraspinatus* and for the second study employing the SDS extraction method of Light & Champion (1984) were *supraspinatus, gastrocnemius, psoas major* and *extensor capri radialus*.

4.2.2 Extraction and Purification of Endomysial Meat Connective Tissue

Seven reagents (described in Chapter 3, section 3.2.2) were investigated for their effectiveness in extracting endomysial collagen and for obtaining a clean endomysial preparation, from a sample of conditioned meat. These were 1 M NaCl, 0.02 M Na_3PO_4, pH 7.4; 0.5 M CH_3COOH; 0.5 M CH_3COOH containing 4 M urea; 6 M urea, 0.05 M tris-Cl, pH 7.4; 1 M $CaCl_2$, 0.05 M tris-C1, pH 7.4; 1 % (w/v) SDS; 1 % (v/v) Triton X-100.

150 g meat, obtained from uncooked conditioned *gastrocnemius* was homgenized in 100 ml ice-cooled $CaCl_2$ for 10 sec. as described in Chapter 3 (section 3.2.2). The filtrate, resulting from filtration of the homogenate through a graded copper grid (section 3.2.2) was centrifuged at 25 000 g x 30 min and the supernatant discarded. The pellet was referred to as the crude endomysial fraction.

Press-dried endomysial fractions were divided into seven sub-fractions. Each was extracted at 20° C with the reagents described above before separating soluble from insoluble endomysial fractions by centrifugation (25 000 g x 20 min) at 10° C. Soluble and insoluble fractions were dialyzed against running water and distilled water, as described in Chapter 3 (section 3.2.2) for extracted perimysial fractions. All fractions were immediately frozen following dialysis and subsequently freeze-dried if necessary, e.g. for total dry weight estimation. The method is summarized in Fig. 4.1.

4.2.3 Endomysial Preparation Employing the Sequential Extraction Method

The muscle was sampled such that 3 x 25 g samples representing the general aspects of the whole muscle were taken. They were cut into 1cm cubes, and each 25 g sample was homogenized in 100 ml ice-cooled 0.05 M $CaCl_2$ for 10 sec at full speed in a Waring Blendor. The homogenate was filtered through a graded copper grid with 1 mm square holes. The insoluble material not passing through the filter was re-homogenized in a further 100 ml ice-cooled 0.05 M $CaCl_2$ and re-filtered. This process was repeated a further two times. All filtrates were combined and centrifuged at 25 000 g x 20 min. The supernatant was discarded and the pellet was referred to as the crude endomysial fraction.

The crude endomysial fraction was stirred in 50 ml 6 M urea, 0.05 M tris-Cl, pH 7.4 for 30 min on a magnetic stirrer at 20° C followed by centrifugation at 25 000 g x 20 min. The supernatant was saved and the pellet was stirred in a further 50 ml 6 M urea, 0.05 M tris-Cl, pH 7.4 and centrifuged as described above. This extraction procedure was repeated for a further time and all supernatants were combined.

The insoluble endomysial fraction from the urea extraction procedure was subjected to a further three extractions in 1% (w/v) SDS, at 20° C, in the manner described above. Soluble endomysial fractions from the urea and SDS extraction procedure were combined. Insoluble and soluble endomysial fractions were dialyzed for 24 h against running water and against two changes of distilled water for a further 48 h. All fractions were immediately frozen following dialysis and subsequently freeze dried if necessary, e.g. for total dry weight estimation. A summary of the procedure is outlined in Fig. 4.2.

4.2.4 Endomysial Preparation Employing the SDS Extraction Method of Light & Champion (1984)

The muscle was sampled such that 4 x 25 g samples representing the general aspects of the whole muscle were taken. These were cut into 1 cm cubes and combined. 50 g was

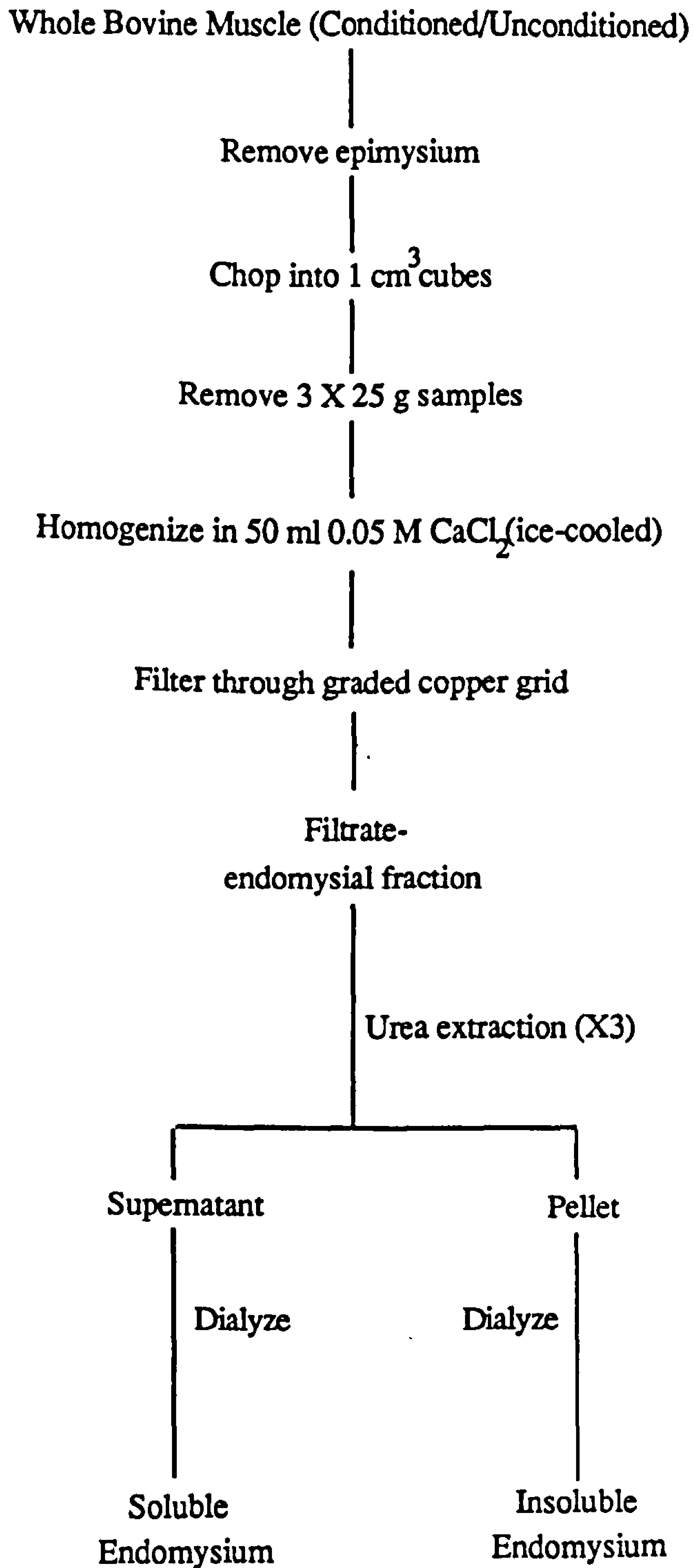


Fig. 4.1 Schematic Representation of Endomysial Connective Tissue Extraction using 6 M urea, 0.05 M tris-Cl, pH 7.4.

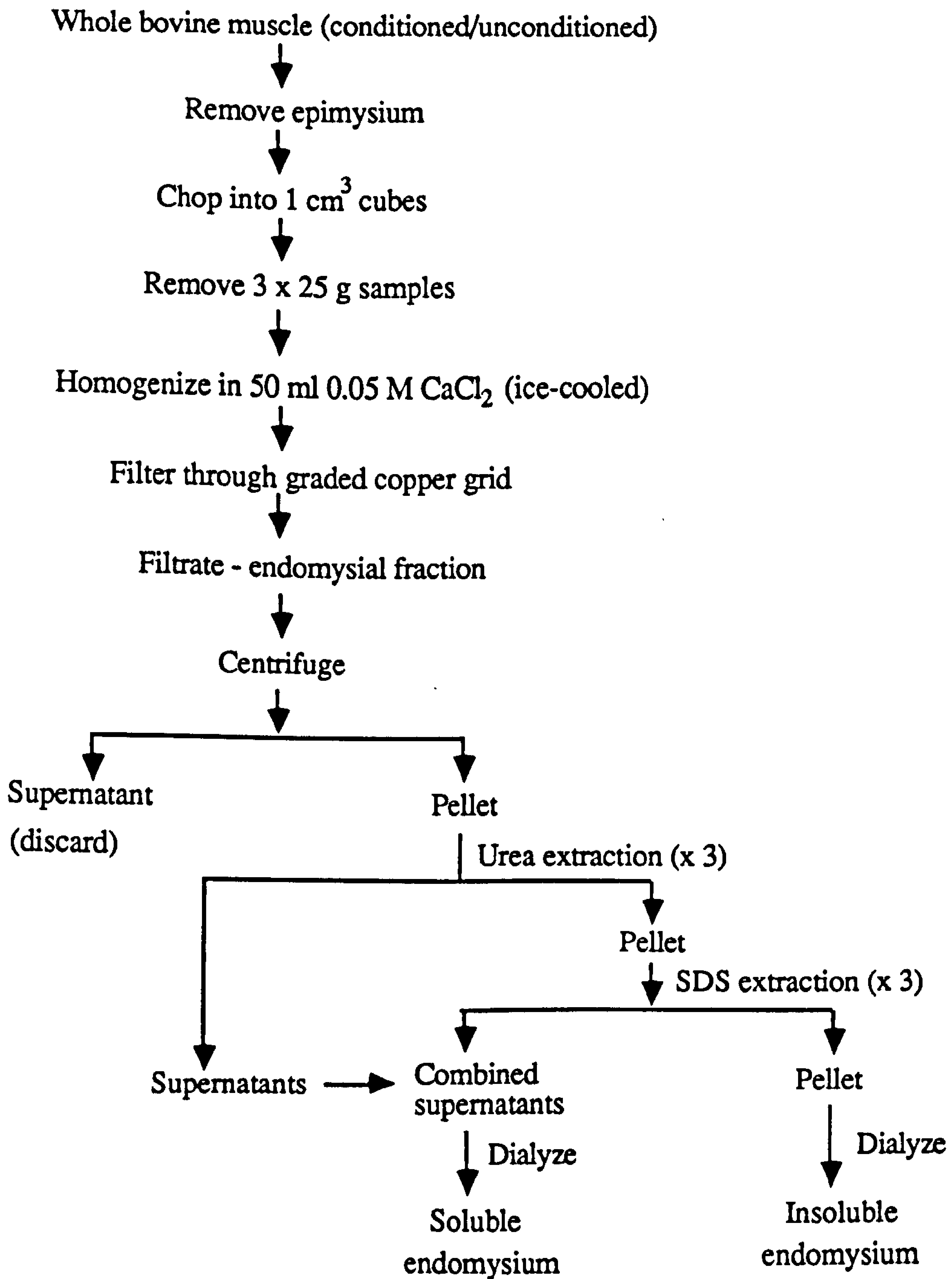


Fig. 4.2 Schematic Representation of Partial Purification of Endomysial Fractions using Sequential Extraction with 6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v)

homogenized in 100 ml of ice-cooled 0.05 M $CaCl_2$ for 10 sec at full speed in a Waring Blendor. The homogenate was filtered through a graded copper grid with 1 mm square holes, then the second 50 g was similarly homogenized and filtered. The combined materials not passing through the filter were re-homogenized in a further 100 ml of ice-cooled 0.05 M $CaCl_2$ and re-filtered. This process was repeated for a further two times, at which point all filtrates were combined. The filtrate was denoted the crude endomysial fraction.

This was left overnight at 4° C whereupon a loose precipitate formed. The supernatant (which contained no endomysial sheaths (Light & Champion, 1984)) was aspirated off and discarded. The pellet was resuspended in 25 mM NaCl containing 2.5 mM-DL-histidine adjusted to pH 7.4 with 1 M tris and stirred in this buffer at 20° C for 5 min. The suspension was centrifuged (2 500 g x 10 min) and the supernatant was discarded. This procedure was repeated a further three times, after which the suspension was incubated in the same buffer at 37° C for 30 min before a further five washes. The final pellet was resuspended in distilled water adjusted to pH 7.5 with 1 M tris and washed for 10 min before centrifugation (2 500 g x 15 min). The endomysial preparation was then extracted in 1 % (w/v) SDS for 30 min and soluble fractions were separated from insoluble fractions by centrifugation at 2 500 g x 15 min. Insoluble endomysium was dialyzed for 24 h against running water, against 40 % (v/v) methanol for 24 h, re-washed in water and dialyzed against distilled water for a further 24 h. Soluble fractions were dialyzed against distilled water for 24 h, against 40 % (v/v) methanol for 24 h and against distilled water for a further 24 h. All fractions were frozen following dialysis and subsequently freeze-dried if necessary, e.g. for total dry weight estimation. A summary of the procedure is outlined in Fig. 4.3.

4.2.5 Quantification of Types I and III Collagen

CNBr peptides prepared from insoluble endomysium obtained from unconditioned and conditioned muscles were resolved on SDS-polyacrylamide gels and were stained with Coomassie Brilliant Blue R-250. Gel tracks were scanned with a densitometer (Light, 1982) and the peak areas of peptides $\alpha1$(I)CB8 and $\alpha1$(III)CB5 were used to calculate the ratio of types I to III collagen.

4.2.6 Determination of Hydroxyproline Content

Hydroxyproline content of soluble endomysial fractions was quantified by HPLC, as described in Chapter 2 (section 2.9) using a conversion factor of 7.14 from

Whole bovine muscle (conditioned/unconditioned)

↓

Remove epimysium

↓

Chop into 1 cm^3 cubes

↓

Remove 100 g samples and combine

↓

Homogenize in 200 ml 0.05 M $CaCl_2$ (ice-cooled)

↓

Filter through graded copper grid

↓

Filtrate - endomysial fraction

↓

Stand overnight at 4° C

↓

Aspirate off supernatant

↓

Resuspend pellet in 25 mM NaCl, 2.5 mM-DL-histidine, pH 7.4

↓ stir for 5 min and centrifuge (x 3)

Incubate pellet in 25 mM NaCl, 2.5 mM-DL-histidine, pH 7.4, at 37° C x 30 min

↓ wash (x 5)

Resuspend pellet in distilled H_2O, pH 7.5

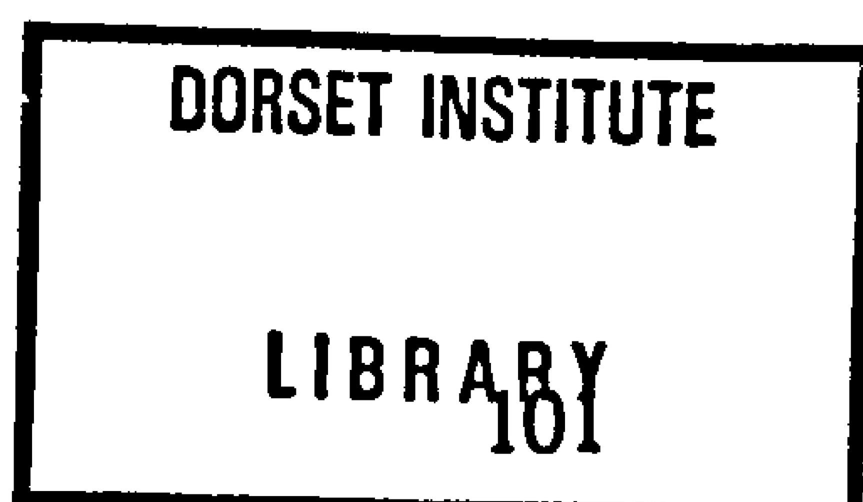
↓ stir for 10 min and centrifuge

Extract pellet in 1 % (w/v) SDS (x 3)

↓

Supernatant	Pellet
↓ Dialyze	↓ Dialyze
Soluble endomysium	Insoluble endomysium

Fig. 4.3 Schematic Representation of Partial Purification of Endomysial Fractions using the SDS Extraction Method of Light & Champion (1984).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hydroxyproline to collagen content.

4.3 RESULTS

4.3.1 Effectiveness of Extractants in Solubilizing Endomysial Collagen

The total amount of extracted material in the endomysial fraction obtained from a sample of conditioned meat, was greatest employing 1 M $CaCl_2$, 0.05 M tris-Cl, pH 7.4 and least employing 1 % Triton X-100 (Table 4.1). SDS and 0.5 M CH_3COOH solubilized almost all (99 % and 95 % respectively) of the endomysial fraction; 4 M urea/0.5 M CH_3COOH, 6 M urea, 0.05 M tris-Cl, pH 7.4 and 1 M $CaCl_2$, 0.05 M tris-Cl, pH 7.4 each solubilized almost half of the endomysial material (58 %, 54 % and 47 % respectively) whereas 1 % Triton X-100 was least efficient in this respect. Much less collagen was present in all solubilized endomysial fractions compared with their perimysial counterparts (Table 4.1) and Chapter 3. SDS, Triton X-100, 6 M urea at neutral pH and 4 M urea/0.5 M CH_3COOH solubilized endomysial fractions yielded similar amounts of collagen, while 1 M NaCl, 0.02 M Na_3PO_4, pH 7.4 and 1 M $CaCl_2$, 0.05 M tris-Cl, pH 7.4 solubilized endomysial fractions contained no measurable collagen. The 0.5 M CH_3COOH solubilized endomysial fraction yielded a small (0.15 %) but quantifiable amount of collagen.

4.3.2 Yields and Collagen Content from the Endomysia of Eight Unconditioned and Conditioned Bovine Muscles using Urea Extraction

Yields of urea extracted soluble and insoluble endomysial fractions obtained from the set of eight unconditioned bovine muscles were compared with the yields from the conditioned muscles (Table 4.2). Statistical analysis using two-way ANOVA indicated no significant difference between the yields of soluble endomysium obtained from eight unconditioned and eight conditioned muscles. No significant difference was observed between the yields of insoluble endomysium obtained from conditioned and unconditioned muscles. Only half of the muscles examined showed an increase in yield of percentage solubilized endomysium in going from the unconditioned to the conditioned state.

Table 4.3 shows the percentage collagen in the soluble endomysial preparations obtained from unconditioned and conditioned muscles. The average collagen content of the soluble endomysial fractions from unconditioned muscles was 0.52 % with the coefficient of variation ranging from 34 to 173 %, while for conditioned muscles the average collagen content was 0.5 % with the coefficient of variation ranging from 78 to 173 %.

Table 4.1 Comparison of the Effectiveness of Methods of Extraction of Solubilized Endomysial Collagen

Extracting Agent	Endomysium wet weight (g)	Soluble Endomysium dry weight (g)	Insoluble Endomysium dry weight (g)	Total Endomysial material (g)	% Soluble Endomysium	Soluble Endomysial Collagen (% of total soluble endomysium)
1	3.5	1.223	0.326	1.549	79	0.00
2.	3.5	1.760	0.053	1.813	97	0.15
3	3.5	1.096	0.397	0.828	45	2.35
4	3.5	0.870	0.741	1.160	54	0.50
5	3.5	1.061	1.191	2.252	47	0.00
6	3.8	1.760	0.010	1.770	99	0.54
7	3.5	0.120	0.750	0.870	14	0.51

1) 1 M NaCl, 0.02 M $Na_3 PO_4$, pH 7.4
2) 0.4 M CH_3COOH
3) 0.5 M CH_3COOH, 4 M urea
4) 6 M urea, 0.05 M tris-Cl, pH 7.4
5) 1 M $CaCl_2$, 0.05 M tris-Cl, pH 7.4
6) SDS (1 % w/v)
7) Triton X-100 (1 % v/v)

Table 4.2 Yields of Endomysial Extracts from Eight Unconditioned and Conditioned Bovine Muscles (Dry Weight) after Urea Extraction

Muscle	Unconditioned	Conditioned	Unconditioned	Conditioned	Unconditioned	Conditioned
	Soluble Endomysium (g)		Insoluble Endomysium (g)		(Soluble Endomysium as % of Total Dry-Weight)	
	($\bar{X} \pm SD$)	($\bar{X} \pm SD$)	($\bar{X} \pm SD$)	($\bar{X} \pm SD$)		
Serratus Ventralis	0.146±0.004	0.890±0.183	1.727±0.273	1.104±0.166	8	45
Gastrocnemius	0.434±0.108	0.820±0.622	1.820±0.751	1.051±0.316	19	44
Psoas Major	0.847±0.133	1.42±0.320	1.563±0.197	1.083±0.339	35	57
Pectoralis Profundus	0.995±0.174	1.10±0.322	1.061±0.493	1.726±0.200	48	39
Sternomandibularis	1.294±0.446	0.757±0.017	0.900±0.012	1.437±0.050	59	35
Gluteus Medius	0.788±0.267	0.873±0.156	1.666±0.126	1.690±0.044	32	34
Exterior Capri Radialus	0.827±0.169	0.821±0.289	1.204±0.254	1.315±0.433	41	38
Supraspinatus	0.851±0.639	0.445±0.050	1.342±0.115	1.815±0.246	39	20

Table 4.3 Collagen Content of Endomysial Extracts from Eight Bovine Muscles after Urea Extraction

Muscle	Unconditioned Soluble (% Collagen) ($\bar{X} \pm SD$)	Conditioned Soluble (% Collagen) ($\bar{X} \pm SD$)
Serratus Ventralis	1.110±0.374	0.163±0.150
Gastocnemius	0.133±0.230	0.294±0.281
Psoas Major	0.683±0.759	0.143±0.248
Pectoralis Profundus	1.013±1.297	0.000 ± 0.000
Sternomandibularis	0.333±0.416	0.400±0.693
Gluteus Medius	0.200±0.346	0.183±0.161
Exterior Capri Radialus	0.453±0.216	1.543±2.389
Supraspinatus	0.247±0.218	1.292±1.013

Statistical analysis using two-way ANOVA indicated no significant difference between the collagen contents of soluble endomysium obtained from unconditioned and conditioned muscles.

Analysis of insoluble endomysial fractions by SDS-polyacrylamide gel electrophoresis gave very poor results (Fig. 4.4) and it was not possible to analyze the gels properly .

4.3.3 Yields and Collagen Content of Endomysial Fractions Obtained by Sequential Extraction with 6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v).

Yields of extracted soluble and insoluble endomysia obtained from a set of five unconditioned bovine muscles were compared with the same five conditioned muscles (Table 4.4).

Statistical analysis using two-way ANOVA indicated that the yield of soluble endomysium obtained from the unconditioned muscles was not significantly different ($p = 0.209$) than the yield of soluble endomysium obtained from the same set of conditioned muscles. The yield of insoluble endomysium extracted from unconditioned muscles was significantly lower ($p < 0.05$) than the yield of insoluble endomysium obtained from the same set of conditioned muscles. The soluble endomysial fraction represented, on average, 46 % of total extracted endomysium in the unconditioned muscles compared with a value of 29 % for conditioned muscles. All muscles examined indicated a decrease in percentage of soluble endomysium on conditioning, while the total amount of endomysium extracted (soluble and insoluble fractions) was greater for conditioned muscles in all cases, than unconditioned muscles. Conditioned *psoas major* yielded the greatest amount of total endomysial material, while for unconditioned muscles, *psoas major* also yielded the greatest quantity of total endomysial material.

The collagen content of all soluble endomysial fractions was negligible, as measured by hydroxyproline analysis by HPLC.

4.3.4 Yields and Collagen Content of Endomysial Fractions Obtained by the SDS Extraction Method of Light & Champion (1984)

Yields of extracted soluble and insoluble endomysia obtained from a set of four unconditioned bovine muscles were compared with the same set of four conditioned muscles (Table 4.5).

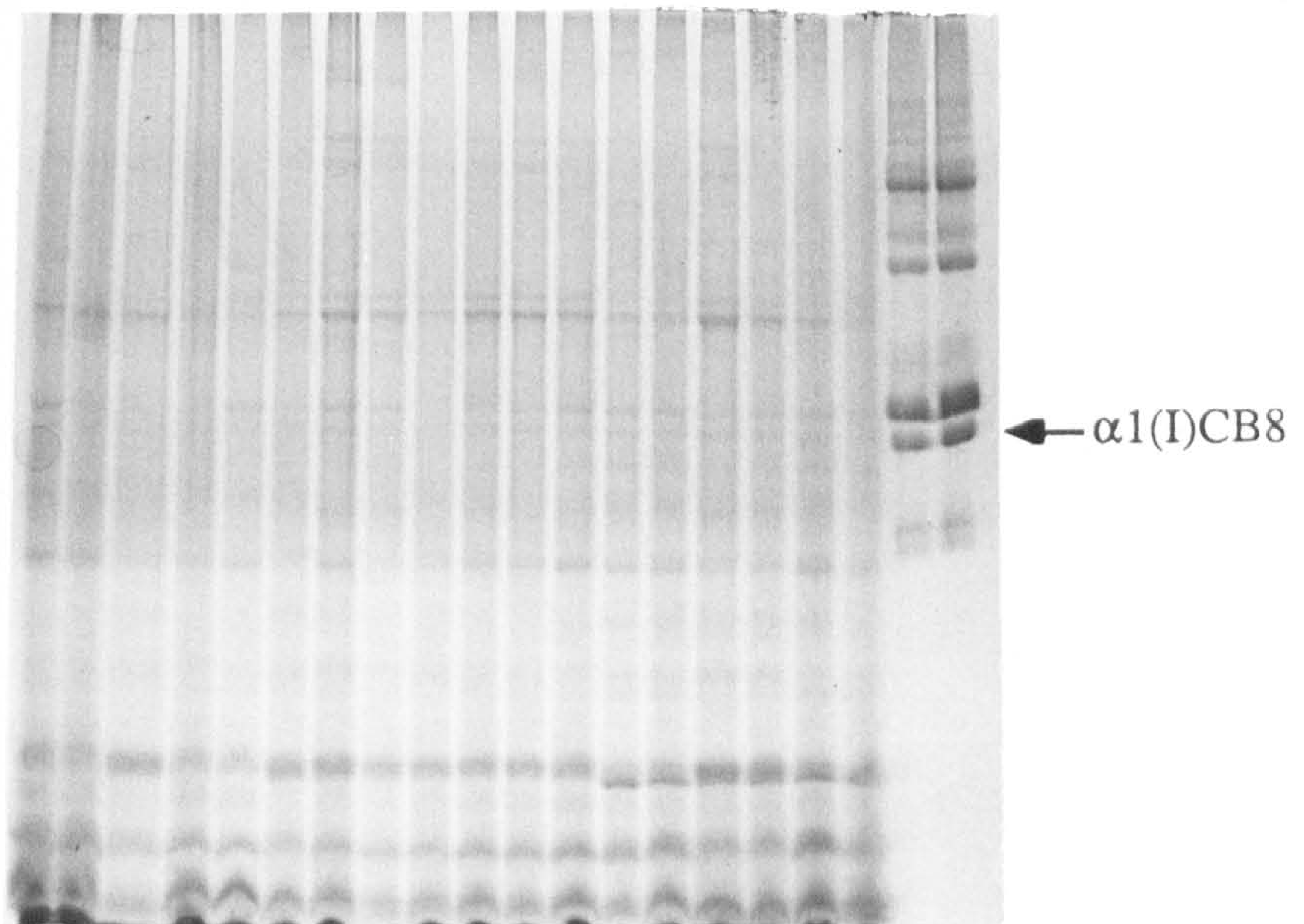


Fig. 4.4 SDS-Polyacrylamide Gel Electrophoretic Analysis of Bovine Muscle Endomysium Obtained by Urea Extraction.

Insoluble endomysium obtained by 6 M urea, 0.05 M tris-Cl, pH 7.4 extraction from unconditioned and conditioned muscles was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Tracks (1), (2) and (3) are CNBr peptide maps of insoluble endomysium from conditioned *supraspinatus* muscle. Tracks (4), (5) and (6) are CNBr peptide maps of insoluble endomysium from unconditioned *supraspinatus* muscle. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble endomysium from conditioned *psoas major* muscle. Tracks (10), (11) and (12) are CNBr peptide maps of insoluble endomysium from unconditioned *psoas major* muscle. Tracks (13), (14) and (15) are CNBr peptide maps of insoluble endomysium from conditioned *extensor capri radialus* muscle. Tracks (16), (17) and (18) are CNBr peptide maps of insoluble endomysium from unconditioned *extensor capri radialus* muscle. Tracks (19) and (20) are rat tail tendon collagen type I digest standards.

Table 4.4 Yields of Endomysial Extracts from Five Unconditioned and Conditioned Bovine Muscles Obtained by Sequential Extraction with 6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v)

Muscle	Soluble endomysium (g) ($\bar{x} \pm$ SD)		Insoluble endomysium (g) ($\bar{x} \pm$ SD)		Soluble endomysium as % of total dry weight	
	Unconditioned	Conditioned	Unconditioned	Conditioned	Unconditioned	Conditioned
Serratus Ventralis	0.87 ± 0.21	1.02 ± 0.23	0.91 ± 0.54	2.3 ± 0.20	49	31
Psoas Major	0.92 ± 0.06	0.80 ± 0.06	2.40 ± 0.15	3.01 ± 0.32	28	21
Sternomandibularis	0.95 ± 0.11	1.17 ± 0.03	0.82 ± 0.15	2.16 ± 0.20	54	35
Extensor capri radialus	0.78 ± 0.07	0.89 ± 0.05	0.66 ± 0.27	2.22 ± 0.04	54	29
Supraspinatus	0.91 ± 0.05	0.84 ± 0.21	1.12 ± 0.42	2.23 ± 0.50	45	27

Table 4.5 Yields of Endomysial Extracts from Four Unconditioned and Conditioned Bovine Muscles (Dry Weight) Obtained by the SDS Extraction Method of Light & Champion (1984)

Muscle	Soluble Endomysium (g)		Insoluble Endomysium (g)		Soluble Endomysium as % of Total Dry Weight	
	Unconditioned	Conditioned	Unconditioned	Conditioned	Unconditioned	Conditioned
Psoas Major	7.50	8.70	0.23	0.09	97	99
Extensor capri radialus	2.30	6.02	0.42	0.22	85	96
Supraspinatus	7.15	6.62	0.15	0.08	98	99
Gastrocnemius	5.50	8.29	0.14	0.36	98	96

The yields of all solubilized endomysial fractions obtained by the SDS extraction method of Light & Champion (1984) were high, and the yields of all insoluble endomysial fractions were low (Table 4.5), compared to the yields obtained from the same muscles employing the sequential extraction method using 6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v) (Table 4.4). The soluble endomysial fraction represented, on average, 37 % of total extracted endomysial material for all muscles examined employing the sequential extraction method using 6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v), while this fraction represented 96 % for all muscles examined, employing the SDS extraction method of Light & Champion (1984).

The yields of soluble endomysium obtained from the conditioned muscles by the SDS extraction method of Light & Champion (1984) were higher in three out of the four cases examined than the same set of unconditioned muscles, the exception was *supraspinatus*. The yields of insoluble endomysium from conditioned muscles were lower in three out of the four cases examined than the same set of unconditioned muscles, the exception was *gastrocnemius*. The soluble fraction represented, on average, 94.5 % of total extracted endomysial material for unconditioned muscles compared to 97.5 % for conditioned muscles.

Table 4.6 shows the collagen content of soluble endomysial fractions obtained from four unconditioned and conditioned bovine muscles. The collagen content of the soluble endomysium obtained from the unconditioned muscles was lower, in all cases examined, than that of the conditioned muscles. Conditioning appeared to have the most profound effect on solublilizing endomysial collagen from *supraspinatus* and the least effect on *psoas major* (Table 4.6).

4.3.5 Analysis of Insoluble Endomysial Fractions Obtained by Sequential Extraction with 6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v)

Insoluble endomysial materials obtained after extraction of unconditioned and conditioned bovine muscles were analyzed after CNBr digestion by SDS - polyacrylamide gel electrophoresis (Figs. 4.5 and 4.6). Although peptide components derived from types I and III collagen were visible, the gels were not clean and it was not possible to properly analyze the gels.

Table 4.6 Collagen Content of Soluble Endomysial Extracts Obtained by the SDS Extraction Method of Light & Champion (1984), from Four Bovine Muscles

Muscle	Unconditioned Soluble (mg collagen/ 100 g meat)	Conditioned Soluble (mg collagen/ 100 g meat)	Collagen Solubilized due to Conditioning (fold increase)
Psoas major	13.5	19.1	1.4
Extensor capri radialus	0.5	9.6	19.2
Supraspinatus	3.6	16.6	4.6
Gastrocnemius	13.8	22.0	1.6

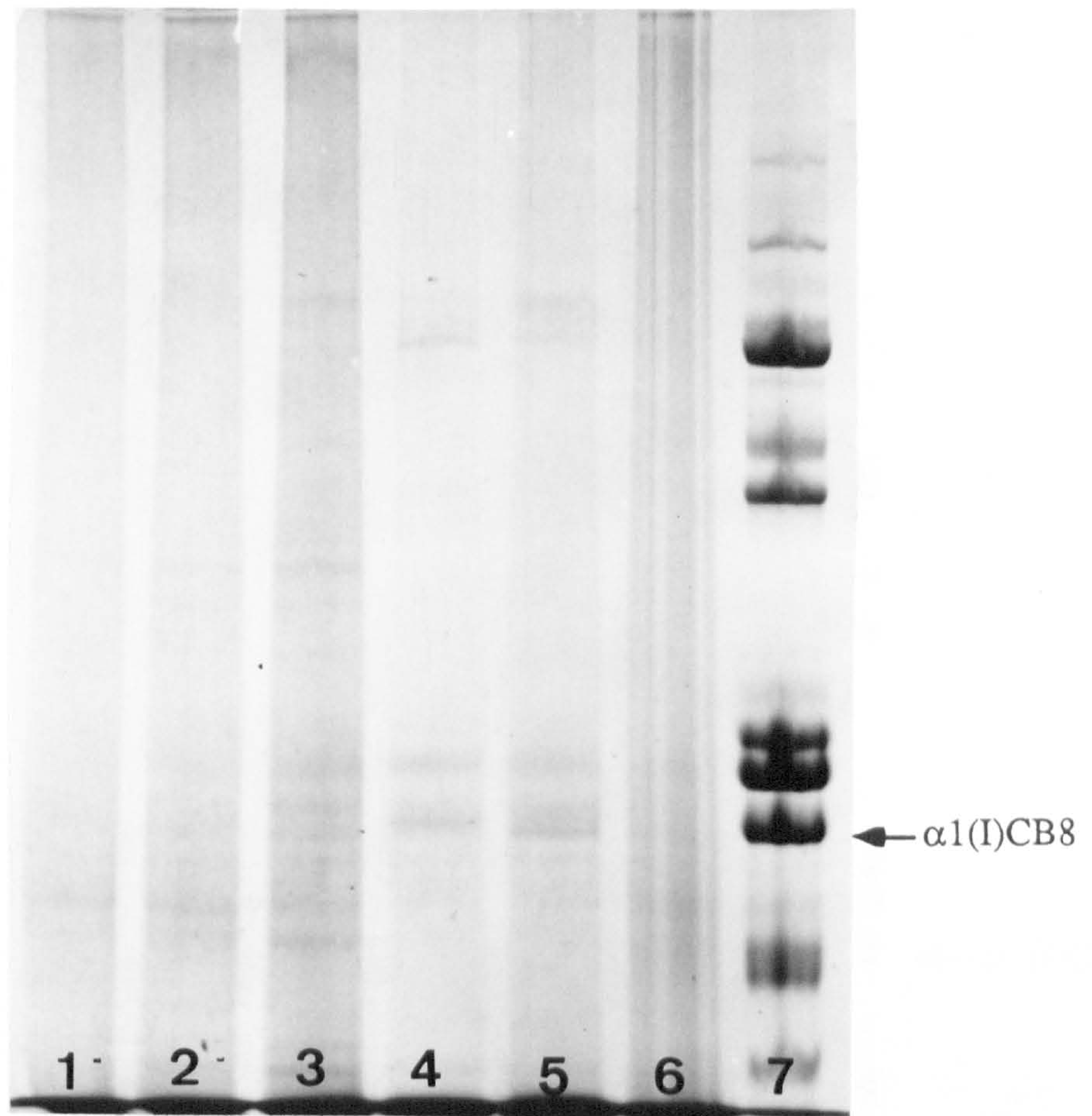


Fig. 4.5 SDS-Polyacrylamide Gel Electrophoretic Analysis of Endomysium from Bovine *Supraspinatus* Obtained by the Sequential Extraction Method.

Insoluble endomysium obtained by sequential extraction with 6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v) from unconditioned and conditioned muscles was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Tracks (1), (2) and (3) are CNBr peptide maps of insoluble endomysium from unconditioned *suprapsinatus* muscle. Tracks (4), (5) and (6) are CNBr peptide maps of insoluble endomysium from conditioned *supraspinatus* muscle. Track (7) is rat tail tendon collagen type I digest standard.

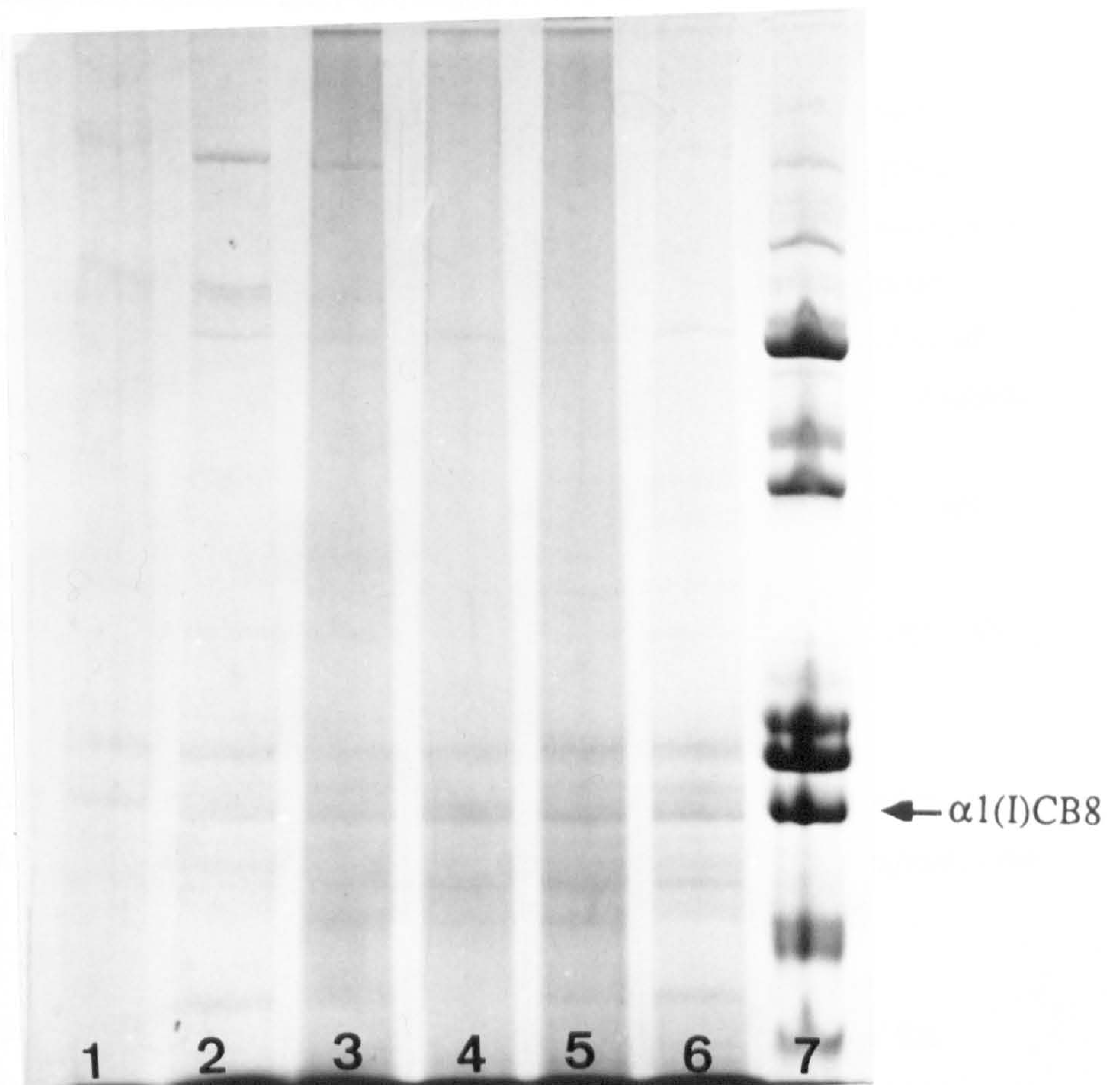


Fig. 4.6 SDS-Polyacryalmide Gel Electrophoretic Analysis of Endomysium from Bovine *Sternomandibularis* Obtained by the Sequential Extraction Method.

Insoluble endomysium obtained by sequential extraction with 6 M urea, 0.05 M tris-Cl, pH 7.4 and SDS (1 % w/v) from unconditioned and conditioned muscles was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Tracks (1), (2) and (3) are CNBr peptide maps of insoluble endomysium from unconditioned *sternomandibularis* muscle. Tracks (4), (5) and (6) are CNBr peptide maps of insoluble endomysium from conditioned *sternomandibularis* muscle. Track (7) is rat tail tendon collagen type I digest standard.

4.3.6 Analysis of Insoluble Endomysial Fractions Obtained by the SDS Extraction Method of Light & Champion (1984)

Insoluble endomysial materials obtained after extraction of unconditioned and conditioned bovine muscles were analyzed after CNBr digestion by SDS - polyacrylamide gel electrophoresis (Figs. 4.7, 4.8, 4.9 and 4.10). The main peptide components observed on analysis of endomysial material were derived from types I and III collagen, as expected (Light & Champion, 1984). While the gels obtained from *psoas major* and *extensor capri radialus* revealed no alterations in the peptide maps obtained after conditioning, those obtained from *gastrocnemius* and *supraspinatus* revealed the appearance of a number of new bands in the conditioned sample, which were absent from the unconditioned samples.

Densitometric scans of the bands obtained from $\alpha1(I)CB8$ and a1(III)CB5 were carried out on SDS-polyacrylamide gels and the ratios of type 1:III collagen analyzed for unconditioned and conditioned *gastrocnemius* and *psoas major* muscles. The results are presented in Table 4.7.

The ratios of types 1:III collagen on the gels obtained from all three unconditioned muscles examined were similar (Table 4.7). On conditioning, this value increased substantially for all muscles examined.

Using the formula for calculation described by Light (1982) for the estimation of the relative % content of type III collagen,it was possible to show an average decrease in

$$\text{Relative \% content of type III collagen} = \frac{\text{Wt of type III peak x 1.5}}{(\text{Wt of type III peak x 1.5 + Wt of type I peak})} \times 100$$

type III content after conditioning from 43.4 % (mean) to 33.9 % (mean) of the total fibrous collagen. This represents a 22 % decrease overall in type III content in the conditioned endomysial fractions compared with the unconditioned fractions.

4.4 DISCUSSION

The results presented in this chapter show that endomysial material is more readily solubilized by urea than perimysial material. Soluble endomysium accounts for 35 to 39 % of total extracted endomysium for unconditioned and conditioned muscles respectively, whereas the soluble perimysium only accounts for 15 to 24.6 % of total extracted perimysium for unconditioned and conditioned muscles respectively (Chapter 3).

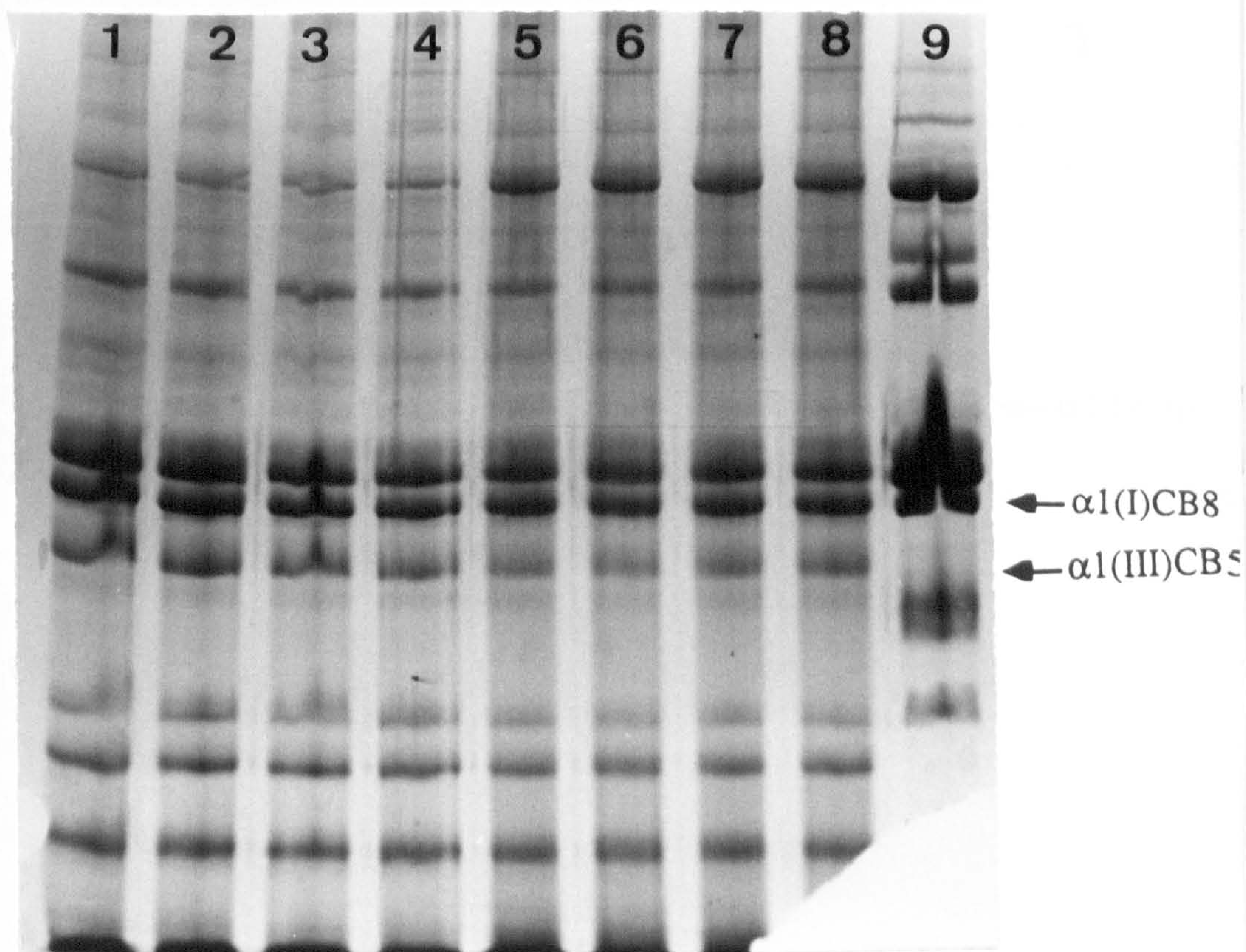


Fig. 4.7 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr Peptides of SDS Washed Endomysium from *Psoas Major*.

Insoluble endomysium obtained by the SDS extraction method of Light & Champion (1984) from unconditioned and conditioned muscles was digested with CNBr and analyzed by SDS-polyacryalmide gel electrophoresis as described in Chapter 2. Tracks (1), (2), (3) and (4) are CNBr peptide maps of insoluble endomysium from unconditioned *psoas major*. Tracks (5), (6), (7) and (8) are CNBr peptide maps of insoluble endomysium from conditioned *psoas major*. Track (9) is rat tail tendon collagen type I digest standard.

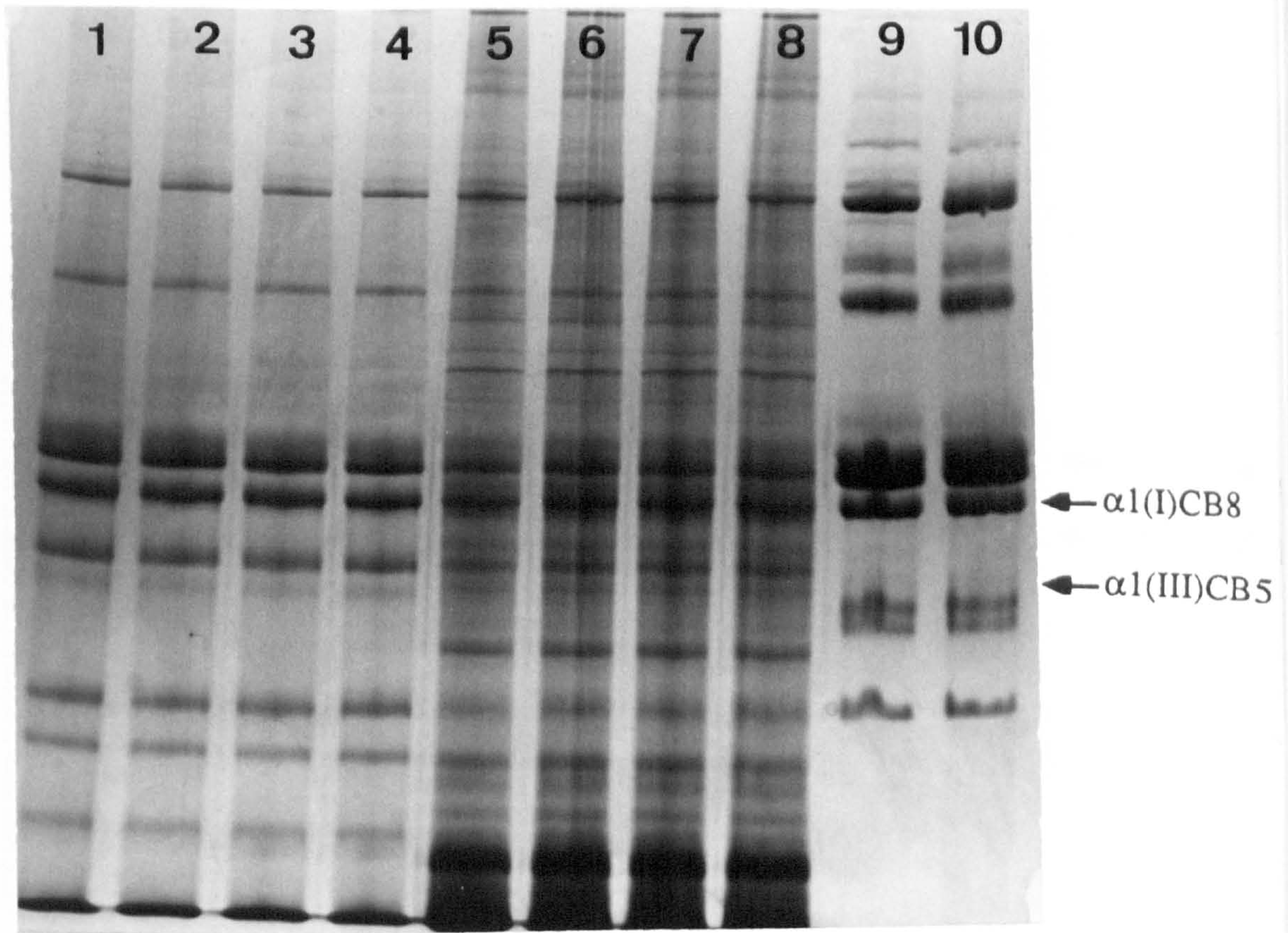


Fig. 4.8 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr-Peptides of SDS-Washed Endomysium from *Gastrocnemius*.

Insoluble endomysium obtained by the SDS extraction method of Light & Champion (1984) from unconditioned and conditioned muscles was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Tracks (1), (2), (3) and (4) are CNBr -peptide maps of insoluble endomysium from unconditioned *gastrocnemius* muscle. Tracks (5), (6), (7) and (8) are CNBr-peptide maps of insoluble endomysium from conditioned *gastrocnemius* muscle. Tracks (9) and (10) are rat tail tendon collagen type I digest standards.

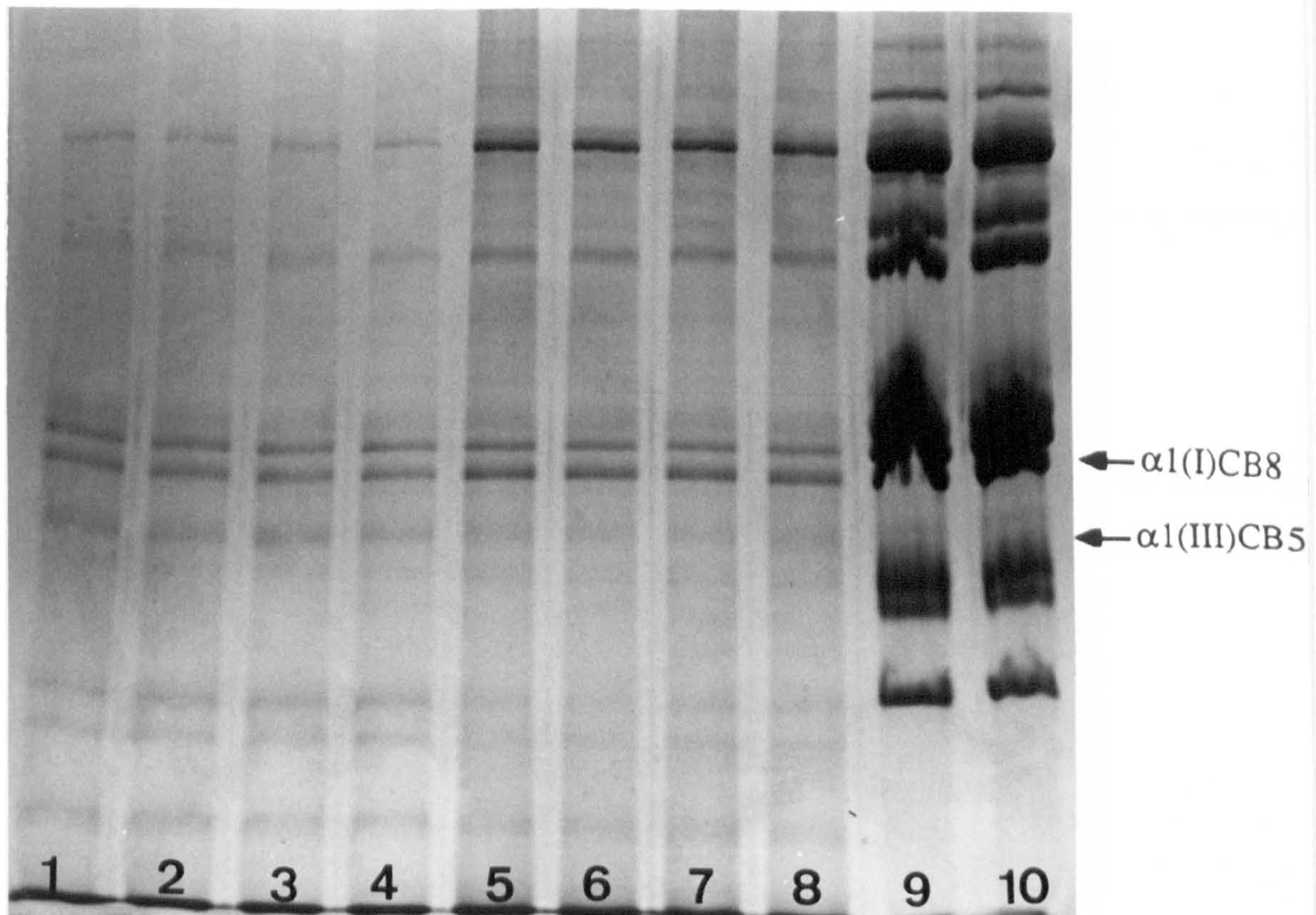


Fig. 4.9 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr-Peptides of SDS-Washed Endomysium from *Extensor Capri Radialus*.

Insoluble endomysium obtained by the SDS extraction method of Light & Champion (1984) from unconditioned and conditioned muscles was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Tracks (1), (2), (3) and (4) are CNBr peptide maps of insoluble endomysium from unconditioned *extensor capri radialus* muscle. Tracks (5), (6), (7) and (8) are CNBr peptide maps of insoluble endomysium from conditioned *extensor capri radialus* muscle. Tracks (9) and (10) are rat tail tendon collagen type I CNBr digest standards.

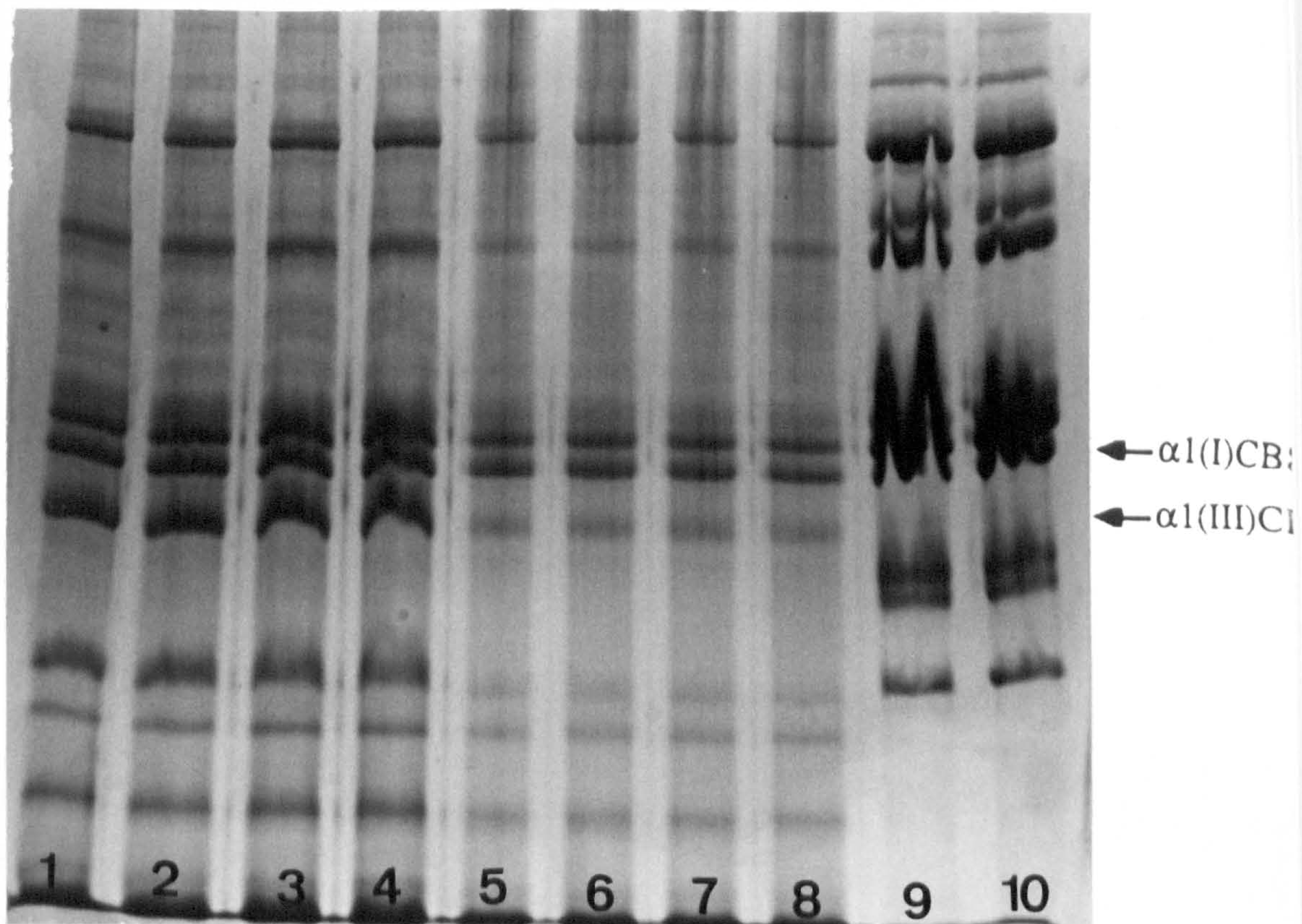


Fig. 4.10 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr-Peptides from SDS-Washed Endomysium from *Supraspinatus*.

Insoluble endomysium obtained by the SDS extraction method of Light & Champion (1984) from unconditioned and conditioned muscles was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2. Tracks (1), (2), (3) and (4) are CNBr peptide maps of insoluble endomysium from unconditioned *supraspinatus* muscle. Tracks (5), (6), (7) and (8) are CNBr peptide maps of insoluble endomysium from conditioned *supraspinatus* muscle. Tracks (9) and (10) are rat tail tendon collagen type I CNBr digest standards.

Table 4.7 Ratio of $\alpha1(I)CB8$ obtained from Unconditioned and Conditioned
$\alpha1(III)CB5$

Endomysial Bovine Muscle Collagen

Muscle	Unconditioned		Conditioned	
	Ratio Type 1:III	% type III relative to type I	Ratio Type 1:III	% type III relative to type I
Gastrocnemius	1.8:1	45.5	3.0:1	33.3
Psoas major	1.9:1	44.1	2.7:1	35.7
Psoas major	2.2:1	40.5	3.1:1	32.6

However, more perimysial collagen is solubilized; 3.19 to 5.64 % (percent collagen in solubilized fractions) for unconditioned and conditioned muscles, respectively, than endomysial collagen; 0.52 % to 0.5 % for unconditioned and conditioned muscles respectively. The high variability obtained in results of soluble perimysial and endomysial collagen content may be accounted for by sampling of connective tissue from different locations in the muscle. Results from endomysial fractions revealed no major changes in terms of solubilized material, solubilized endomysial collagen or CNBr peptide spectrum of insoluble endomysium due to conditioning. It was concluded that the method of extraction of endomysium with 6 M urea, 0.05 M tris-Cl, pH 7.4 was unsuitable for purification of endomysial collagen and for separation of soluble and insoluble endomysium. The endomysial preparations obtained were contaminated with attached perimysial fibres and non-collagenous material, resulting in extracts with such low collagen contents that accurate quantification was not possible.

Therefore, a second extraction, employing SDS was introduced into this procedure (the sequential extraction method) and its usefulness in separating soluble from insoluble endomysium and in producing a clean insoluble endomyusial preparation was investigated.

It may be expected that the total yields of endomysium would be greater employing the sequential extraction method because SDS binds to proteins forming a complex with a constant ratio of SDS to protein (about 1.4:1 by weight). However, when the results of endomysial yields obtained by the urea and sequential extraction methods were compared for equivalent muscles, it was found that this was not so for all cases examined. The total yield of endomysium (soluble and insoluble) obtained from unconditioned muscles was less in 80 % of the muscles examined, for the sequential extraction method employing SDS, than for the method employing urea alone. The total yield of endomysium obtained from conditioned muscles was greater in all five muscles examined by both extraction methods for the sequential method employing SDS, than for the method employing urea alone. However, when the yields of soluble and insoluble fractions obtained from the same muscles by both methods were examined, it was found that it was the yields of insoluble endomysia from conditioned muscles obtained by the sequential extraction method employing SDS that were remarkably higher than their unconditioned counterparts. Considering the data in Table 4.2 and 4.4 it is evident that sequential extraction resulted in an average increase in total insoluble material averaging 176 % for the 5 conditioned muscles also investigated by the urea method. On the other hand insoluble endomysial residues prepared from the same five unconditioned muscles by sequential extraction showed a decrease in mean weight to 87 % of urea extracted insoluble endomysia. The reasons for this are not well understood but this observation

may suggest that the conditioning effect on the insoluble endomysium remaining was such that the matrix was damaged in such a way that the preliminary urea extraction made it possible for SDS to be bound more easily to its constituent proteins than was possible for the undamaged insoluble endomysium obtained from unconditioned muscles. However, there is no evidence to support this hypothesis and resolution of the problem must await further work.

The results of the study employing the sequential extraction method for purification of endomysial fractions revealed no statistically significant difference in yields of soluble endomysial material or in the collagen content of the solubilized endomysial fractions. The soluble endomysial fraction represented, on average, 46 % of total extracted endomysial material in the unconditioned muscles, compared with a value of 29 % for conditioned muscles. The lower value obtained for the soluble endomysial fraction (expressed as a percentage of total extracted endomysium for conditioned muscles), resulted from the abnormally high yields of insoluble endomysia obtained from conditioned muscles which employed the sequential procedure, and which distorted the results as discussed above. It was concluded that the sequential extraction method for isolation of endomysial fractions was unsuitable for the purpose of investigating conditioning effects on the solubilization of endomysial fractions from bovine muscle, due to the fact that accurate quantification of yields of the endomysial fractions was prevented by employing SDS in this procedure. Furthermore, the method proved inadequate for quantification of solubilized endomysial collagen. As observed previously in Chapter 3 for gross-SDS-solubilized connective tissue fractions, the collagen content of the solubilized endomysial fractions, extracted by the sequential extraction method, which employed SDS in the procedure, resulted in high yields of solubilized endomysial material, relative to the low collagen contents. Therefore, this protein fraction was diluted to such an extent that it was unquantifiable in the soluble endomysial fractions obtained by seqential extraction. Although the insoluble endomysial fractions obtained using the sequential extraction method were more purified than those obtained using the procedure which employed urea extraction alone, the CNBr peptide maps of the insoluble endomysial collagen were of poor quality and were unsuitable for analysis. Therefore, it was impossible to reach any valid conclusions on the effects of conditioning on insoluble endomysial collagen, using the sequential method of endomysial extraction.

Results from the SDS extraction method of Light & Champion (1984) showed that the percentage solubilized endomysium was greater for all 4 conditioned muscles examined when compared to the same unconditioned muscles. This fraction represented, on average 97.5 %, for conditioned muscles, compared to a value of 94.5 % for

unconditioned muscles. It should be noted here that the SDS extraction method was much more efficient at solubilizing endomysial material than either the urea or sequential methods of endomysial extraction. The urea extraction method solubilized 35 % and 39 % of total endomysial material for unconditioned and conditioned muscles, respectively, while the sequential extraction method solubilized 46 % and 29 % of total endomysial material, for unconditioned and conditioned muscles, respectively. However, the corresponding fractions represented close to 100 % for the SDS method of endomysial extraction. The collagen content of the soluble endomysia obtained from the four conditioned muscles was higher in all cases examined than that of the unconditioned muscles. The two highest quality muscles examined in the study, *psoas major* and *gastrocnemius*, indicated the highest yields of soluble collagen before and after conditioning, while the percentage collagen solubilized due to conditioning was lower for these muscles than for the lower quality muscles studied. The average percentage endomysial collagen solubilized from the four muscles studied was 0.13 ± 0.11 % for unconditioned muscles and 0.22 ± 0.05 % for conditioned muscles. These values are much lower than the average percentage perimysial collagen solubilized from unconditioned and conditioned muscles by urea extraction (Chapter 3), which were found to be 1 ± 0.8 % and 3.4 ± 3.3 % for unconditioned and conditioned muscles, respectively. Conditioned *psoas major* and *gastrocnemius* muscles showed increases of about 1.5-fold over unconditioned muscles, while *supraspinatus* and *gastrocnemius* indicated much higher increases of 5-19-fold over the unconditioned muscles. The fact that there are variations in the extent of endomysial collagen solubilization suggest that it is random and uncontrolled, as shown for perimysium in Chapter 3. The higher quality muscles examined yielded relatively high quantities of proteolyzed soluble perimysial (Chapter 3) and endomysial collagen on conditioning, which might suggest a correlation between high yields of solubilized intra-muscular connective tissue collagen and tenderness of meat. However, as discussed in Chapter 3, some poor quality muscles showed similar changes, therefore, it appears that there is little correlation between known muscle quality and solubility of intra-muscular collagen, on conditioning.

The endomysial collagenous residue which remained insoluble after conditioning was purified by the SDS extraction method of Light & Champion (1984). Changes observed on the peptide maps obtained by SDS-polyacrylamide gel electrophoresis appeared to be muscle specific. *Gastrocnemius* and *supraspinatus* revealed the appearance of a number of new peptide bands compared to the unconditioned insoluble endomysium, while *psoas major* and *extensor capri radialus* revealed no alterations in the peptide maps obtained after conditioning. However, it was unclear whether the new peptide material evident on SDS-polyacrylamide gels was collagenous, and further work with two-dimensional

electrophoretic analysis of unconditioned and conditioned endomysial material attempts to resolve this problem in Chapter 6.

Analysis of the CNBr peptide patterns obtained, by comparison of collagen types I:III ratios of insoluble endomysia from unconditioned and conditioned muscles, indicated that the endomysial fraction was modified on conditioning. The percentage type III collagen relative to type I collagen in endomysia obtained from unconditioned muscles ranged from 40.5 to 45.5, which decreased on conditioning to 32.6 to 35.7. These results seem to indicate that type III collagen is preferentially destroyed during conditioning. This may indicate that type III collagen has a key role in determining textural properties, as sensitivity to proteolytic attack may be seen as a pre-requisite for 'good' or effective conditioning. This result is supported by earlier work by Bailey et al (1979) who, in an attempt to discover the roles of the two major fibrous forms of this protein in determining meat texture, showed a correlation between a higher content of type III collagen and toughness and Deethardt & Tuma (1971) who showed that reticulin fibres (which may be types III collagen (Nowack et al., (1976)) showed greater resistance to cooking than other collagenous fibres. However, these results tend to conflict with those of Wu et al. (1982) who suggests that type III collagen is more resistant to proteolytic attack during conditioning than type I collagen. Light (1987) indicates a role for type III collagen in contributing to the overall toughness of meat. Although it may not be more resistant to proteolytic attack, as suggested earlier (Wu et al., 1981), type III collagen may have a preferential role to play in determining some textural properties of meat. Lovell et al. (1987) showed that type III collagen in human skin becomes more resistant to pepsin degradation with age. However, in young humans (birth to 25 years) type III collagen was consistently solubilized by pepsin.

In the present study, animals less than 2 years of age were used, equivalent in age to young humans (less than 15 years of age). The results of this study suggest that type III collagen is more susceptible to proteolysis during conditioning than type I collagen. This may indicate that topographical and steric forces have an important role to play in the susceptibility of type III collagen to proteolysis during conditioning. The type III collagen triple helix is absent from the surface of mature large diameter fibrils (Nowack et al., 1976; Keene et al., 1987). However, type III collagen was observed on fibrils of small diameter, which suggest that on maturation, they may have been buried within the interior of the fibril, making them less resistant to proteolytic attack, due to their location.

Early attempts to isolate collagen from muscle employed extraction methods with urea, acetate and dilute sodium hydroxide (Gallop & Seifter, 1963; Kauffman et al., 1964;

Jackson & Cleary, 1967). These methods proved inadequate for the removal of interfering muscle proteins without denaturing the collagenous proteins. Subsequently, McClain devised a simple procedure for isolating intramuscular connective tissue in the native state (McClain, 1969). The procedure was based on the selective fragmentation of the muscle tissue and involved grinding frozen samples at two different speeds at -20° C. The intramuscular tissues were then isolated by filtering the blended samples through a stack of three different sizes of sieves. Utilizing this method only 60 to 70 % of the total collagen in muscle tissue could be isolated, while the isolated collagen was still contaminated with structural proteins from muscle (McClain, 1974). Furthermore, this method yielded a final preparation of perimysium and endomysium in unknown proportions. Fujii & Murota (1982) described a method for the preparation of washed muscle connective tissue without the use of protein denaturants. This method employed rapid stirring of the homogenate of skeletal muscle with a magnetic stirrer. Fibrous material entangled during rapid stirring was recovered by passing the homogenate through a sieve and then sequentially extracted with Hasselbach-Schneider solution and 0.6 M KI/0.06 M $Na_2S_2O_3$. Greater than 90 % of the total collagen in muscle was recovered with this method and was shown to be highly purified. However, the method again yielded a mixture of both perimysium and endomysium.

A method that can preparatively differentiate between the perimysium and endomysium which combined an original method of McCollester (1962) for the preparation of endomysial 'ghosts', extraction solvents of Hasselbach & Schneider (1951) and Laurent et al. (1981) was described by Light & Champion (1984). Connective tissue thus prepared may be washed with salt and treated with pepsin to liberate soluble native collagen, or washed with SDS to produce a very clean insoluble collagenous product. The procedure employed careful filtration and included SDS in the washing procedures after the homogenization process described by McCollester (1962) and allowed the bulk separation of the perimysium from the endomysial segments and their associated finer fibres. An electron-microscopal survey of collagen fibre size definition based on the work of Rowe (1978) who showed a difference in collagen fibre diameter in endomysium and perimysium was used to define the different connective tissue pools obtained. Quantification of fibre diameter confirmed the efficiency of the preparative method for perimysium and endomysium. Recoveries of 60 to 90 % of total collagen in muscle tissues were obtained using this method.

Crude extraction methods using SDS alone or urea were not successful in the present study, largely due to the presence in extracts of excess non-collagenous (presumably intracellular) material which was variable in amount, thus affecting total yield estimates.

Furthermore, quantification of the 'diluted' soluble collagen was impeded employing these methods of endomysial extraction. It was only by sequential use of hypotonic solutions of $CaCl_2$ and histidine, to remove most, if not all, intracellular material, followed by SDS to solubilize more intractable collagen fragments that a successful result was obtained in these studies.

Weakening of the lysosomal membranes post-mortem, results in the release of lysosomal cathepsins (Dutson & Lawrie, 1974; Wu et al., 1981). The endomysium is that connective tissue fraction which represents the first collagenous barrier for lysosmal cathepsin attack once these proteases have breached the sarcolemma. Therefore, the contribution of the endomysial connective tissue fraction to the overall textural properties of meat may be expected to be substantial. However, little or no evidence is available in the literature to show the direct *in situ* effect of these proteases on the isolated endomysial component of bovine connective tissue in conditioned meat.

Results of this study suggest that post-mortem effects lead to subtle modifications occurring in the endomysium of bovine muscles due to the conditioning process. These post-mortem effects were evident as higher yields of solubilized endomysial material and solubilized endomysial collagen, in conditioned muscles than unconditioned muscles, and significant alterations to the collagen component of the insoluble endomysium remaining after conditioning. Such modifications to the endomysial fraction of meat after conditioning may be expected to have a profound effect on determining the perceived changes in textural quality of conditioned meat.

CHAPTER 5

INVESTIGATION OF 2-D ELECTROPHORESIS SYSTEMS AND THEIR SUITABILITY FOR USE IN INVESTIGATING INSOLUBLE MEAT COLLAGEN

5.1 INTRODUCTION

In Chapters 3 and 4 evidence has been provided which shows that conditioned meat yielded significantly greater quantities of solubilized perimysial and endomysial collagen than unconditioned meat, although in both cases the bulk of meat collagen remained insoluble after conditioning. This increased solubilization of perimysial and endomysial collagen in conditioned meat suggested that proteolytic damage was inflicted during the conditioning process. One-dimensional SDS-polyacrylamide gel electrophoresis provided little or no evidence for such damage in the insoluble perimysium and endomysium remaining after conditioning. Therefore, there was a need for more sensitive techniques to determine if such changes in the insoluble collagen fractions occurred during conditioning.

High resolution of complex mixtures of proteins can be achieved by two-dimensional (2-D) gel electrophoresis. 2-D electrophoretic protein-mapping techniques have been developed which use a charge separation (isoelectric focussing) in the first dimension and a size separation (SDS-polyacrylamide gel electrophoresis) in the second dimension (O'Farrell, 1975). Since its introduction, this method has become one of the most widely used methods for separation of complex protein mixtures. The principal reason for its success is that it separates denatured proteins according to two different independent parameters. Separation in the first dimension is based on charge, which is reflected by the isoelectric point (pI), followed by molecular weight separation in the second dimension. Consequently, individual proteins are separated as discrete spots on the gel. O'Farrell (1975) demonstrated the potential of this technique for separation of *Escherichia coli* proteins which was capable of resolving over 1000 proteins with isoelectric points in the pH range 4 to 7. However, when the pH gradient was extended beyond pH 7, the few slightly basic proteins which entered the gel were poorly resolved (O'Farrell et al., 1977) and the pH gradient could not be further extended to include very basic proteins.

An alternative procedure was developed for the first dimension by O'Farrell et al. (1977) which, unlike IEF, resolved basic as well as acidic proteins, and was referred to as nonequilibrium pH gradient electrophoresis (NEPHGE). The main differences between

NEPHGE and IEF are that in the former the samples are applied to the acidic side of the gel and the product of voltage and time is smaller than in IEF. Under such conditions, the pH gradient does not reach full equilibrium. As a result, the proteins are not totally focussed at their isoelectric point as they are in IEF gels. Nevertheless, most proteins in NEPHGE gels are separated according to their charges. NEPHGE was combined with SDS-polyacrylamide gel electrophoresis resulting in a high resolution 2-D procedure, NEPHGE-SDS. The resolving power of NEPHGE-SDS was demonstrated for *Escherichai coli* proteins and eucaryotic proteins (from African green monkey kidney).

Finlayson & Chrambach (1971) reported the successful focussing of collagen CNBr cleavage peptides and subsequently the technique was combined with SDS-polyacrylamide gel electrophoresis to obtain high resolution of collagen CNBr cleavage peptides from the α1-chain of collagen types I, II and III from rabbit fetal skin fibroblasts and adult articular chondrocytes (Benya, 1981) and from CNBr cleavage peptides from human types I, II, III and V collagen (Cole & Chan, 1981). The latter employed buffers, solutions and procedures as described by O'Farrell et al. (1977). 2-D electrophoretic studies have shown that CNBr cleavage peptides of collagen exist in multiple charged forms (Benya, 1981; Cole & Chan, 1981). Charge heterogeneity was also evident after isoelectric focussing of α1 (I) peptides by Finlayson & Chrambach (1971) and Butler et al. (1967) observed that α1(I)CB6, CB7 and CB8 peptides were heterogeneous, as each peptide could be resolved into several components by rechromatography on carboxy-methyl-cellulose. Charge heterogeneity was not detected when collagen CNBr cleavage peptides were analyzed by SDS-polyacrylamide gel electrophoresis alone (Cole & Chan, 1981). The separation of the multiple charged forms of the CNBr cleavage peptides from collagen using NEPHGE-SDS was potentially an elegant method for studying conditioning effects on bovine perimysial and endomysial collagen and this chapter presents an investigation of its usefulness in this application.

In addition, a second 2-D protein mapping technique was studied in this respect. Cleveland et al. (1977) devised an elegant method to map in one dimension the peptides of almost any given protein. The method involved limited proteolysis in the presence of SDS and the partial digestion products were analyzed by SDS-polyacrylamide gel electrophoresis. Under a particular set of proteolysis conditions, a protein yielded a reproductible pattern of partial digestion products. The method of Cleveland et al. (1977) was adapted for the analysis of heterogeneous protein samples (Bordier & Crettol-Jarvinen, 1979). Using this procedure, the proteins of the mixture were separated in the first dimension by SDS-polyacrylamide gel electrophoresis. The proteins of the sample were subsequently subjected to limited proteolysis in the gel and a second dimension

electrophoresis at right angles to the first gel. The peptides of each individual protein were resolved as a series of spots located below the original position of the undigested protein/peptide spot. The method was demonstrated for separation of protein P23 and P23* of bacteriophage T_4, the proteins of *Dictyostelium discoideum* membranes, the proteins of human erythrocyte membranes and ^{35}S-labeled proteins of *D. discoideum* synthesized *in vivo* and *in vitro* in a cell-free wheat germ extract using *Saphylococcus aureus* strain V8 protease (Bordier & Crettol-Jarvinen, 1979). The resolving power of this method depends on the molecular weight distribution, relative amount, and protease susceptibility of the individual proteins of the sample. This chapter investigates the potential of this method for studying conditioning effects on bovine perimysial and endomysial collagen and describes a modification of the 2-D method of Bordier & Crettol-Jarvinen (1979) for resolution of collagen CNBr cleavage peptides, employing the proteases, trypsin, chymopapain and chymotrypsin.

5.2 MATERIALS AND METHODS

All electrophoresis reagents including ampholines, Ultrapure grade of urea and proteases were purchased from BDH Chemicals Ltd. or Sigma Chemicals Ltd., both of Poole, Dorset, U.K. Solutions of acrylamide were filtered prior to use.

5.2.1 Preparation of Insoluble Perimysium and Endomysium

Unconditioned and conditioned muscles were prepared as outlined in Chapter 2 (section 2.3). Insoluble perimysium was extracted and purified as outlined in Chapter 3 (section 3.2.3) employing 6 M urea, 0.05 M tris-Cl, pH 7.4 as extractant while insoluble endomysium was purified as outlined in Chapter 4 (section 4.2.3) employing SDS as extractant. Both insoluble perimysial and endomysial CNBr cleavage peptides were exhaustively dialyzed prior to 2-D analysis.

5.2.2 Proteases Employed in 2-D Electrophoretic Analysis

All enzymes selected for the SDS-polyacrylamide gel electrophoresis-proteolysis technique had pH optima in the range 6-8. These were trypsin (EC 3.4.21.4) which is specific for the carboxyl side of arg and lys residues and it has a pH optimum of 7.8, and chymotrypsin (EC 3.4.21.1) (MW 22 600) with a pH optimum of 7.8 and is specific for trp, phe, tyr (leu, met, Asn, his) residues.

Chymopapain (EC 3.4.22.6) was also investigated. Chymopapain is a sylphydryl protease found in large quantity in papaya latex, existing in a number of forms, along with papain and a lysozyme. The molecular weight of the sub units ranges from 22 - 36 000. Chymopapain has an optimum pH of 7.0 - 7.5 (Ebata & Yasunobu, 1962), is activated by cysteine and cyanide and inhibited by iodoacetate, p-chloromercuribenzoate and DFP (Ebata & Yasunobu, 1962).

5.2.3 NEPHGE-SDS

Non-equilibrium pH-gradient electrophoresis (NEPHGE) in polyacrylamide tube gels was carried out as the first dimension of two-dimensional analysis using the procedure described by O'Farrell et al., (1977). Samples (exhaustively dialyzed CNBr-cleavage peptides) were dissolved in sample buffer (9 M urea, 0.5 % (w/v) SDS and 0.2 % (v/v) ampholines) at a concentration of 20 mg/ml. 10 μl of 0.02 M NaOH was added to 50 μl of sample to convert homoserine lactone into homoserine (Finlayson & Chrambach, 1971) and 30-45 μl of this solution was applied to the top of the gel. The gel mixture was composed of 9.2 M urea, 2 % (v/v) Nonidet P-40 (NP-40), 4 % (w/v) acrylamide/ bis-acrylamide (from a stock solution containing 28.4 % (w/v) acrylamide and 1.6 % (w/v) bis- acrylamide) and 2 % (v/v) ampholines, consisting of either 1.8 % (v/v) ampholines pH 3.5-10 and 0.2 % (v/v) ampholines pH 5-8 or 2 % (v/v) ampholines pH 3.5-10. Gels were polymerized with 20 μl of a 10% (w/v) aqueous solution of ammonium persulphate and 14 μl of TEMED per 10 ml of gel mixture. The gels were overlayed with water and allowed to polymerize for 1 to 2 h. The lower reservoir was filled with 0.02 M NaOH and the tubes were placed in the electrophoresis chamber. Samples were loaded and overlayed with 20 μl overlay solution (8 M urea and a mixture of 1.8 % (v/v) ampholines pH 3.5-10 and 0.2 % (v/v) ampholines 5-8) and the tubes were filled with 0.01 M phosphoric acid. The upper reservoir was filled with 0.01 M phosphoric acid. The gels were electrophoresed at 500 V for 3 h with the cathode on the bottom and the anode on the top. At the end of the run, the gels were extruded from the tubes, equilibrated for 2 h in SDS sample buffer (10 % (v/v) glycerol, 2.3 % (w/v) SDS and 0.0625 M tris-Cl, pH 6.8) and used immediately or stored frozen at -20° C.

Equilibrated electrofocusing gels from the first dimension were subjected to SDS-polyacrylamide gel electrophoresis in the second dimension in 10 % (w/v) acrylamide slabs as described above. Electrofocussing gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. The course of the electrophoresis was followed by means of tracking dye (bromophenol blue). Gels were stained with Coomassie Brilliant Blue R-250 and destained as described in Chapter 2 (section 2.8.4).

5.2.3.1 Measurement of pH Gradient

The isoelectric focussing gels were cut into 5 mm sections once isoelectric focussing was complete. This was evidenced by formation of sharp bands from a sample of applied haemoglobin, indicating completion of the process. Gel sections were placed in individual vials containing 2 ml degassed H_2O. Vials were capped and shaken for 5 to 10 min, prior to measurement of pH using a standardized microelectrode.

5.2.4 SDS-Polyacrylamide Gel Electrophoresis - Proteolysis Technique

30-50 µl of CNBr digests of perimysial and endomysial samples, dissolved in sample buffer at a concentration of 5 mg/ml and 10 mg/ml respectively, were resolved in the first dimension in 10% (w/v) polyacryamide tube gels containing SDS and tris buffers as described in Chapter 2 for one-dimensional SDS-polyacrylamide gel electrophoresis (section 2.8.3). Following electrophoresis, the tube gels were incubated in 50 ml of 125 mM tris-HCl, pH 6.8, containing 0.1% (w/v) SDS for 40 min at room temperature. Tube gels were used immediately for second dimension analysis or stored frozen at -20° C. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs containing 0.1 % (w/v) SDS as described above. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 300 to 400 µl of enzyme solution (section 5.2.3.1) in gel sample buffer (section 2.8.2) was applied to the top of the gel, carefully overlaid with electrode buffer and current was applied until the bromophenol blue marker was visible just below the first dimension gel. The current was switched off and the system was incubated for various times whilst proteolysis occurred in the first dimension gel. Second dimension analysis was then continued as described in Chapter 2 for one-dimensional SDS-polyacrylamide gel electrophoresis (section 2.8.3). Gels were stained with Coomassie Brilliant Blue R-250 and destained as described in Chapter 2 (section 2.8.4).

5.2.4.1 Conditions of Proteolysis Employed in the Second Dimension

All enzymes selected for this study had pH optima in the range 6 to 8. Trypsin was dissolved in gel sample buffer at a concentration of 5 mg/ml (0.2 units/ml) and 300 µl of this solution was applied to the top of the resolving gel prior to the second dimension analysis. Proteolysis was allowed to continue for times ranging from 0 to 24 h.

Chymopapain was dissolved in gel sample buffer at concentrations of 3.33 mg/ml (4.33 units/ml) and 50 mg/ml (66.7 units/ml). 300 µl of these solutions was applied to the top

of the resolving gel prior to the second dimension analysis. Proteolysis was allowed to continue for times ranging from 2 to 24 h.

Chymotrypsin was dissolved in gel sample buffer at a concentration of 1.33 mg/ml (200 units/ml) and 400 µl of this solution was applied to the top of the resolving gel prior to the second dimension analysis. Proteolysis was allowed to continue for times ranging from 2 to 3 h.

As a control two-dimensional separation of CNBr cleavage peptides from insoluble perimysium was carried out, without proteolysis, first in 10 % (w/v) acrylamide tube gels followed by separation at right angles in 10 % (w/v) acrylamide slab gels as a control.

5.3 RESULTS

5.3.1 NEPHGE-SDS

Two approaches were investigated for the resolution of purified CNBr cleavage peptides from insoluble perimysium obtained from unconditioned and conditioned bovine muscles, involving modifications of the first dimension (NEPHGE), while the second dimension (SDS-polyacrylamide gel electrophoresis) remained the same as that outlined in Chapter 2 (section 2.8.3).

5.3.1.1 First Dimension (NEPHGE) using Ampholines pH 3.5-10

The first approach involved the use of ampholines with pH range of 3.5-10, for separation of CNBr cleavage peptides from insoluble perimysium, in the first dimension. An example of the final pH gradient obtained is shown in Fig. 5.1. The pH gradient was formed after electrophoresis for 1500 V h. The pH gradient was relatively stable in the more acidic pH range, but not at the basic end of the gel.

The resolution of CNBr cleavage peptides from insoluble perimysium obtained from unconditioned *serratus ventralis* bovine muscle is shown in Fig. 5.2. The left side of the gel pattern represents the top (acidic end) of the first dimension gel. The resolution of the collagen CNBr cleavage peptides produced poor results (Fig. 5.2) employing ampholines with the pH range 3-10. One peptide on the gel had more than one charged form of the same molecular weight, while the second was detected as a single spot. Most collagen CNBr peptides in the applied sample were not detected.

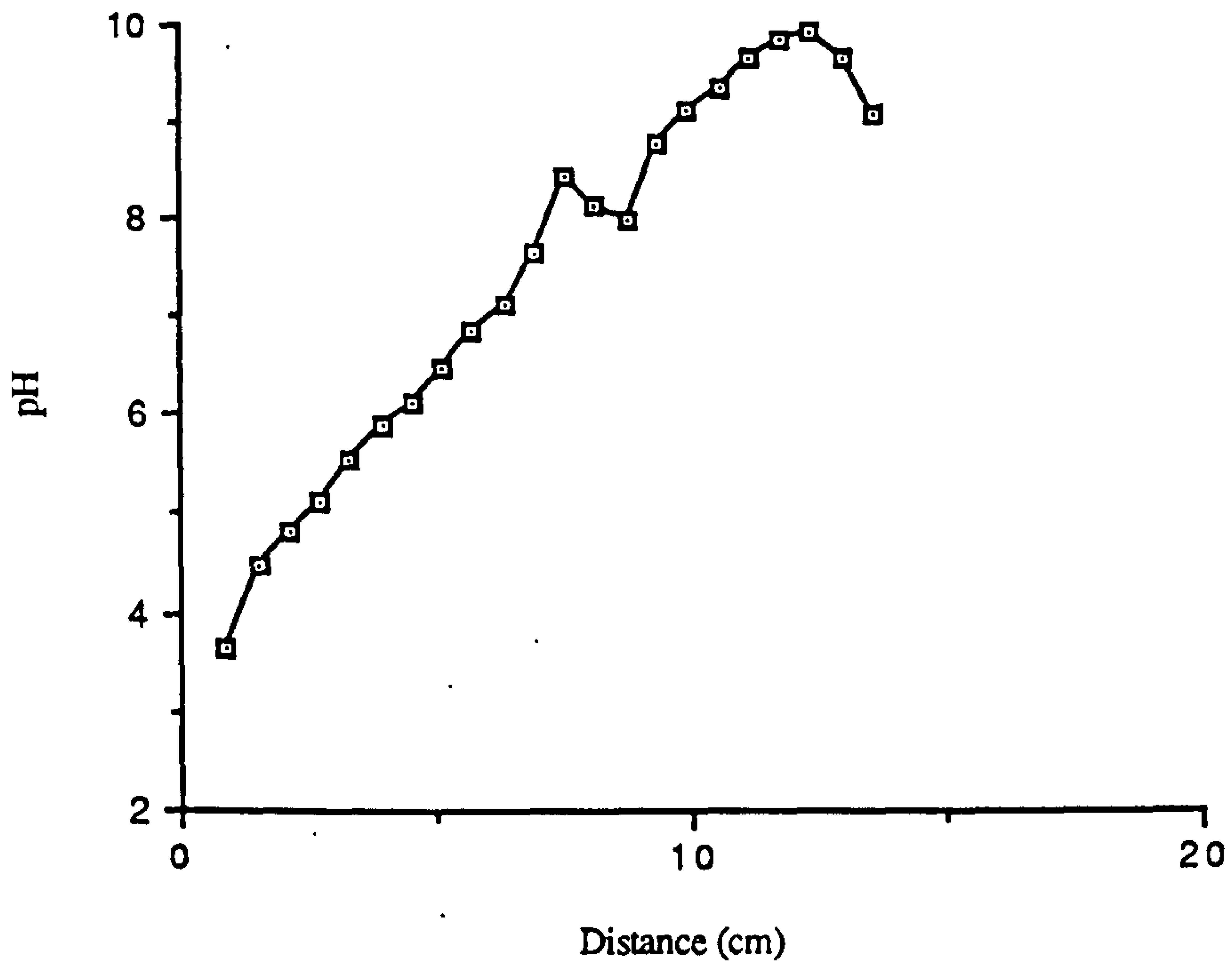


Fig. 5.1 Formation of pH Gradient in First Dimension NEPHGE Tube Gels (pH 3.5 - 10 Ampholines).

Fig. 5.2 2-D NEPHGE Separation of CNBr-Peptides from Insoluble Perimysium (pH 3.5 - 10 Ampholines).

Exhaustively dialyzed CNBr-cleavage peptides, extracted from unconditioned *serratus ventralis* bovine muscle using 6 M urea, 0.05 M tris-Cl, pH 7.4, were subjected to electrophoresis in the first dimension for 3 h at 500 V, on a pH 3.5 - 10 gradient (comprised of 2 % (v/v) ampholines pH 3.5 - 10). First dimension gels were electrophoresed in the second dimension on 10 % (w/v) acrylamide SDS slab gels using conditions as described in Chapter 2.

5.3.1.2 First Dimension (NEPHGE) using Ampholines pH 3-10 and pH 5-8

The second approach involved the use of ampholines with pH range of 3.5-10 and 5-8, for separation of CNBr cleavage peptides from insoluble perimysium, in the first dimension. An example of the final pH gradient obtained is shown in Fig. 5.3. The final pH gradient was formed after 1500 V h. The pH gradient obtained using ampholines pH 3.5-10 and pH 5-8 was stable throughout its entire range (Fig. 5.3).

The resolution of CNBr cleavage peptides from insoluble perimysium obtained from unconditioned *gastrocnemius* bovine muscle is shown in Fig. 5.4. The left side of the gel pattern represents the top (acidic end) of the first dimension gel. The CNBr cleavage peptides were well separated (Fig. 5.4) using a combination of pH 3.5-10 and 5-8 ampholines, relative to the results obtained using pH 3.5-10 ampholines alone (Fig. 5.2). Most peptides detected had more than one charged form of the same molecular weight (Fig. 5.4).

5.3.2 SDS-Polyacrylamide Gel Electrophoresis-Proteolysis Technique

CNBr cleavage peptides from insoluble perimysium obtained from conditioned *gluteus medius* bovine muscle were separated by SDS-polyacrylamide gel electrophoresis, first in a 10 % (w/v) acrylamide tube gel, followed by electrophoresis at right angles in a 15 % (w/v) acrylamide slab gel (Fig. 5.5). The electrophoretic separation of the peptides of the sample was performed in a first dimension from left to right and resolution of the separated products was performed in a second dimension from top to bottom all subsequent gels were run in the same way. The diagonal line of spots extending from top left to lower right of Fig. 5.5 was formed by the 2-D separation of the peptides of the sample and represents the undigested control to the subsequent figures in this chapter.

Fig. 5.6 shows the 2-D peptide pattern obtained from partial digestion with 300 µl trypsin solution (5 mg/ml, 0.2 units/ml) of CNBr cleavage peptides obtained from unconditioned *extensor capri radialus* bovine muscle. The stacking phase, during which digestion of the peptide products by trypsin took place, was not halted. The partial proteolysis products of the individual peptides were found below the diagonal under the position of the undigested protein. However, only very limited digestion of the peptides took place, evident as a series of spots below the undigested peptides (Fig. 5.6).

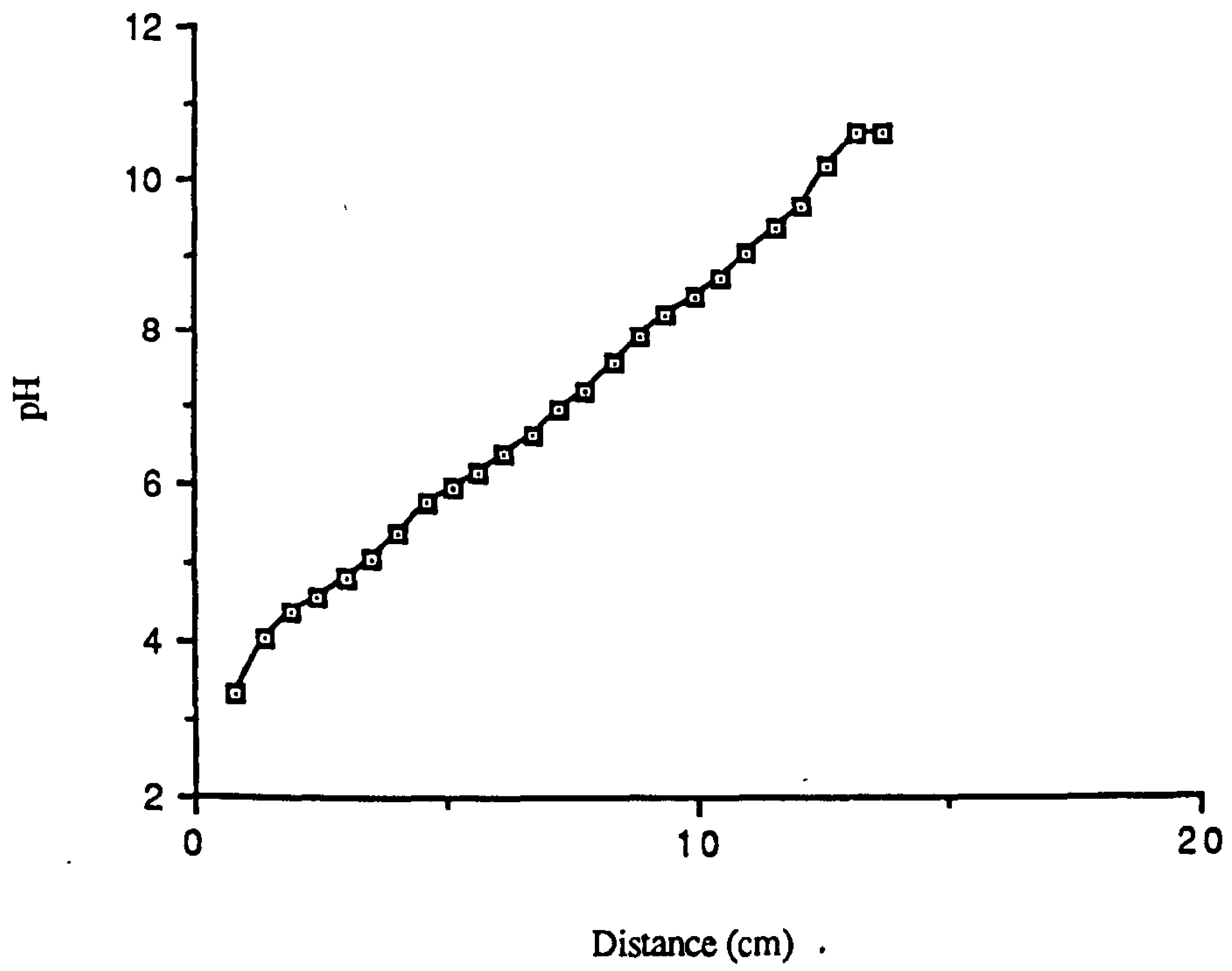


Fig. 5.3 Formation of pH Gradient in First Dimension NEPHGE Tube Gels (pH 3.5 - 10 and 5 - 8 Ampholines).

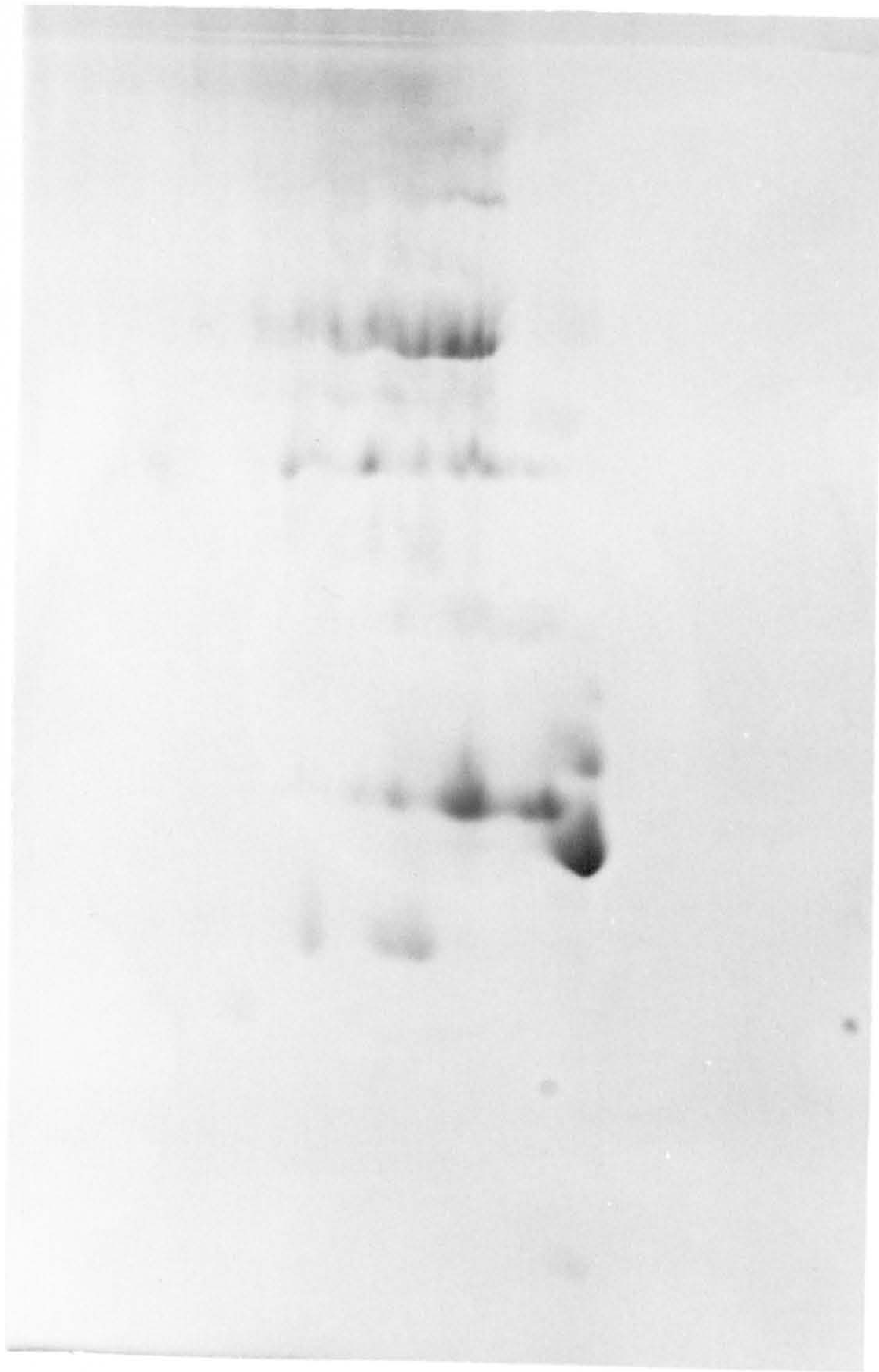


Fig. 5.4 2-D NEPHGE Separation of CNBr-Peptides from Insoluble Perimysium (pH 3.5 - 10 and 5 - 8 Ampholines).

Exhaustively dialyzed CNBr-cleavage peptides, extracted from unconditioned *gastrocnemius* muscle using 6 M urea, 0.05 M tris-Cl, pH 7.4, were subjected to electrophoresis in the first dimension for 3 h at 500 V, on a pH 3.5 - 10 gradient (comprised of 2 % (v/v) ampholines, consisting of 1.8 % (v/v) ampholines pH 3.5 - 10 and 0.2 % (v/v) ampholines pH 5 - 8). First dimension gels were electrophoresed in the second dimension on 10 % (w/v) acrylamide slab gels using conditions as described in Chapter 2.

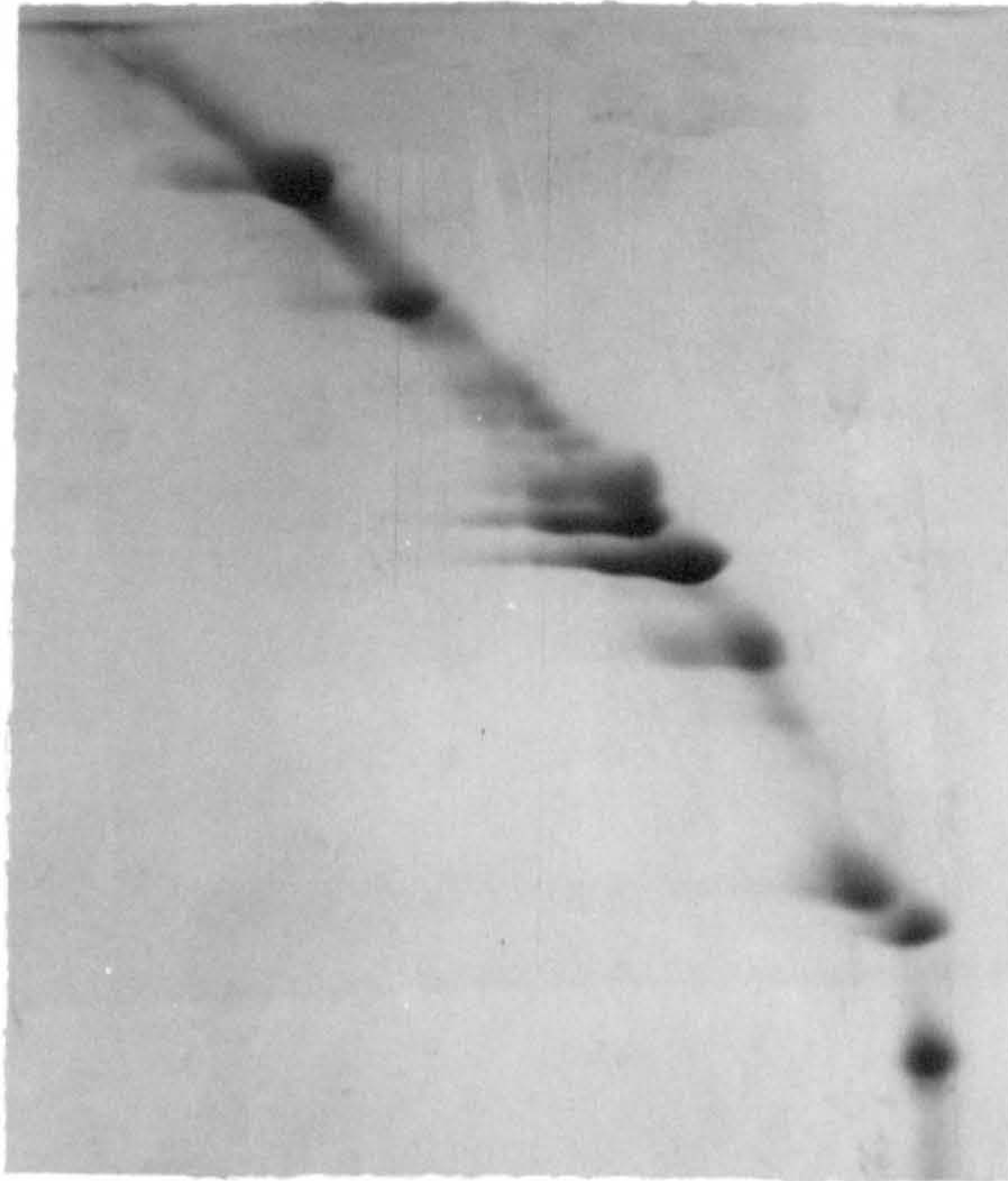


Fig. 5.5 2-D SDS-Polyacrylamide Gel Electrophoretic Separation of CNBr-Peptides from Insoluble Perimysium.

CNBr digests of 6 M urea washed perimysial samples, obtained from conditioned *gluteus medius* bovine muscle, dissolved in sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels, and second dimension electrophoresis was carried out in 15 % (w/v) acryalmide slabs using conditions as described in Chapter 2.

Fig. 5.6 2-D SDS-Polyacryalmide Gel Electrophoretic Separation of CNBr-Peptides following Trypsin Digestion (5 mg/ml) from Insoluble Perimysium.

CNBr digests of 6 M urea washed perimysial samples, obtained from unconditioned *extensor capri radialus* bovine muscle, dissolved in sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 300 μl of trypsin (5 mg/ml, 0.2 units/ml) in gel sample buffer was applied to the top of the gel and second dimension electrophoresis was carried out immediately in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

Fig. 5.7 shows the 2-D peptide pattern obtained from partial digestion with 300 µl trypsin solution (5 mg/ml, 0.2 units/ml) of CNBr cleavage peptides obtained from conditioned *gluteus medius* bovine muscle. The duration of trypsin digestion in the stacking phase was 24 h. Digestion of the peptide material throughout the entire pattern took place, evident as a series of products found below the diagonal under the position of the undigested protein.

Fig. 5.8 shows the 2-D peptide pattern obtained from partial digestion with 300 µl chymopapain solution (3.33 mg/ml, 4.33 units/ml) of CNBr cleavage peptides obtained from conditioned *supraspinatus* bovine muscle. The duration of the stacking phase was 24 h. Extensive proteolysis of all peptide material took place and the diagonal line of spots extending from top left to middle right in the parent sample was almost completely digested.

Fig. 5.9 shows the 2-D peptide pattern obtained from partial digestion with 300 µl chymopapain solution (3.33 mg/ml, 4.33 units/ml) of CNBr cleavage peptides obtained from unconditioned *supraspinatus* bovine muscle. The duration of the stacking phase was 2 h. Partial digestion products were generated from all the major peptides of the parent sample (Fig. 5.9).

Fig. 5.10 shows the 2-D peptide pattern obtained from partial digestion with 300 µl chymopapain solution (50 mg/ml, 66.7 units/ml) of CNBr cleavage peptides obtained from unconditioned *gluteus medius* bovine muscle. In this figure, the electrophoretic separation of the peptides of the sample was performed in the first dimension from right to left and resolution of the separated products was performed in the second dimension from top to bottom. The duration of the stacking phase was 2 h. Extensive proteolysis of all peptide material took place and the diagonal line of spots extending from top right to middle left in the parent undigested sample was completely destroyed. A series of dark lines extended from top to bottom of the gel, corresponding to the area through which the chymopapain solution travelled in the second dimension.

Fig. 5.11 shows the 2-D peptide pattern obtained from partial digestion with 400 µl chymotrypsin solution (1.33 mg/ml, 200 units/ml) of CNBr cleavage peptides obtained from unconditioned (left) and conditioned (right) *gluteus medius* bovine muscle. The partial proteolysis products of the individual peptides were found below the diagonal under the position of the undigested protein. The duration of the stacking phase was 3 h. Extensive digestion of peptide material throughout the entire pattern took place.

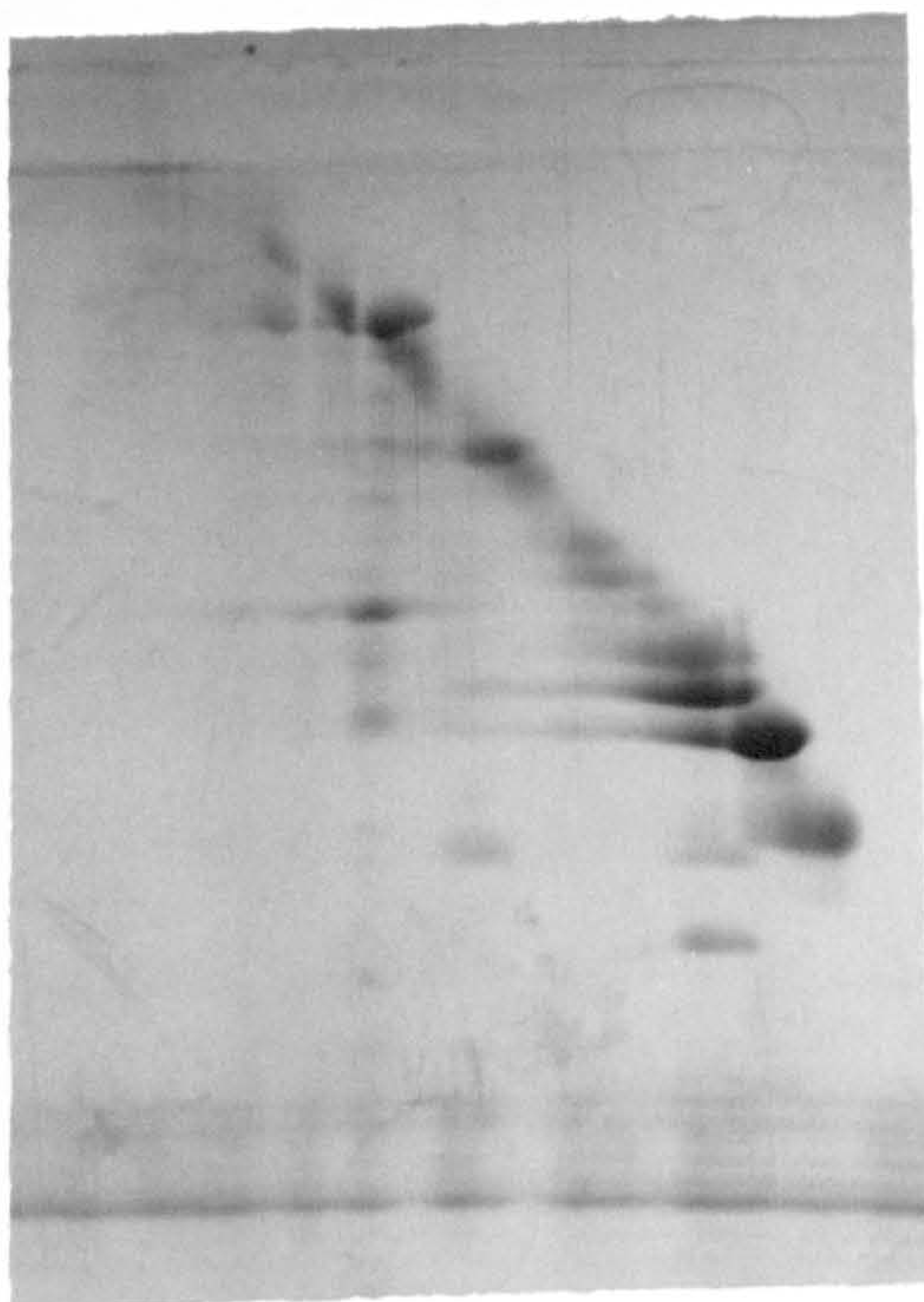


Fig. 5.7 2-D SDS-Polyacrylamide Gel Electrophoretic Separation of CNBr-Peptides following Trypsin Digestion (5 mg/ml) for 24 h from Insoluble Perimysium.

CNBr digests of 6 M urea washed perimysial samples, obtained from conditioned *gluteus medius* bovine muscle, dissolved in sample buffer at a concentration of 5 mg/ml, were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 300 μl of trypsin (5 mg/ml, 0.2 units/ml) in gel sample buffer was applied to the top of the gel, and the system was incubated for 24 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

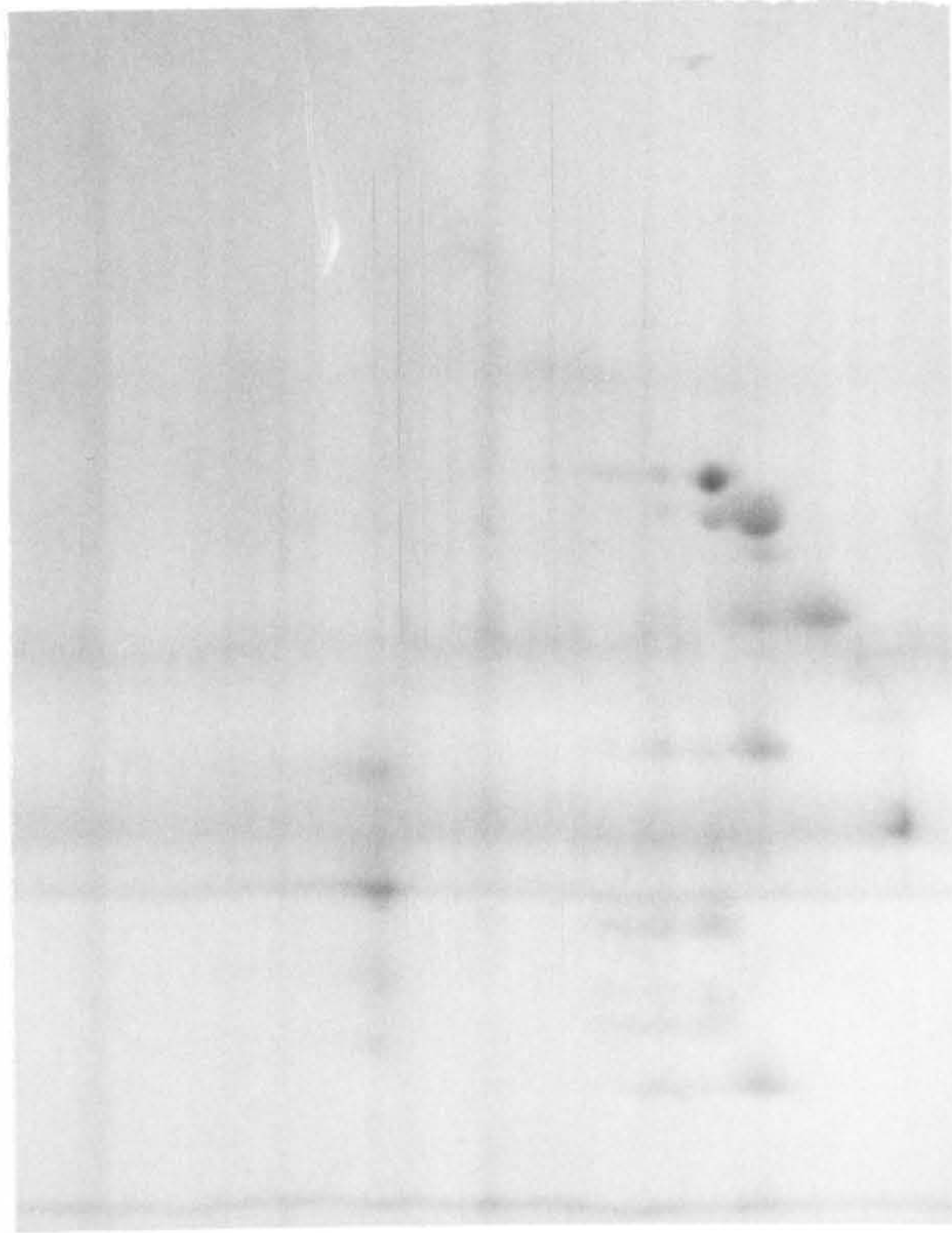


Fig. 5.8 2-D SDS-Polyacrylamide Gel Electrophoretic Separation of CNBr-Peptides following Chymopapain Digestion (3.33 mg/ml) for 24 h from Insoluble Perimysium.

CNBr digests of 6 M urea washed perimysial samples, obtained from conditioned *supraspinatus* bovine muscle, dissolved in sample buffer at a concentration of 5 mg/ml, were resolved in the first dimension in 10% (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acryalmide stacking gels applied to the top of the resolving gels. 300 μl of chymopapain solution (3.33 mg/ml, 4.33 units/ml) in gel sample buffer was applied to the top of the gel, and the system was incubated for 24 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

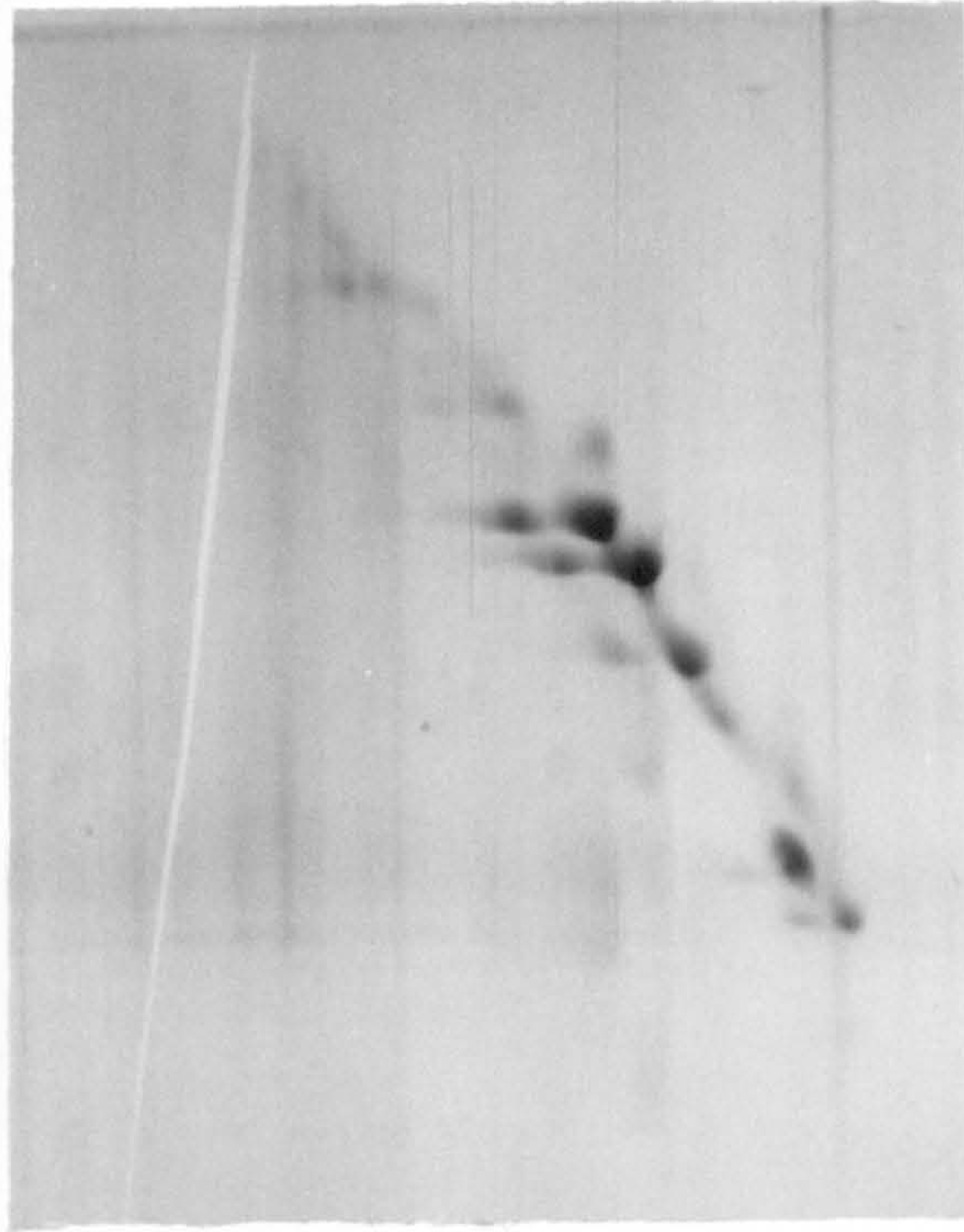


Fig. 5.9 2-D SDS-Polyacryalmide Gel Electrophoretic Separation of CNBr-Peptides following Chymopapain Digestion (3.33 mg/ml) for 2 h from Insoluble Perimysium.

CNBr digests of 6 M urea washed perimysial samples, obtained from unconditioned *supraspinatus* bovine muscle, dissolved in sample buffer at a concentration of 5 mg/ml, were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 300 µl of chymopapain solution (3.33 mg/ml, 4.33 units/ml) in gel sample buffer was applied to the top of the gel, and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

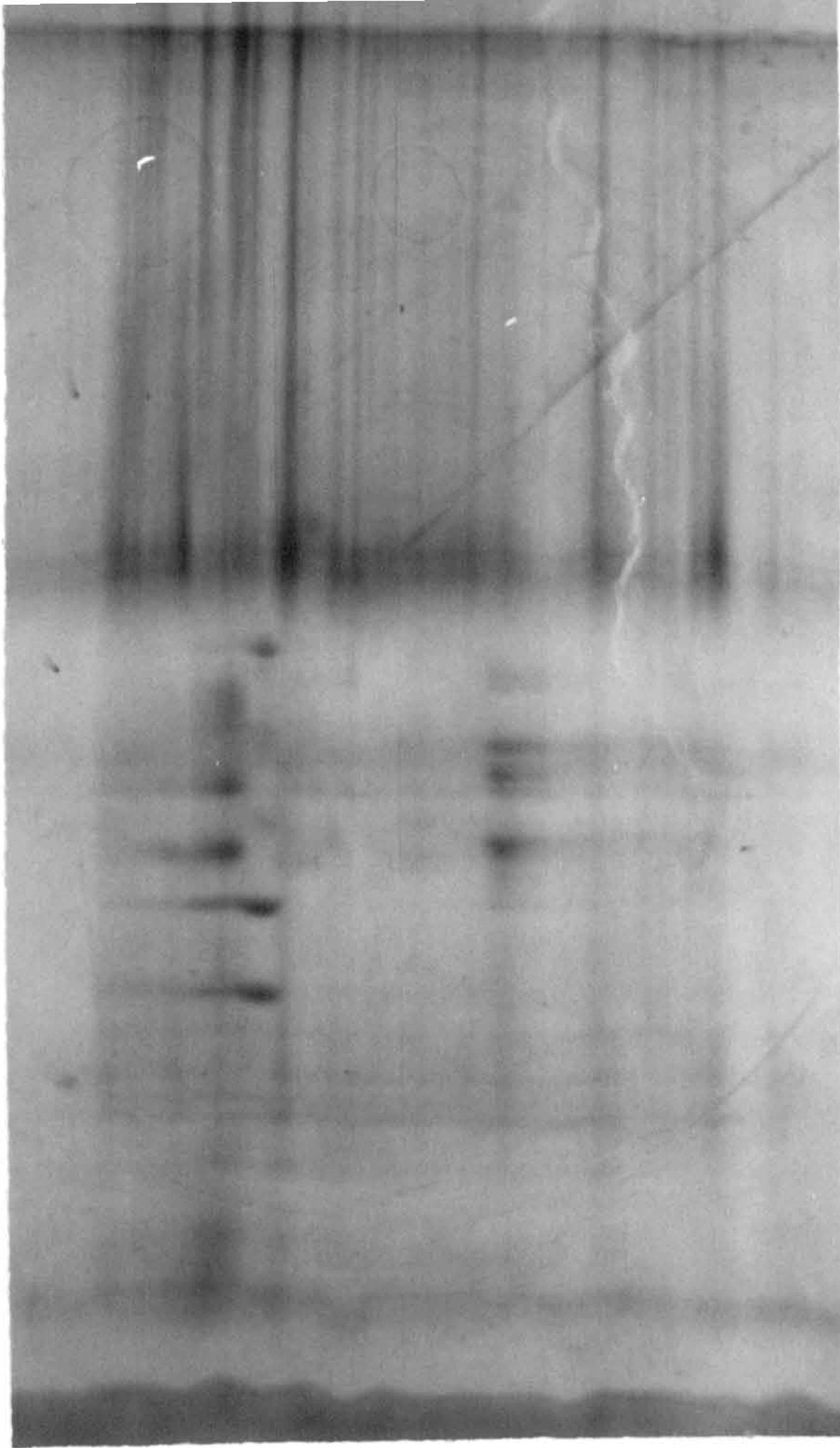


Fig. 5.10 2-D SDS-Polyacrylamide Gel Electrophoretic Separation of CNBr-Peptides following Chymopapain Digestion (50 mg/ml) for 2 h, from Insoluble Perimysium.

CNBr digests of 6 M urea washed perimysial samples, obtained from unconditioned *gluteus medius* bovine muscle, dissolved in sample buffer at a concentration of 5 mg/ml, were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 300 μl of chymopapain solution (50 mg/ml, 66.7 units/ml) in gel sample buffer was applied to the top of the gel, and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

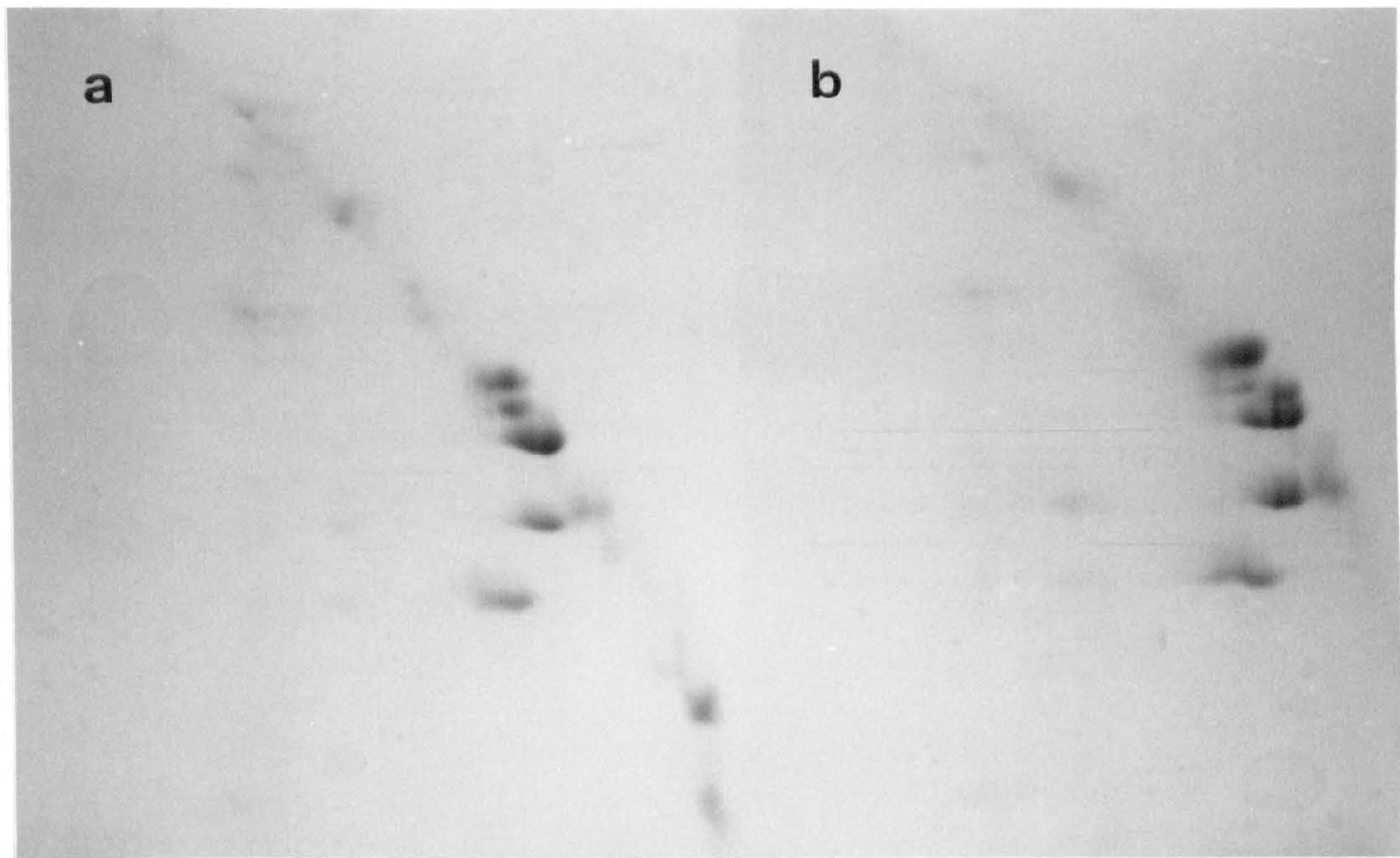


Fig. 5.11 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium using Chymotrypsin Digestion for 3 h, from (a) Unconditioned and (b) Conditioned *Gluteus Medius* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis.

CNBr digests of perimysial samples, dissolved in sample buffer at a concentration of 5 mg/ml, were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 400 μl of chymotrypsin solution (1.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel, and the system was incubated for 3 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

Figs. 5.12 shows the 2-D peptide pattern obtained from partial digestion with 400 μl chymotrypsin solution (1.33 mg/ml, 200 units/ml) of CNBr cleavage peptides obtained from unconditioned (left) and conditioned (right) *extensor capri radialus* bovine muscle. The duration of the stacking was 2 h. It can be seen that many of the peptides generated from digestion of the unconditioned samples were found in the pattern generated by the conditioned samples. Furthermore, the extent of proteolysis of each individual parent peptide was also reproducible, as evidenced by the relative intensities of comparable peptide spots generated in both samples which were very similar.

Two identical samples of perimysial CNBr-cleavage peptides were analyzed on the same gel to test the reproducibility of the method. Fig. 5.13 shows the 2-D peptide pattern obtained from partial digestion with 400μl chymotrypsin solution (1.33 mg/ml) of CNBr cleavage peptides from perimysium obtained from *serratus ventralis*, conditioned for 1 day (left and right). The duration of the stacking phase was 2 h. Identical peptide maps were obtained from the two samples of CNBr-cleavage peptides obtained from *serratus ventralis* showing the viability of the method.

CNBr cleavage peptides derived from a mixture of purified type I + III collagen was used as a molecular weight standard, to identify the separated peptides resulting from secondary chymotrypsin digestion of CNBr-cleavage peptides from perimysium obtained from unconditioned *extensor capri radialus* (Fig. 5.14). Intense bands derived from the chymotrypsin (MW 22 600) interfered with the resolution of CNBr peptides in the same molecular weight range. These peptides, which included α1(I)CB7, α1(I)CB8, α2(I)CB4 and α1(III)CB5 and lower molecular weight components, were poorly resolved. Best resolution was obtained for the high molecular weight peptides of the perimysial digest. High resolution of the polymeric peptide poly α1(I)CB6, α2(I)CB3, 5 and peptides with molecular weights over 30 000 was obtained.

5.4 DISCUSSION

Electrophoresis is a method widely used in the analysis of biological macromolecules. Since the first application of polyacrylamide gel as a supporting medium for electrophoresis (Raymond & Weintraub, 1959) and especially since the publication of the fundamental work of Ornstein (1964) and Davis (1964), polyacrylamide gel electrophoresis has become one of the most widely used methods in biochemistry. The high resolving power of the method allows not only the separation of mixtures containing a large number of macromolecular species, but also the characterization (charge, molecular weight, conformation) of macromolecules. Polyacrylamide gel, being an inert supporting

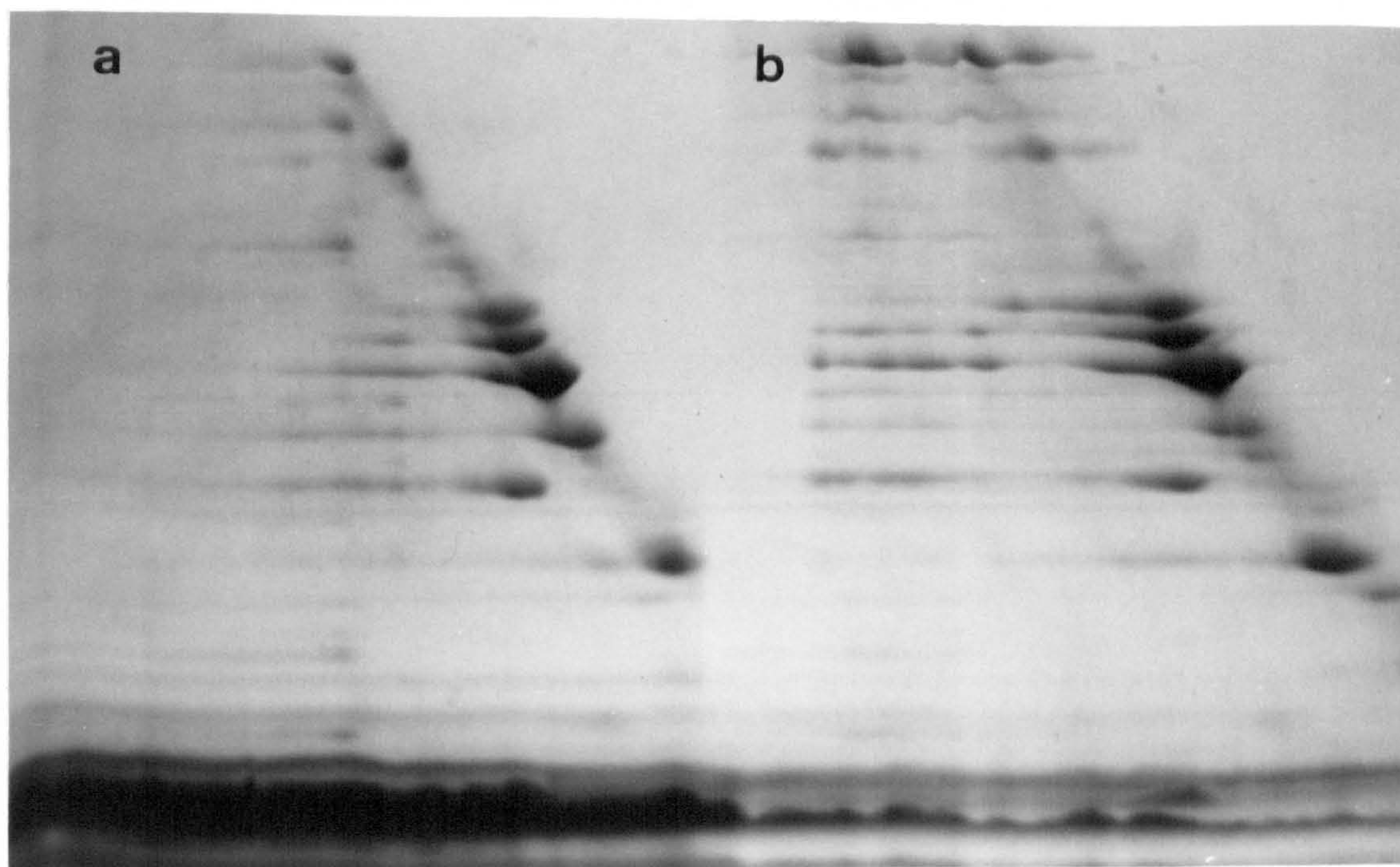


Fig. 5.12 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium using Chymotrypsin Digestion for 2 h, from (a) Unconditioned and (b) Conditioned *Extensor Capri Radialus* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis.

CNBr digests of perimysial samples, dissolved in gel sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 400 μl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel, and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2. The arrows show the presence of two new sets of peptides and absence of two peptides in the conditioned sample when compared to the unconditioned sample.

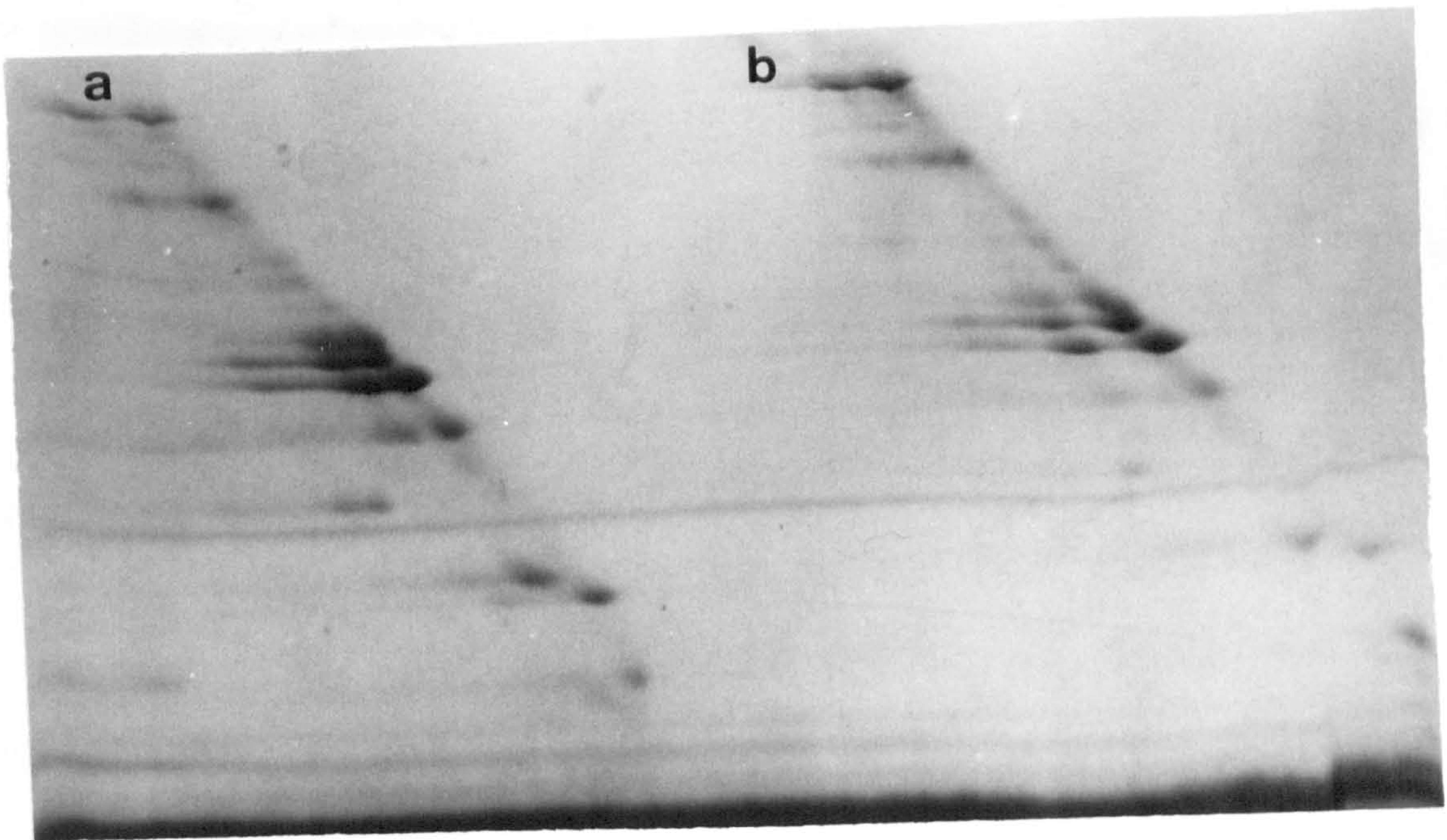


Fig. 5.13 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium using Chymotrypsin Digestion for 2 h, from *Serratus Ventralis* Bovine Muscle, Conditioned for 1 day, (a) and (b), by 2-D SDS-Polyacrylamide Gel Electrophoresis.

CNBr digests of perimysial samples, dissolved in gel sample buffer, at a concentation of 5 mg/ml, were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 400 µl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel, and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

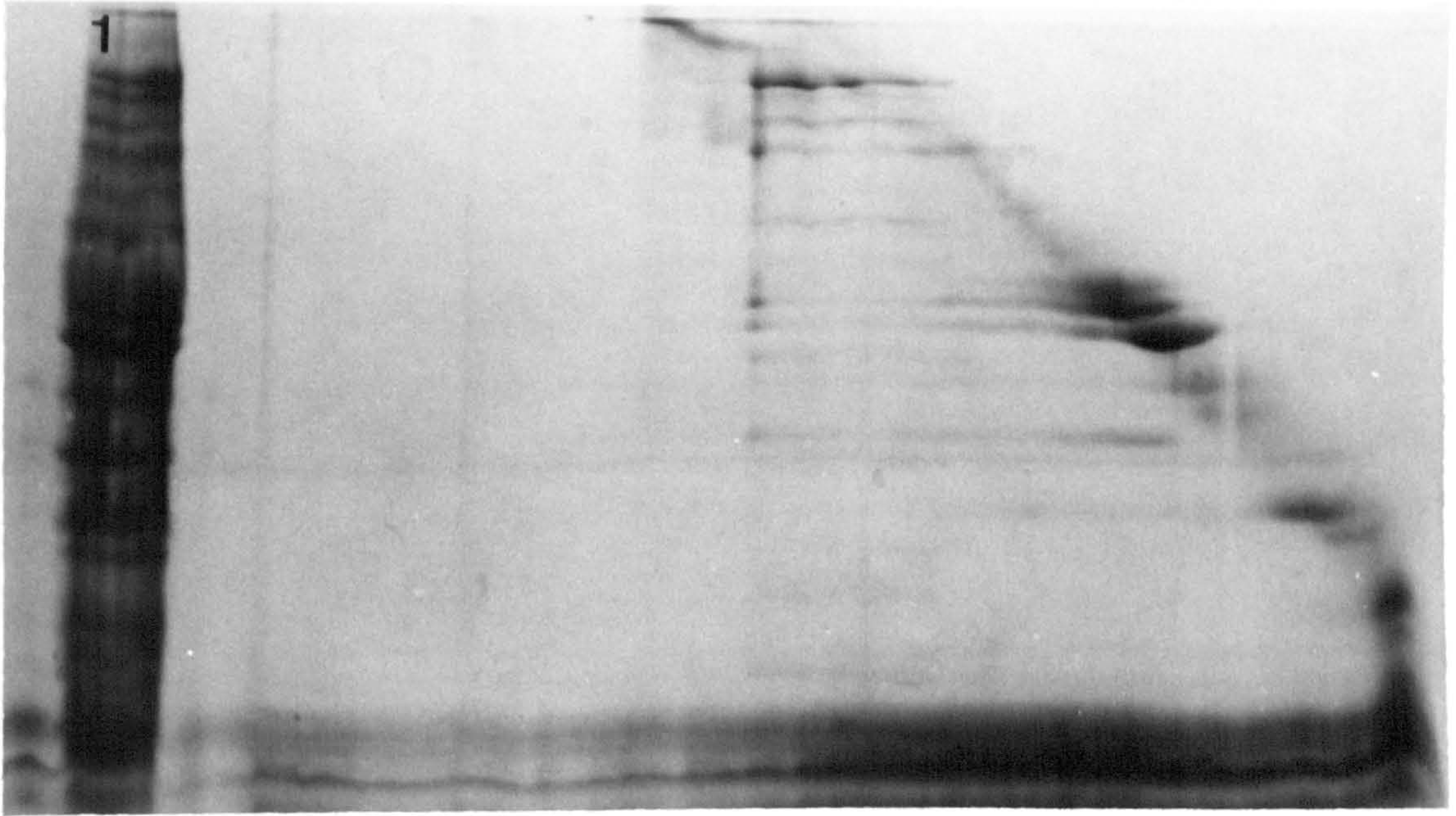


Fig. 5.14 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium using Chymotrypsin Digestion for 2 h from Unconditioned *Extensor Capri Radialus* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis.

CNBr digests of perimysial samples, dissolved in gel sample buffer, at a concentration of 5 mg/ml, were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) polyacrylamide stacking gels applied to the top of the resolving gels. The first dimension gel was incubated for 2 h with 400 µl of chymotrypsin solution (3.33 mg/ml, 200 units/ml). Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2. Track (1) shows CNBr peptides from purified type I + III collagen (5 mg/ml) used as a molecular weight standard.

medium, is also often used in isoelectric focussing of macromolecules. Electrophoresis is the term given to the migration of charged particles under the influence of a direct electric current. It is a single phase system and depends upon the relative mobilities of ions under identical electrical conditions.

The effectiveness of the electrophoretic separation of proteins is influenced by a number of different factors including the actual charge of the molecules, the sieving properties of the support used for electrophoresis, and the presence of denaturing agents. This variability endows electrophoretic methods with unique resolving power. However, when a complex mixture of numerous proteins has to be analyzed, some proteins may overlap each other. A second electrophoresis may then lead to an improved separation. This idea suggested the introduction of two-dimensional techniques.

Two 2-D electrophoretic techniques were investigated in the present study for their usefulness in separating collagenous peptides derived from bovine connective tissue and for highlighting conditioning effects in these samples.

The first, isoelectric focussing, is one of the newest methods in the family of procedures based on the behaviour of molecules in an electric field. During isoelectric focussing, a pH gradient is created between the anode and the cathode. The charged molecules which are, at the beginning, distributed in the medium or applied as a single band, move according to their actual charge towards the oppositely charged electrode, and will migrate to the point where the pH of the gradient is the same as their isoelectric pH. They will remain there, as long as the gradient and potential differences are maintained. If the molecules wandering in the pH gradient are amphoteric, their charge is continuously changing during this process and finally the molecules will reach a position where their net charge will equal zero. This occurs when the molecules arrive at a zone of the pH gradient where the pH is equal to their isoelectric points. Thus, molecules having the same isoelectric point are concentrated in a narrow band. If the pH gradient is stable, no further changes occur and the molecules will remain concentrated, since spreading or diffusion is inhibited by the electric field. Historically, the first application of this principle was described by Ikeda & Suzuki (1912) who separated glutamic acid from hydrolyzates of plant proteins. Williams and Waterman (1929) clearly identified the basic principle of IEF, and they are regarded as the real inventors of the method.

The use of polyamino-polycarboxylic acids, which show a significant electrical conductivity at their pI values, enables the formation of a stable pH gradient. These compounds were originally developed by LKB-Produkter AB under the trade name of

Ampholine carrier ampholytes. Ampholines are available with a wide range of pI values, a feature dependent upon their precise chemical nature and which permits the formation of pH gradients varying from pH 2.5 to pH 11, or over narrower pH ranges. They have relative molecular masses below 1000 and show all the qualities necessary for isoelectric focussing techniques, including high buffering capacity, high solubility and good, although minimal conductivity at their pI values.

In the current work, a pH gradient was produced by incorporating a mixture of Ampholines with appropriate pI values in polyacrylamide gels. Phosphoric acid was used at the anode and sodium hydroxide at the cathode, the pH of these being approximately the same as the pI values of the two extremes of the Ampholine range. Thus, basic proteins led in the separation from left to right, and the left sides of the gel patterns represented the tops of the first dimension gels.

The pH gradient formed, incorporating pH 3.5-10 Ampholines only, was relatively stable in the more acidic pH range, but was unstable over the entire basic end of the gel, while the pH gradient formed for Ampholines with pH ranges of pH 3.5-10 and pH 5-8 was stable throughout the entire pH range but was disrupted slightly at the extreme basic end of the gel. The collapse of the basic end of the pH gradient is not clearly understood, but may be partly due to cathodic migration (Chrambach et al., 1973), which is especially severe in the presence of urea. Resolution of CNBr cleavage peptides employing Ampholines with a pH range of 3.5-10 only ,was poor, but was significantly improved by additional incorporation of Ampholines with a pH range of 5-8. In addition to the pH range of the Ampholines, time of electrophoresis, length of the gel and minor changes in the sample mixture can change the resolution and distribution of proteins (O'Farrell et al., 1977) so that these parameters must be carefully controlled in the first dimension. It was possible to obtain reproducible results, using Ampholines with a pH range of 3.5-10 (1.8 % v/v) and 5-8 (0.2 % v/v), 7.5 cm gel length, and exhaustively dialyzed CNBr cleavage peptides derived from bovine perimysial connective tissue, extracted by the method outlined in Chapter 2 (section 2.4.2) employing urea in the procedure. CNBr cleavage peptides from endomysial fractions employed in this study were extracted using SDS, by the method of Light & Champion (1984) as described in Chapter 4. Therefore, the results obtained with the modified Ampholine gradient (Fig. 5.3) were considered good enough for application to the study of differences between insoluble collagens from conditioned and unconditioned meats (see Chapter 6).

The second 2-D electrophoretic technique involved SDS-polyacrylamide gel electrophoresis in the first dimension and then the electropherogram thus produced was

applied, without fixation and staining, as a starting zone for electrophoresis in the second dimension (perpendicular to the first dimension). If the two electrophoretic runs are performed under identical conditions, the migration rates of different proteins will be the same in both directions and therefore, the spots of the separated molecules will be arranged on a diagonal line. Such systems do not result in an increase in resolution except for that caused by the longer migration distance. In order to obtain superior resolution, it is necessary to change at least one of the characteristics of the electrophoretic conditions in the second run. In the second 2-D technique employed here, molecular weight was the criterion changed in the second dimension, brought about by partial proteolysis of the separated 'parent' peptides from the first dimension and therefore gel pore size was altered in the second run.

The extent of proteolysis of the separated peptides from the first dimension was found to be determined by the type of protease employed for digestion, the amount of protease used and by the duration of the stacking process. It was important to identify a suitable protease, which brought about limited digestion of collagen CNBr cleavage peptides in a relatively short time, to make this a viable 2-D electrophoretic method of peptide separation, suitable for routine analysis of conditioning effects on bovine connective tissue collagen. Three proteases were investigated for their effectiveness in bringing about limited proteolysis of collagen CNBr cleavage peptides.

The first, trypsin, was investigated using two incubation times for proteolysis. When the trypsin solution was run through the second dimension gel without halting the stacking phase, it was found that insufficient digestion of the 'parent' peptides from the first dimension took place. However, when the stacking phase, during which digestion of the 'parent' peptide material took place, was halted for 24 h, a series of peptide products was evident below the diagonal under the position of the undigested protein. However, because 24 h incubation was considered too long for a routine analytical method this enzyme was not used further.

Chymopapain was investigated for its effectiveness in causing partial proteolysis over 24 h and was found to cause extensive proteolysis of all collagenous material. On reducing the time period of the stacking phase from 24 h to 2 h, partial digestion of the 'parent' peptide material took place. It was decided, however, that the chymopapain preparation used was unsuitable because it produced a series of dark lines on the gel which interfered with interpretation of collagen derived peptides. This defect was highlighted by increasing enzyme concentration, but was evident even at the lower concentration which produced a sufficient degree of proteolysis.

The third protease investigated in this study, chymotrypsin, produced excessive digestion of the 'parent' collagen CNBr cleavage peptide material after 3 h of digestion in the stacking phase. Reduction of the stacking phase time period to 2 h resulted in partial proteolysis of all peptide material. Unconditioned and conditioned samples from the first dimension were analyzed simultaneously on the same gel, ensuring identical electrophoretic conditions and degree of proteolysis, which made the method ideal for the purposes of comparing CNBr cleavage peptides derived from unconditioned and conditioned bovine muscles.

Comparison of unconditioned with conditioned perimysium, obtained from *extensor capri radialus*, revealed increased incidence of high molecular weight material in the conditioned sample, suggesting that during the conditioning process, proteolytic damage was inflicted in the high molecular weight aggregates, present in the CNBr digest, which allowed more extensive action by chymotrypsin, prior to second-dimension separation of the peptides. In addition to these changes observed in CNBr digests from insoluble perimysium, appearance of at least one other peptide, in the α1(I)CB6 region and loss of at least two peptides from the conditioned sample resulted due to conditioning. These results are discussed in Chapter 6 and prove that the 2-D method of separation of CNBr-cleavage peptides does have the potential to differentiate changes in the connective tissue fraction of meat, as discussed in the next chapter.

The method was shown to be reproducible by comparison of two identical samples, under identical electrophoretic conditions and degree of proteolysis on the same gel. It was found that best resolution was obtained for the high molecular weight peptides (polymeric peptide α2(I)CB3,5 and peptides with molecular weight over 30 000. Chymotrypsin (MW 22 600) would overtake the higher molecular weight CNBr-peptides in the electrophoretic system, and proteolysis took place during the halted stacking phase

Cathepsins have been implicated in the extracellular proteolysis of collagen in meat (see Chapter 1) and are thought to act at the N and C-terminal non-helical regions of the collagen molecule. As these regions are implicated in the production of high moleuclar weight aggregates in mature fibres (see Chapter 1) and produce high M_r peptides on CNBr-digestion, the proteolytic 2-D electrophoretic method appeared to be an excellent one for investigating these peptides. In the following chapter, the two methods of 2-D separation of CNBr-cleavage peptides investigated here, are applied to the investigation of the effects of conditioning on collagen derived from bovine connective tissue.

CHAPTER 6

THE EFFECTS OF PROTEASES ON INSOLUBLE PERIMYSIAL AND ENODMYSIAL COLLAGEN IN MODEL SYSTEMS AND ON CONDITIONING

6.1 . INTRODUCTION

As discussed in Chapter 3, previous work on the effects of conditioning on the connective tissue fraction of meat has been contradictory, and little or no direct evidence exists to show that proteolysis of collagen takes place during conditioning. However, direct evidence has been provided in Chapters 3 and 4 to show that conditioned meat yielded significantly greater quantities of solubilized perimysial and endomysial collagen than unconditioned meat. This increased solubilization of connective tissue collagen in conditioned meat suggested that proteolytic damage was inflicted during the conditioning process. However, when analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis, the insoluble perimysium remaining after conditioning was found to be unchanged although minor damage to insoluble endomysial preparations was observed in some of the muscles investigated. Furthermore, it was unclear whether the new peptide material observed in the insoluble conditioned endomysial fraction on one-dimensional SDS-polyacrylamide gel electrophoresis was derived from the collagenous component. However, it is important to be able to identify residual damage to the conditioned connective tissue matrix because such damage may have profound effects on the contribution of collagen to textural changes in meat during the conditioning process. The work outlined in this chapter is concerned with application of the methods outlined in Chapter 5 for investigation of connective tissue collagen, to detection of proteolytic changes to bovine connective tissue collagen.

Acid proteases (cathepsins) released into the extracellular matrix are believed to be responsible for proteolysis of intramuscular collagen, during the conditioning process as discussed in Chapter 1 (section 1.5.4). First, *in vitro* tests were carried out, using model protease systems to investigate the damage inflicted on perimysial collagen, using two-dimensional gel electrophoresis techniques. 2-D electrophoretic results from *in vitro* treatment of perimysial collagen with cathepsins, were compared with those obtained from perimysium obtained from conditioned meat. Correlations between the extent of damage caused by both methods of proteolysis was possible. Secondly, results of the investigation of the effects of conditioning on the high molecular weight region of perimysial and endomysial collagen are presented. The results indicate that this region is

significantly affected by conditioning and that the extent of damage is different in different muscles.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of Muscles and Connective Tissue Extracts

Unconditioned and conditioned muscles were prepared as outlined in Chapter 2. Insoluble perimysium was extracted and purified using 6 M urea, 0.05 M tris-Cl, pH 7.4, as outlined in Chapter 2 (section 2.4) and insoluble endomysium was extracted and purified by 1 % (w/v) SDS, as outlined in Chapter 4 (section 4.2.4).

6.2.2 Preparation of Spleen Extract

80 g fresh bovine spleen was minced and homogenized in 9 volumes 50 mM sodium acetate buffer, pH 5.0 (ice-cooled) containing 1 mM EDTA and 0.2 % (v/v) Triton X-100 for 1 min at full speed in a Polytron homogenizer. The spleen extract was stirred for 4 h at 4° C, before centrifugation at 25 000 x g for 20 min to remove debris. The supernatant was denoted crude cathepsin.

6.2.3 Cathepsin Assays

The assays employed were as described by Mason et al. (1984) using the fluorimetric assays developed by Barrett (1980) for determination of cathepsins B and H activities using the substrates Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec. Stock substrates were dissolved at 10 mM final concentration in dimethyl sulphoxide and were stored at 4° C. Measurement of combined cathepsin B and L activities was based on hydrolysis of Z-Phe-Arg-NHMec and cathepsin B activity was based specifically on hydrolysis of Z-Arg-Arg-NHMec with an incubation medium of 100 mM-sodium phosphate buffer, pH 6.0, containing 1 mM EDTA, 10 mM cysteine (freshly dissolved), 10 μM NHMec substrate and enzyme in a final volume of 3.2 ml. Incubation was at 37° C for 10 min and the reaction was started by addition of substrate. Initial rates at 37° C were determined from continuous recordings, using excitation and emission wavelengths of 340 nm and 460 nm respectively.

6.2.4 Incubation of Perimysium with Pepsin

1 g (wet weight) of press dried, freshly extracted unwashed or urea washed unconditioned insoluble perimysium was dispersed in 28 ml 0.5 M CH_3COOH. Pepsin was added and incubated with the substrate at 4° C at a substrate to enzyme ratio of 100:1 for times ranging from 0 to 24 h. Activity was stopped by increasing the pH to 8 with concentrated NaOH. Soluble fractions were separated from insoluble fractions by centrifugation, dialyzed against distilled water and freeze-dried.

6.2.5 Incubation of Perimysium with Spleen Extract

3 ml spleen extract (specific activity of cathepsin B + L, 0.12 $\mu mol^{-1} min^{-1} ml^{-1}$ and cathepsin B, 0.05 $\mu mol^{-1} min^{-1} ml^{-1}$) was added to 1 g (wet weight) of press dried, freshly extracted unwashed or urea washed unconditioned insoluble perimysium, dispersed in 14 ml 0.09 M sodium citrate buffer, pH 5.5, containing 1mM EDTA. pH 5.5 was chosen to simulate the pH of conditioned meat and was not necessarily the pH optimum of the extracted cathepsins. Cysteine (10 mM) was included, and incubation was carried out at 4° C for times ranging from 0 to 24 h. The final activity of cathepsin B added was 0.75 $nmol^{-1} min^{-1} mg^{-1}$ collagen. Cathepsin activity was stopped by increasing the pH to 8 with concentrated NaOH. Soluble fractions were separated from insoluble fractions by centrifugation, dialyzed against distilled water and freeze-dried.

6.2.6 Incubation of Perimysium with Trypsin

1 g (wet weight) of press dried, freshly extracted unwashed or urea washed unconditioned insoluble perimysium was dispersed in 11 ml 0.67 M NaH_2PO_4, pH 7.6. Trypsin was incubated with this substrate at 25° C, using a substrate to enzyme ratio of 200:1 for times ranging from 0 to 24 h. Soluble fractions were separated from insoluble fractions by centrifugation, dialyzed against distilled water and freeze-dried.

6.2.7 CNBr Digestion, SDS-Polyacrylamide Gel Electrophoresis and Hydroxyproline Estimation

CNBr digestion of insoluble perimysial and endomysial fractions was carried out in 70 % (v/v) formic acid at 30° C as outlined in section (2.7). One-dimensional SDS-polyacrylamide gel electrophoresis was carried out on CNBr-cleavage peptides of extracted insoluble perimysium and endomysium as outlined in Chapter 2 (section 2.8). Two-dimensional electrophoresis, employing NEPHGE-SDS and SDS-polyacrylamide gel

electrophoreis-proteolysis techniques were carried out on CNBr cleavage peptides of insoluble perimysium and endomysium as described in Chapter 5 (sections 5.2.2. and 5.2.3). Hydroxyproline content was quantified by HPLC as described in Chapter 2 (section 2.9). Percentage collagen content was thus estimated in total insoluble perimysium, insoluble residues remaining after enzyme digestion and in solubilized fractions.

6.3 RESULTS

6.3.1 Solubilization of Perimysium with Model Protease Systems

Table 6.1 shows the solubilization of perimysial material from unwashed and urea washed perimysium by pepsin, cathepsin and trypsin. In most cases the solubilization of perimysium had plateaued by 2 to 3 h. After 24 h the total yield of solubilized perimysial material by the three enzymes varied from 12 to 30 % of the original while pepsin and trypsin treatments yielded almost twice as much material from unwashed than from urea washed perimysium. After the same time period, cathepsin treatment of the two preparations gave about the same yield. Trypsin solubilized the highest proportion of unwashed perimysium but this contained the smallest amount of collagen solubilized by any of the three enzymes. A similarly small amount of collagen was solubilized from the urea washed sample by trypsin. The yields of unwashed perimysial collagen by pepsin and cathepsin were similar after 24 h and in each case was less than half the yield of collagen released from urea washed perimysium at this time (Table 6.1).

Fig. 6.1 shows the time course of solubilization of collagen from urea washed and unwashed insoluble perimysium by pepsin. The total amount of material solubilized and the collagen content of the solubilized fractions increased from 0 to 24 h. There was a good correlation between the amount of collagen solubilized and the time of pepsin treatment ($r = 0.81$ and $r = 0.95$ for unwashed and 6 M urea washed perimysium, respectively). The amount of pepsin solubilized collagen liberated from unwashed and 6 M urea washed insoluble perimysium after 24 h showed very little difference (Fig. 6.1); 10.2 mg collagen solubilized from unwashed perimysium compared with 14.0 mg collagen solubilized from 6 M urea washed insoluble perimysium.

The spleen cathepsin preparation had a similar effect on the insoluble perimysium as did pepsin (Fig. 6.2). Material was solubilized continuously over the 24 hr period and the collagen content of the solubilized fraction steadily rose. The correlation between solubilized collagen and time of cathepsin treatment was $r = 0.90$ for unwashed

TABLE 6.1 Percentage of Perimysium and Perimysial Collagen Solubilized by Pepsin, Cathepsin and Trypsin

Time (h)	Pepsin				Cathepsin				Trypsin			
	Unwashed		Urea washed		Unwashed		Urea washed		Unwashed		Urea washed	
	collagen* (%)	perimysium (%)	collagen* (%)	perimysium (%)	collagen* (%)	perimysium (%)	collagen* (%)	perimysium (%)	collagen* (%)	perimysium (%)	collagen* (%)	perimysium (%)
0	2.1	15.0	0.0	0.0	2.1	6.0	0.0	0.0	0.4	12.0	0.8	8.0
1	4.0	15.0	5.0	3.0	3.3	11.0	1.9	11.0	0.5	16.0	1.2	13.0
2	4.5	26.0	11.0	5.0	4.7	12.0	1.6	13.0	-	-	-	-
3	7.9	25.0	14.0	5.5	4.8	11.0	2.0	18.0	0.6	19.0	1.6	17.0
4	-	-	19.0	8.5	-	-	12.9	18.0	-	-	-	-
5	9.0	25.0	21.0	9.0	7.3	12.0	19.2	14.0	0.7	31.0	1.6	16.0
24	11.3	28.0	27.5	12.0	10.1	13.0	27.8	18.0	0.6	30.0	2.3	16.0

* percentage collagen in solubilized fraction

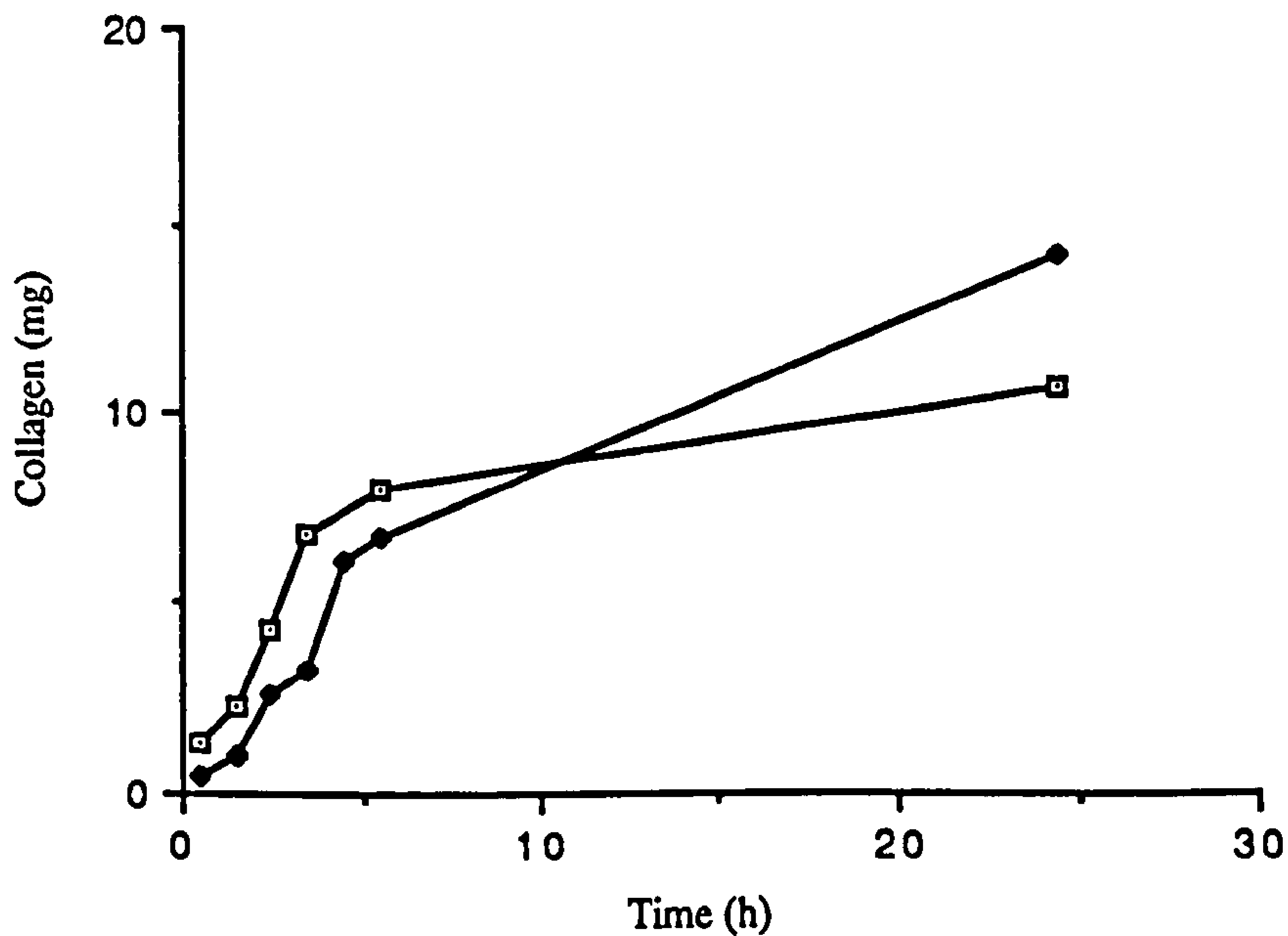


Fig. 6.1 Solubilization of Collagen from Pepsin Treated Unwashed and 6 M Urea-Washed Freshly Excised Unconditioned Insoluble Bovine Perimysium.

Partially purified perimysium (unwashed (□) and washed (♦) with 6 M urea, 0.05 M tris-Cl, pH 7.4) from unconditioned bovine muscle was incubated at 4° C with pepsin at a substrate to enzyme ratio of 100:1 for times ranging from 0 to 24 h. Enzyme activity was stopped by increasing the pH to 8 with concentrated NaOH. Hydroxyproline was quantified in soluble fractions by HPLC using a conversion factor of 7.14 to convert hydroxyproline content to collagen content, assuming an average hydroxyproline content of 14 % in meat collagens.

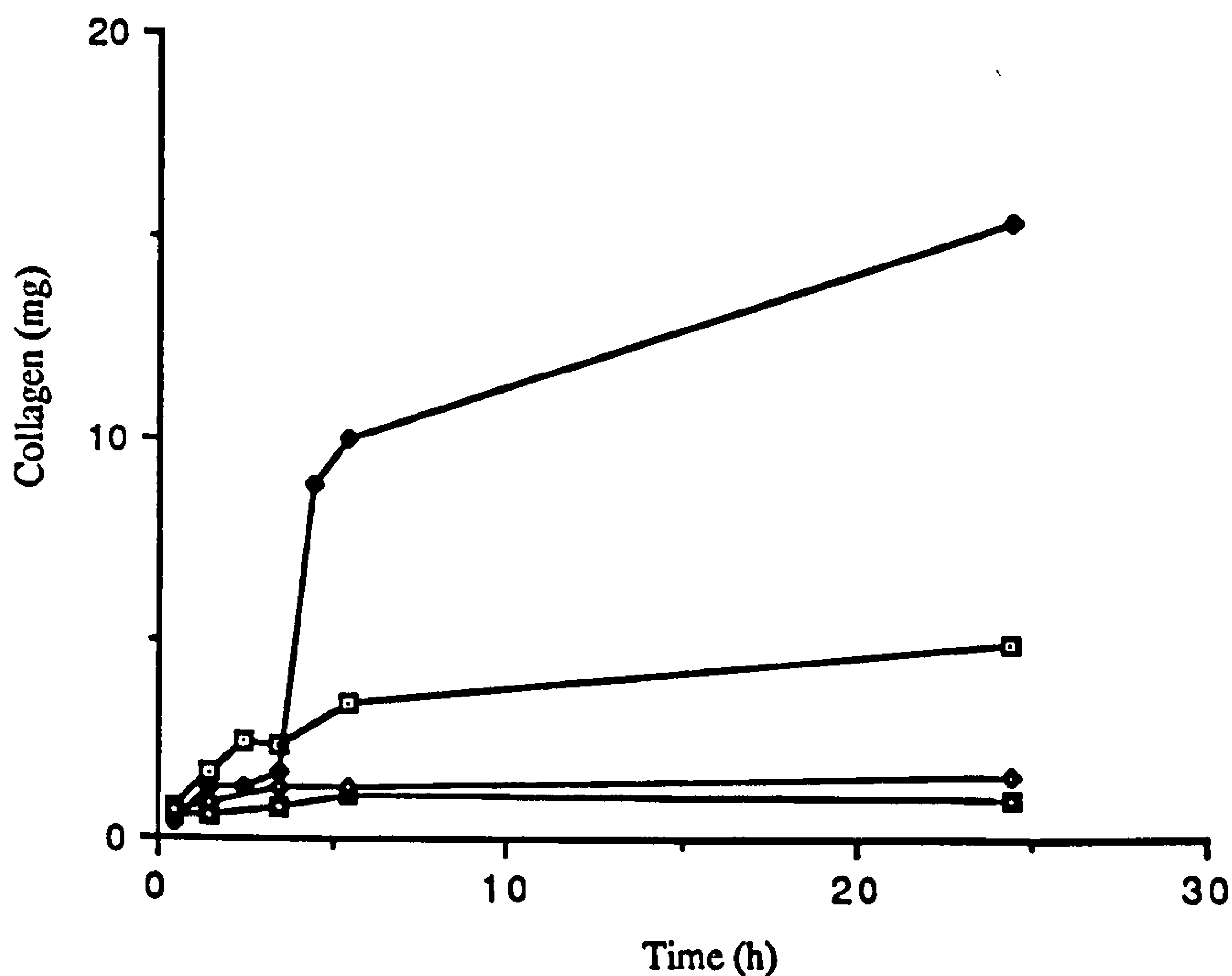


Fig. 6.2 Solubilization of Collagen from Cathepsin and Trypsin -Treated Unwashed and 6 M Urea -Washed Freshly Excised Unconditioned Insoluble Perimysium.

Partially purified perimysium, unwashed and washed with 6 M urea, 0.05 M tris-Cl, pH 7.4 from unconditioned bovine muscle was incubated for times ranging from 0 to 24 h with either a bovine spleen cathepsin preparation or trypsin. Cathepsin activity was stopped by increasing the pH to 8 and trypsin activity was stopped by increasing the pH to 10 with concentrated NaOH. Hydroxyproline was quantified in soluble fractions by HPLC using a conversion factor of 7.14 to convert hydroxyproline content to collagen content, assuming an average hydroxyproline content of 14 % in meat collagens.

- ◻ - incubation of unwashed perimysium at 4° C with spleen extract in the presence of cysteine (10 mM).
- ♦ - incubation of 6 M urea washed perimysium at 4° C with spleen extract in the presence of cysteine (10 mM).
- ◼ - incubation of unwashed perimysium at room temperature with trypsin (in buffers as specified in section 6.2.6) using a substrate to enzyme ratio of 200:1.
- ◆ - incubation of 6 M urea washed perimysium at room temperature with trypsin using a substrate to enzyme ratio of 200:1.

perimysium and r = 0.85 for 6 M urea washed perimysium. In this case, however, there was a more marked difference in the extent of collagen solubilization between the urea washed and unwashed perimysium. The total amount of collagen solubilized using cathepsin was greater for 6 M urea washed insoluble perimysium than for unwashed material; the 6 M urea washed samples yielded 15.0 mg solubilized collagen after 24 h treatment compared with only 4.5 mg solubilized collagen from the unwashed sample. This was markedly different from the results obtained using pepsin treatment.

Trypsinization of perimysial fractions was used to ascertain whether 6 M urea washing denatured perimysial collagen extensively enough to affect its properties as a protease substrate. When trypsin solubilization of collagen from unwashed and 6 M urea washed insoluble perimysium was monitored from 0 to 24 h the quantity of collagen in the solubilized fractions was shown to be similar from both unwashed and urea washed preparations at each stage of proteolysis (Fig. 6.2). The quantity of trypsin solubilized collagen, at all stages of incubation, released from both 6 M urea washed and unwashed perimysium was considerably less than that liberated by cathepsin from these substrates (Fig. 6.2).

6.3.2 One-Dimensional SDS-Polyacrylamide Gel Analysis of Pepsin and Cathepsin Treated Insoluble Perimysium

Pepsin treatment of 6 M urea washed unconditioned insoluble perimysium for 0 to 24 h resulted in little damage to the insoluble collagenous residue remaining, as evidenced by SDS-polyacrylamide gel electrophoretic analysis of the CNBr-cleavage peptides (Fig. 6.3). The CNBr peptide spectrum of insoluble perimysium treated with cathepsin for up to 4 h did not alter significantly from that of the untreated control (Fig. 6.4). There was some increase in intensity of material in the low molecular weight range but it was not obvious that this resulted from damage to collagen. The same substrate treated with cathepsin for 5 h showed a similar increase in low molecular weight material on polyacrylamide gels but no other apparent changes. However, the sample of perimysium treated with cathepsin for 24 h showed the most profound changes in the CNBr peptide pattern, revealing loss of high molecular weight material as well as a significant reduction in quantity of all peptides when compared with the untreated control.

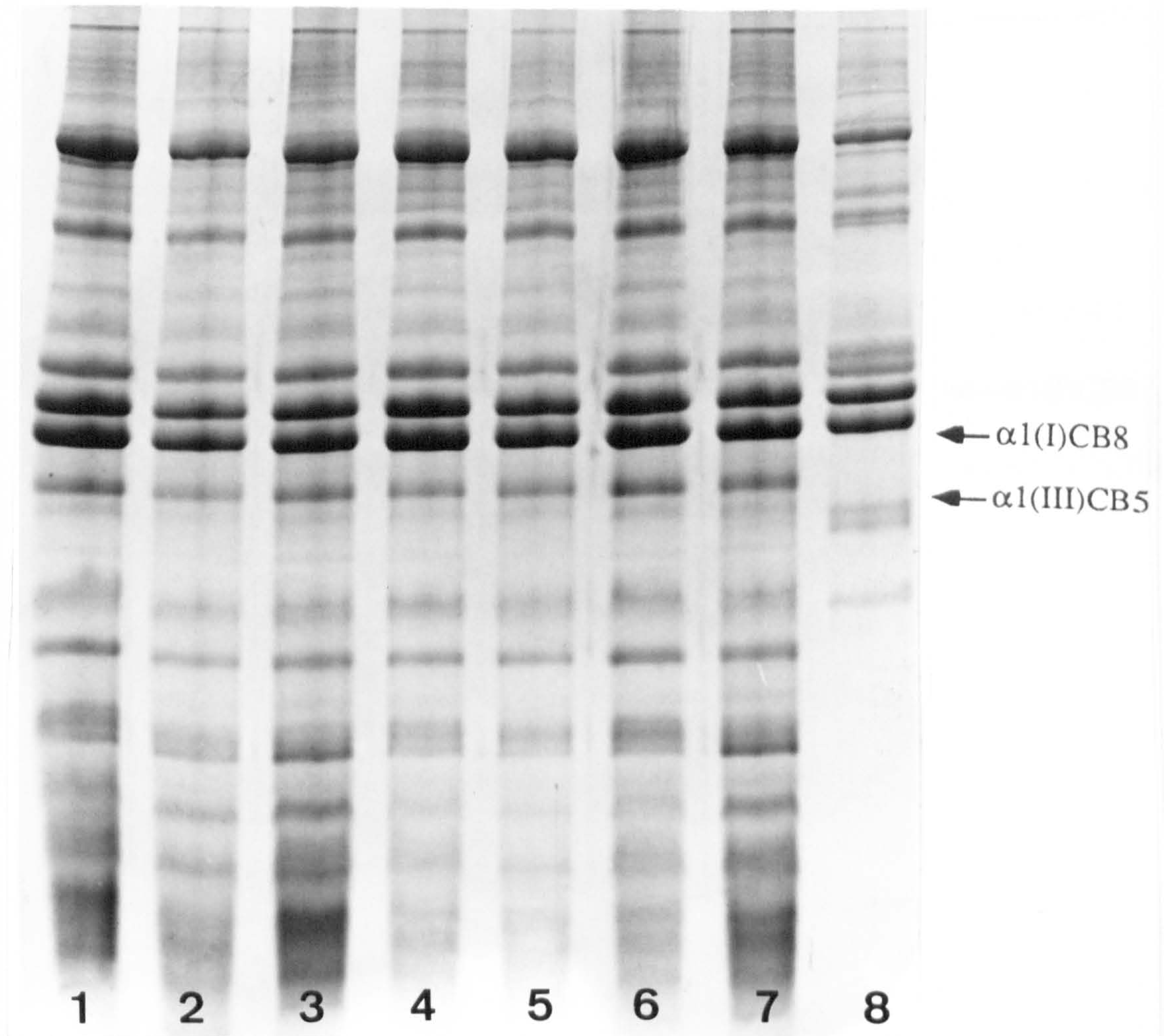


Fig. 6.3 SDS-Polyacrylamide Gel Electrophoresis of CNBr -Peptides of Residual Insoluble Material after Pepsin Treatment of 6 M Urea -Washed Perimysium.

Insoluble perimysium from unconditioned bovine muscle, washed with 6 M urea, 0.05 M tris-Cl, pH 7.4 was incubated with pepsin at 4° C, using a substrate to enzyme ratio of 100:1 and the insoluble residues at various time points were analyzed by SDS-polyacrylamide gel electrophoresis after CNBr digestion as described in Chapter 2. Track (1), incubation time of 0 h, track (2), incubation time of 1 h, track (3), incubation time of 2 h, track (4), incubation time of 3 h, track (5), incubation time of 4 h, track (6), incubation time of 5 h, track (7), incubation time of 24 h, track (8), rat tail tendon collagen type I CNBr digest standard.

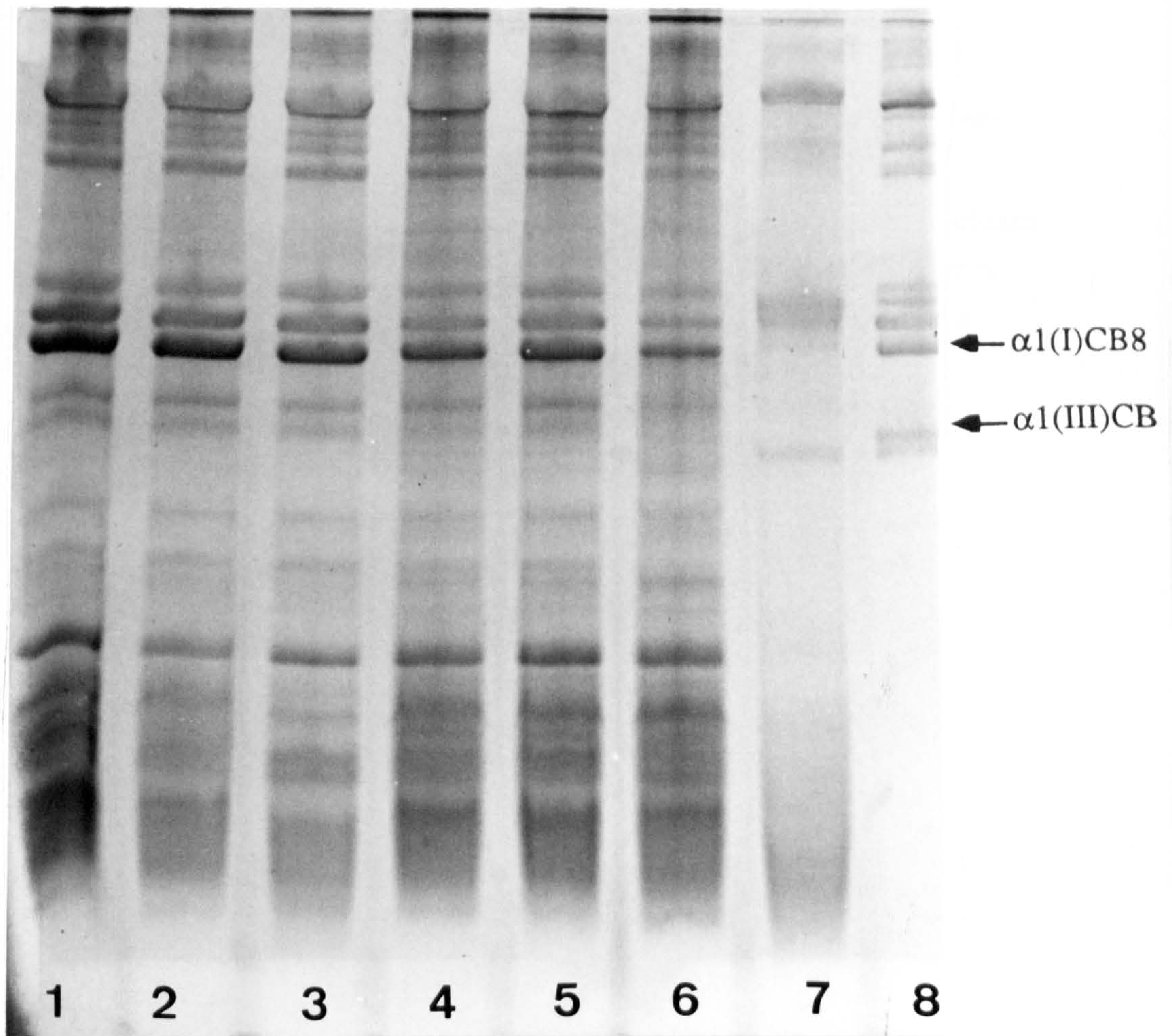


Fig. 6.4 SDS-Polyacrylamide Gel Electrophoresis of CNBr-Peptides of Residual Insoluble Material after Cathepsin Treatment of 6 M Urea -Washed Perimysium.

Insoluble perimysium from unconditioned bovine muscle, washed with 6 M urea, 0.05 M tris-Cl, pH 7.4 was incubated with spleen extract, at 4° C, and the insoluble residues at various time points were analyzed by SDS-polyacrylamide gel electrophoresis after CNBr digestion as described in Chapter 2. Track (1), incubation time of 0 h, track (2), incubation time of 1 h, track (3), incubation time of 2 h, track (4), incubation time of 3 h, track (5), incubation time of 4 h, track (6), incubation time of 5 h, track (7), incubation time of 24 h, track (8), rat tail tendon collagen type I CNBr digest standard.

6.3.3 2-D Gel Analysis of CNBr Digested Insoluble Perimysial Material after Cathepsin Treatment and Normal Conditioning

NEPHGE was combined with SDS-polyacrylamide gel electrophoresis to produce two-dimensional separations of CNBr-cleavage peptides. Exhaustively dialyzed CNBr-cleavage peptides from insoluble perimysium used in these analyses were found to contain on average greater than 95 % collagen. Figs. 6.5 and 6.6 show the distribution of total CNBr-cleavage peptides obtained from untreated perimysium from fresh muscle (as a control) and 6 M urea washed insoluble perimysium treated with cathepsin for 24 h, respectively. Two-dimensional resolution of the cathepsin treated sample showed the absence of several peptide spots and the appearance of at least one new spot when compared with the control (Figs. 6.5 a,b and 6.6 a,b and Table 6.2).

Fig. 6.7 shows the distribution of CNBr-cleavage peptides from 6 M urea washed insoluble perimysium obtained from conditioned *gastrocnemius* muscle. As in the case of the model cathepsin system, there were distinct differences between the peptide map of the conditioned sample and that of the unconditioned sample (Fig. 6.5). Some peptide spots, which were seen on the control, were absent from the conditioned sample. In addition, there were new spots on the conditioned sample which did not appear in the control. These differences are highlighted in Fig. 6.7 b and summarized in Table 6.2.

The peptides labelled i, l, m, k, o were lost from conditioned insoluble perimysium and peptides labelled a, c, e, f, h', i, m, n, were lost from 24 h cathepsin incubated insoluble perimysium when compared with the unconditioned (control) sample. The peptides lost from both conditioned and cathepsin treated perimysium were, therefore, i and m. New spots were generated in both conditioned and cathepsin treated perimysium, however, no new spots generated were common to both conditioned and cathepsin treated insoluble perimysium. New spots generated in the conditioned sample were denoted g', s, t, u, p,q and in the cathepsin treated sample spots labelled x, y, g" were generated which were not present in the unconditioned sample.

Figs. 6.8 a and b show the results of two-dimensional analysis of unconditioned (left) and conditioned (right) perimysium respectively, from *extensor capri radialus* using the 2-D gel protease system. Comparison of the two results showed that many additional peptides could be seen resulting from chymotrypsin digestion of the high molecular weight material in the conditioned sample (arrowed in Fig. 6.8 b) which did not appear in the unconditioned sample. A band in the molecular weight range 40 000 - 50 000 (highlighted with an arrow) appeared in the conditioned sample but was hardly visible on

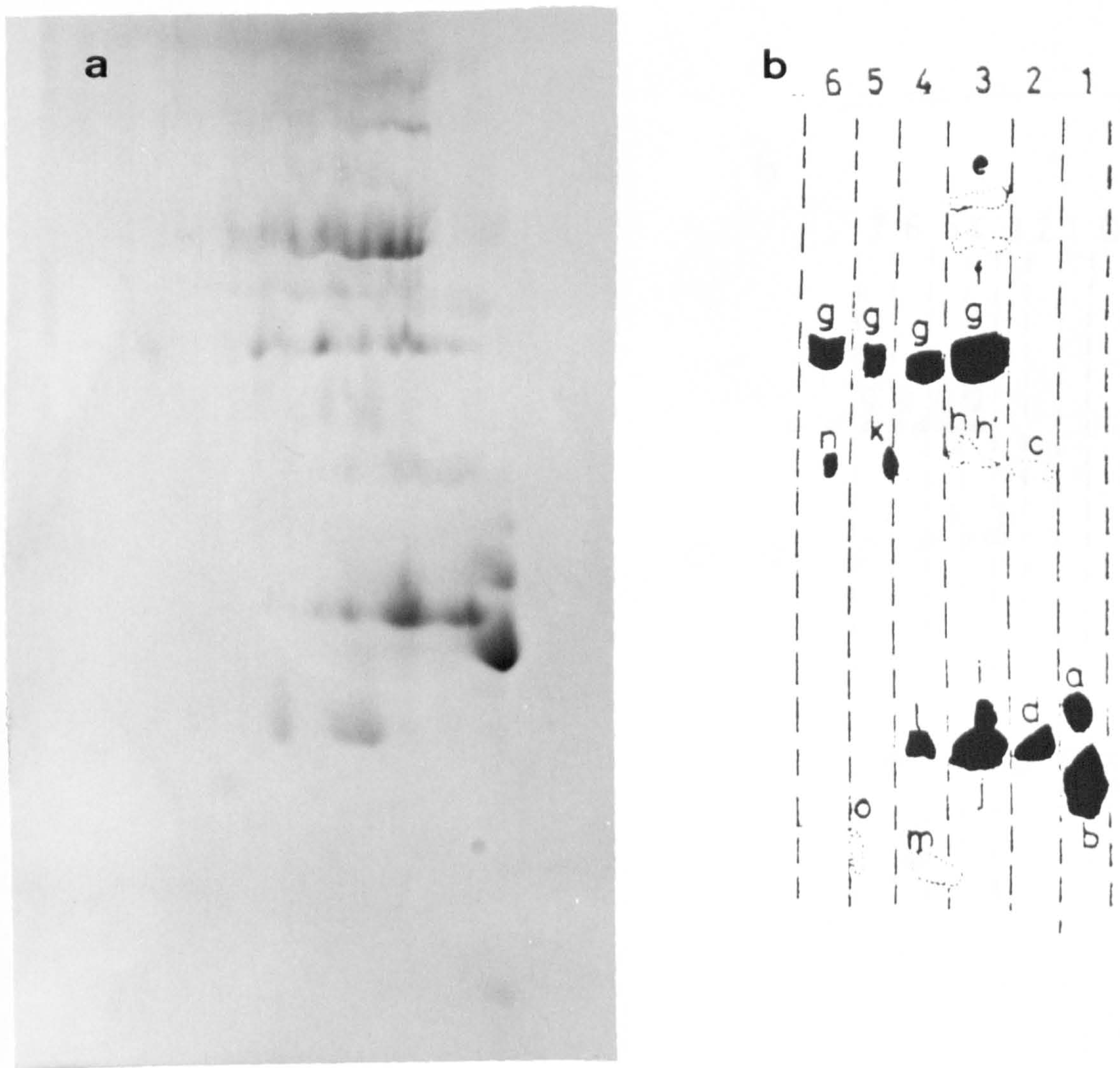


Fig. 6. 5 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium from Unconditioned *Gastrocnemius* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis

Exhaustively dialyzed CNBr-cleavage peptides, extracted from unconditioned *gastrocnemius* muscle using 6 M urea, 0.05 M tris-Cl, pH 7.4, were subjected to electrophoresis in the first dimension for 3 h at 500 V, on a pH 3.5-10 gradient (comprised of 2 % (v/v) ampholines, consisting of 1.8 % (v/v) ampholines pH 3.5-10 and 0.2 % (v/v) ampholines pH 5-8). First dimension gels were electrophoresed in the second dimension on 10 % (w/v) acrylamide SDS slab gels using conditions described in Chapter 2.

(a) Photograph of peptide map obtained after 2-D analysis,

(b) Diagrammatic representation of the peptide map from (a).

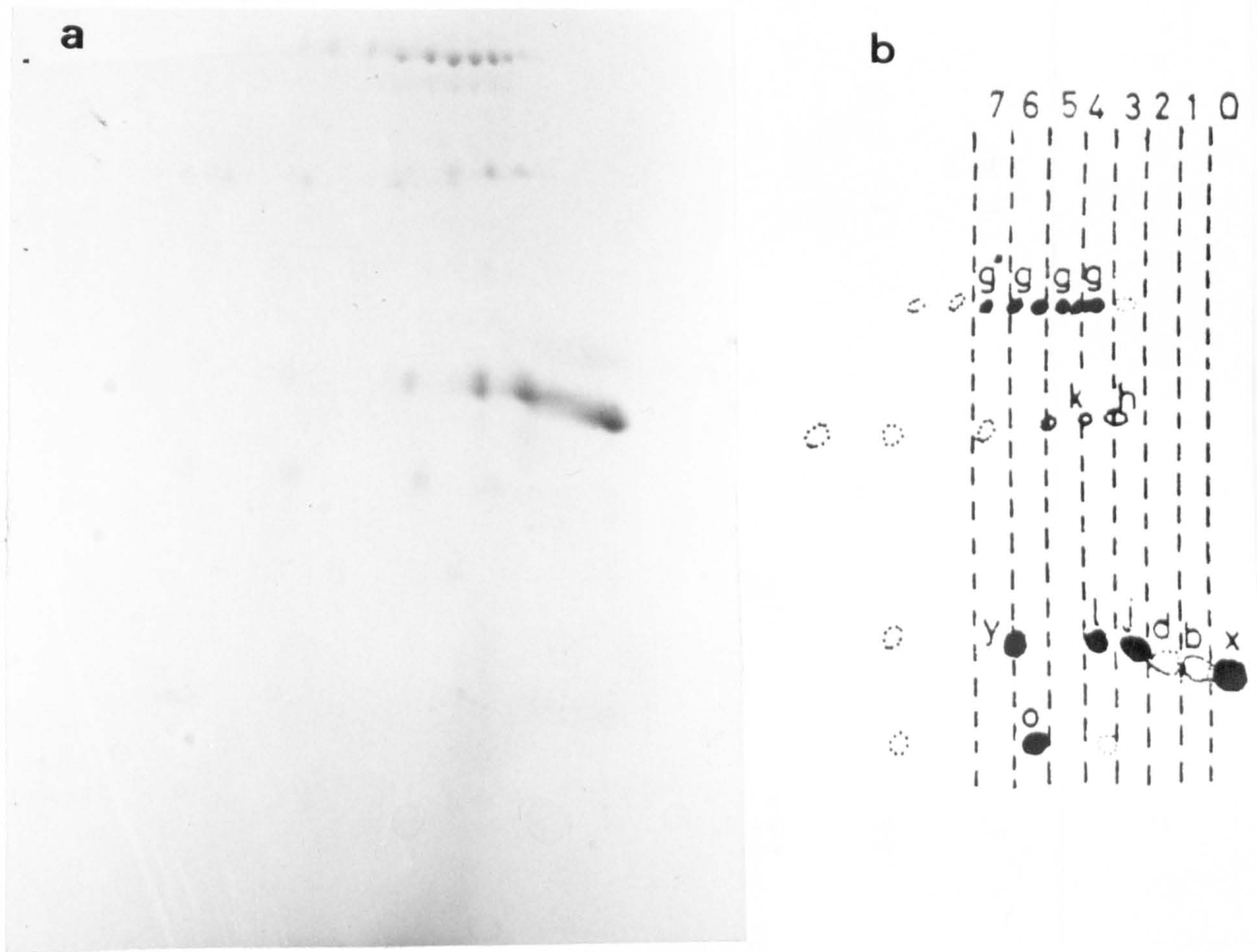


Fig. 6.6 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium from Freshly Excised Unconditioned Bovine Muscle, Treated with Cathepsin for 24 h, by 2-D SDS-Polyacrylamide Gel Electrophoresis.

Exhaustively dialyzed CNBr-cleavage peptides, extracted from freshly excised unconditioned muscle using 6 M urea, 0.05 M tris-Cl, pH 7.4, were subjected to electrophoresis in the first dimension for 3 h at 500 V, on a pH 3.5-10 gradient (comprised of 2 % (v/v) ampholines, consisting of 1.8 % (v/v) ampholines pH 3.5-10, and 0.2 % (v/v) ampholines pH 5-8). First dimension gels were electrophoresed in the second dimension on 10 % (w/v) acrylamide SDS slab gels using conditions described in Chapter 2.

(a) Photograph of peptide map obtained after 2-D analysis,

(b) Diagrammatic representation of the peptide map from (a).

Table 6.2 Summary of Data from CNBr Peptide Maps of Insoluble Perimysium

Column	Unconditioned	Conditioned	Cathepsin Treated	Peptides missing in test sample*	New peptides in test sample*
0	-	-	x	-	x
1	a,b	a,b	b	a	-
2	c,d	c,d,p,g'	d	c	g',p
3	e,f,g,h, h'i,j	e,f,g,h, h',t,s,j,u	g,h,j	e,f,h',i	t,s,u
4	g,l,m	g	g,l	m	-
5	g,k,o	g	g,k,o	-	-
6	g,n	g,n	g,y	-	y
7	-	q	g''	-	g'',q

* test sample denotes conditioned and cathepsin treated

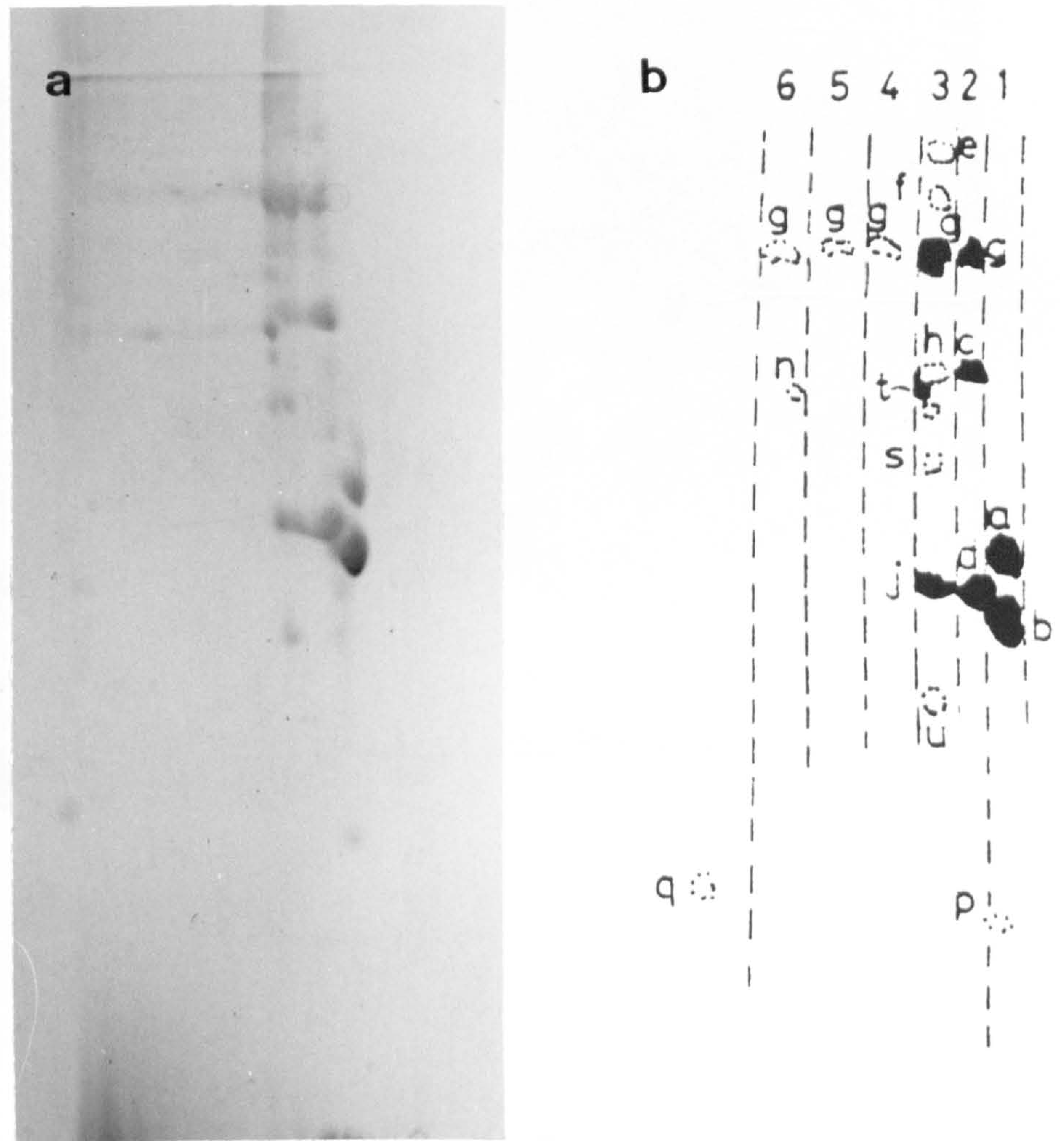


Fig. 6.7 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium from Conditioned *Gastrocnemius* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis.

Exhaustively dialyzed CNBr-cleavage peptides, extracted from conditioned *gastrocnemius* muscle using 6 M urea, 0.05 M tris-Cl, pH 7.4, were subjected to electrophoresis in the first dimension for 3 h at 500 V, on a pH 3.5-10 gradient (comprised of 2 % (v/v) ampholines, consisting of 1.8 % (v/v) ampholines pH 3.5-10 and 0.2 % (v/v) ampholines pH 5-8). First dimension gels were electrophoresed in the second dimension on 10 % (w/v) acrylamide SDS slab gels using conditions described in Chapter 2.

(a) Photograph of peptide map obtained after 2-D analysis,

(b) Diagrammatic representation of the peptide map from (a).

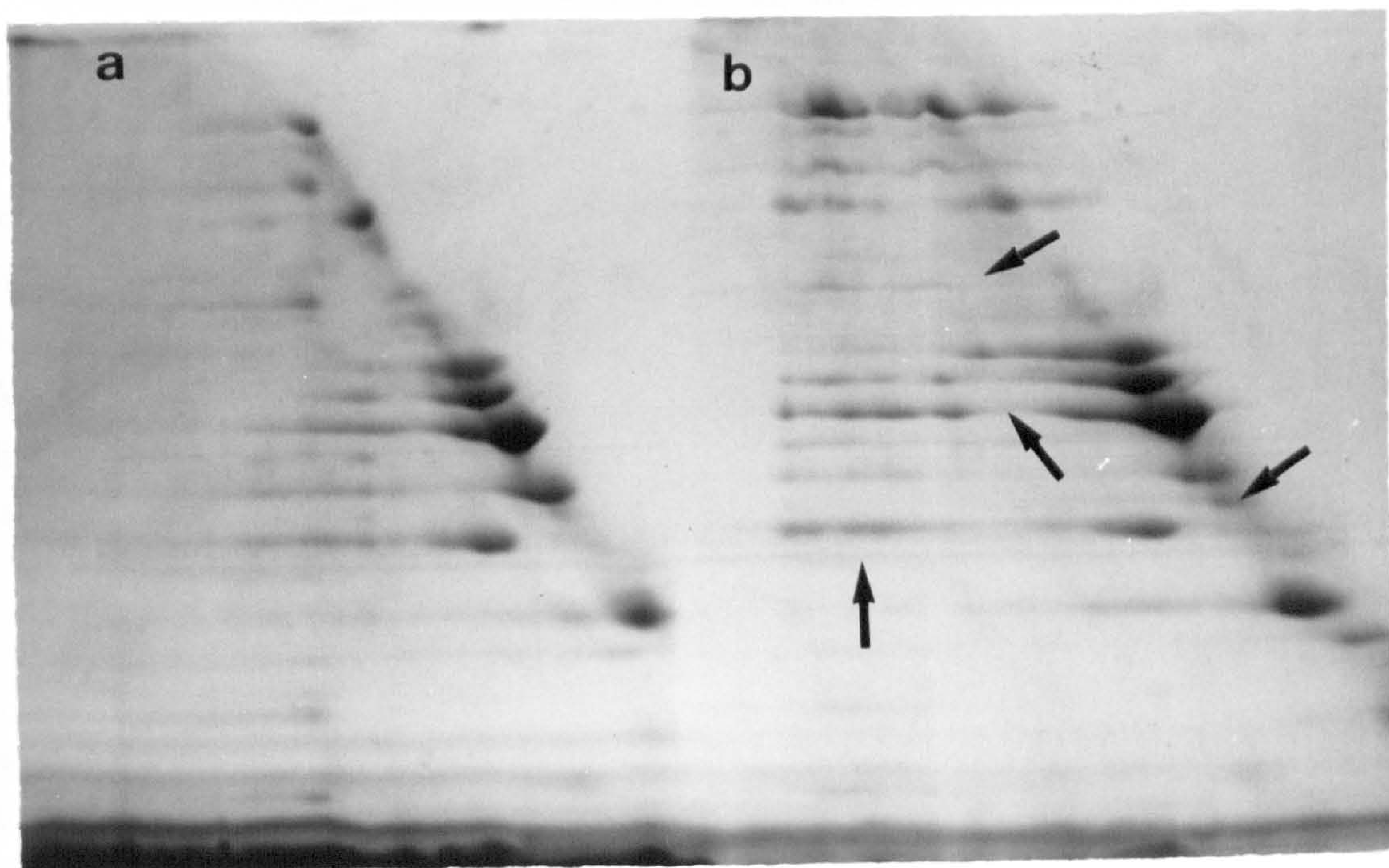


Fig.6.8 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium from (a) Unconditioned and (b) Conditioned *Extensor Capri Radialus* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis.

CNBr digests of perimysial samples, dissolved in sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10% (w/v) polyacryamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 400 µl of chymotrypsin solution (3.33 mg/ml, 200 units/ml)) in gel sample buffer was applied to the top of the gel, and the system was incubated for 2 h whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acryamide slabs using conditions as described in Chapter 2. The arrows show the presence of two new sets of peptides and absence of two peptides in the conditioned sample when compared to the unconditioned sample.

the unconditioned gel. There were also at least two additional peptides which were evident in the unconditioned sample but were absent from the conditioned perimysial digest. Similar changes were noted due to conditioning, or 2-D analysis of perimysium obtained from other muscles. Figs. 6.9 and 6.10 show the results of 2-D analysis of unconditioned (left) and conditioned (right) perimysium obtained from *gastrocnemius* and *gluteus medius* muscles, respectively. It was noted that the extent of differences observed was greater in some muscles than in others. However, comparisons of unconditioned with conditioned perimysium, obtained from these muscles revealed increased incidence of peptides resulting from chymotrypsin digestion of the high molecular weight material (highlighted with an arrow), results which were similar to those obtained for *extensor capri radialus* above (Fig. 6.8).

6.3.4 2-D Gel Analysis of CNBr Digested Insoluble Endomysial Material

Although the gels obtained from 2-D analysis of endomysium were inferior to those obtained for perimysium, comparison of unconditioned with conditioned samples revealed alterations in this fraction, due to conditioning.

Figs. 6.11 and 6.12 show the results of analysis of unconditoned (left) with conditioned (right) insoluble endomysium, obtained from *gastrocnemius* and *psoas major* muscles, respectively, using the 2-D protease system. There was evidence of a new peptide in the molecular weight range 40 000 - 50 000 (Fig. 6.11b) (highlighted with an arrow). Insoluble endomysium obtained from conditioned muscles showed increased incidence of proteolysis of the high molecular weight material (Figs. 6.11 and 6.12).

6.4 DISCUSSION

The use of urea washed perimysium in this study was prompted by the need for a substrate with as high a collagen content as possible to allow the measurement of effects on this particular connective tissue component. Other solvents and extractants may have been less effective in yielding a relatively clean substrate, whilst others could have led to unacceptable changes in the perimysium, rendering it useless as a physiological substrate in these *in vitro* studies (see Chapter 3). The use of unwashed and urea washed substrates with pepsin and spleen cathepsin preparations and the action of trypsin on both showed that the urea washed substrate behaved substantially like the unwashed collagen in terms of the extent and rate of collagen solubilization and did not exhibit undue signs of denaturation. Trypsin does not degrade native collagen, so this was a useful tool for investigating the effect of extraction solvents on denaturation of collagen in perimysium.

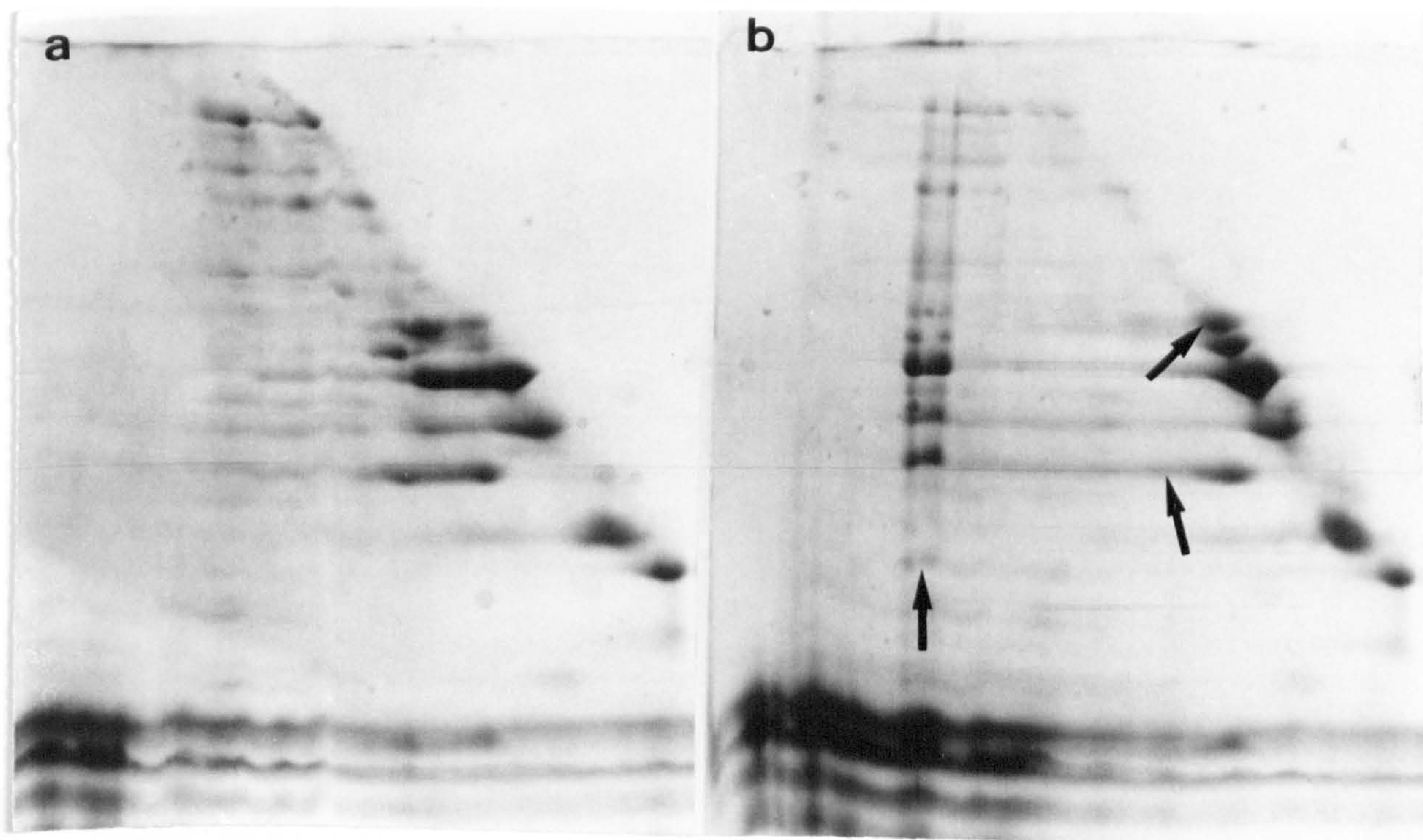


Fig. 6.9 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium from (a) Unconditioned and (b) Conditioned *Gastrocnemius* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis.

CNBr digests of perimysial samples, dissolved in sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 400 μl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel, and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

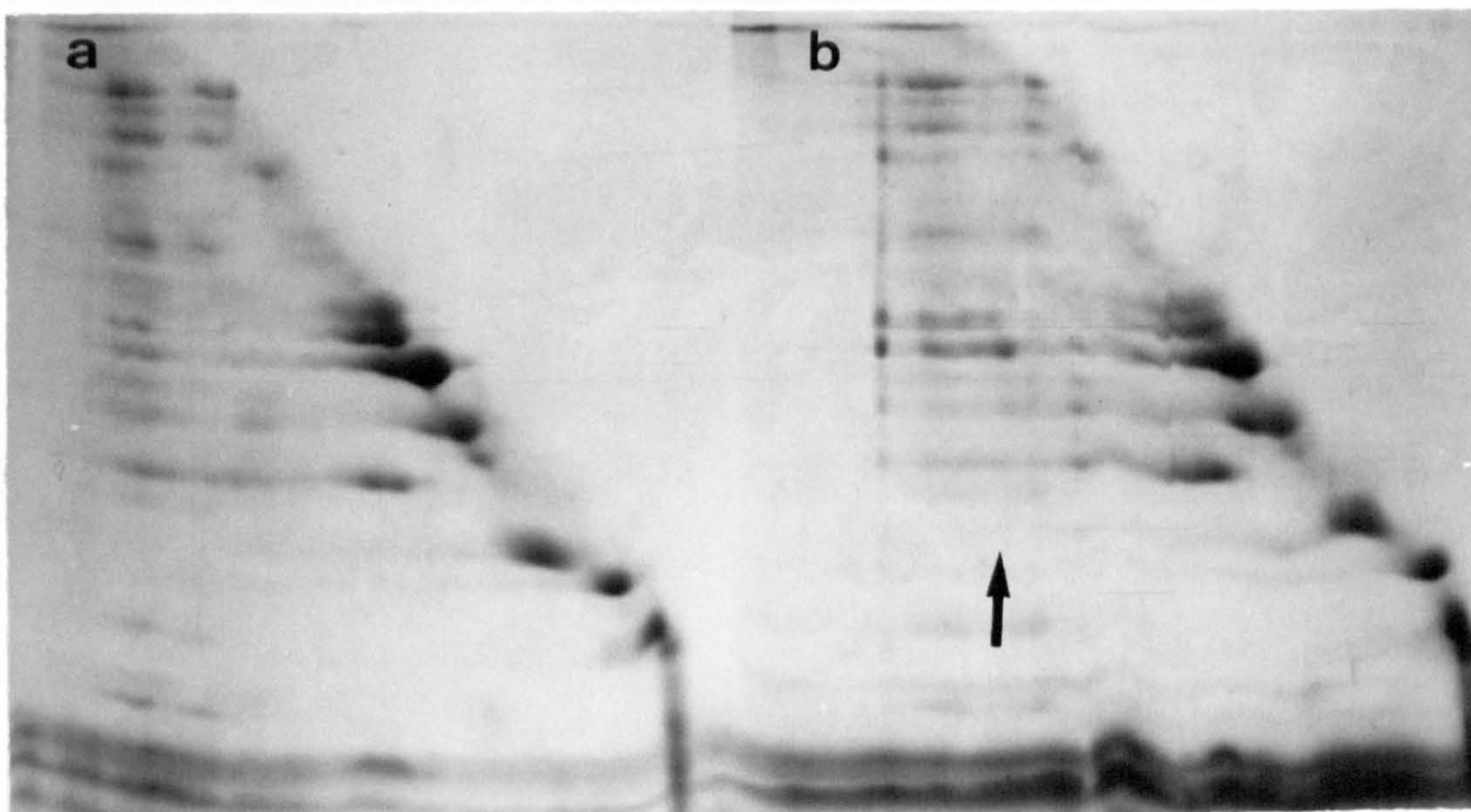


Fig. 6.10 Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium from (a) Unconditioned and (b) Conditioned *Gluteus Medius* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoreis.

CNBr digests of perimysial samples, dissolved in sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 400 μl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

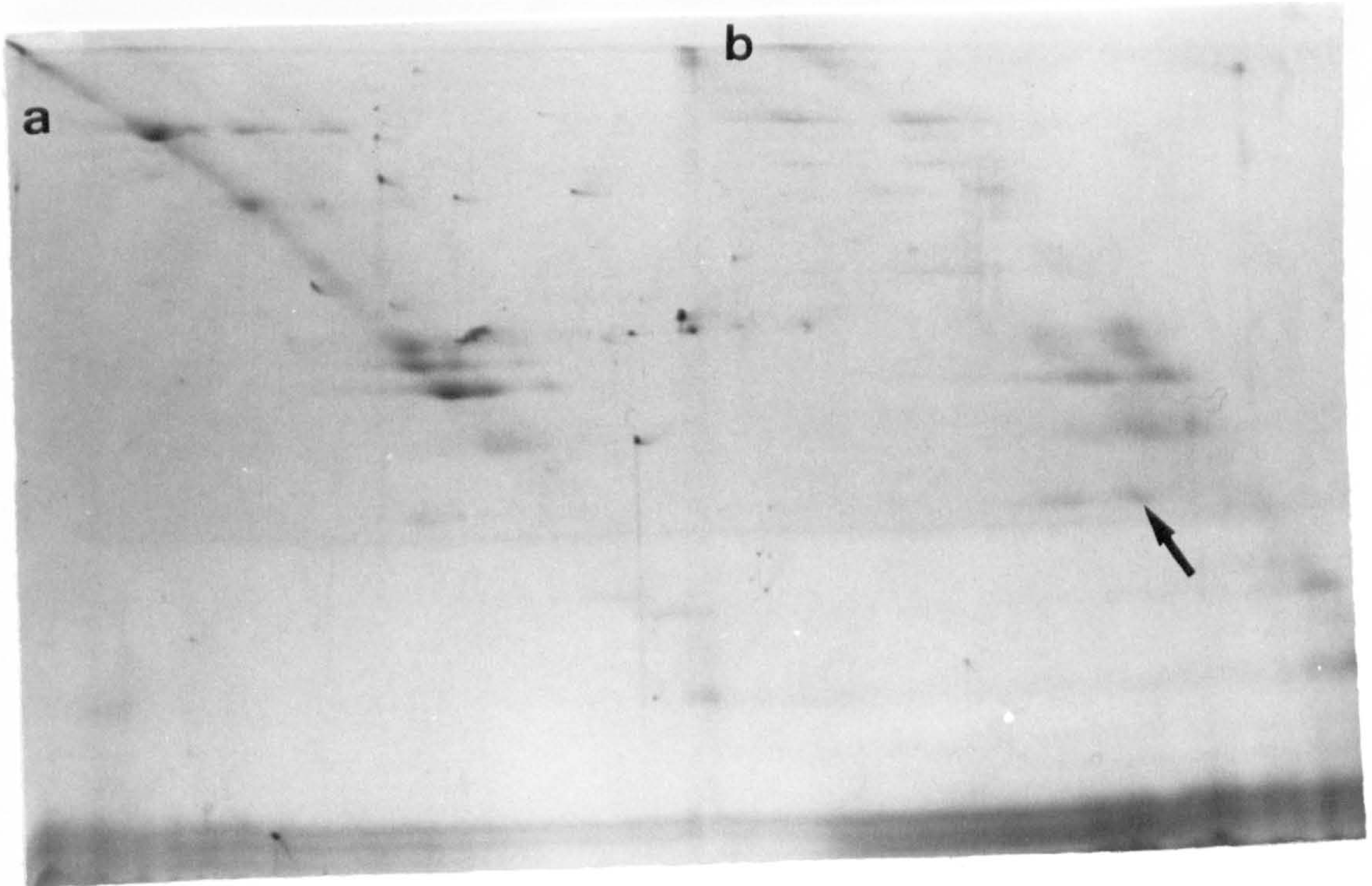


Fig. 6.11 Peptide Mapping of SDS-Washed Insoluble Endomysium from (a) Unconditioned and (b) Conditioned *Gastrocnemius* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis.

CNBr digests of endomysial samples, dissolved in sample buffer at a concentration of 10 mg/ml, were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 400 μl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

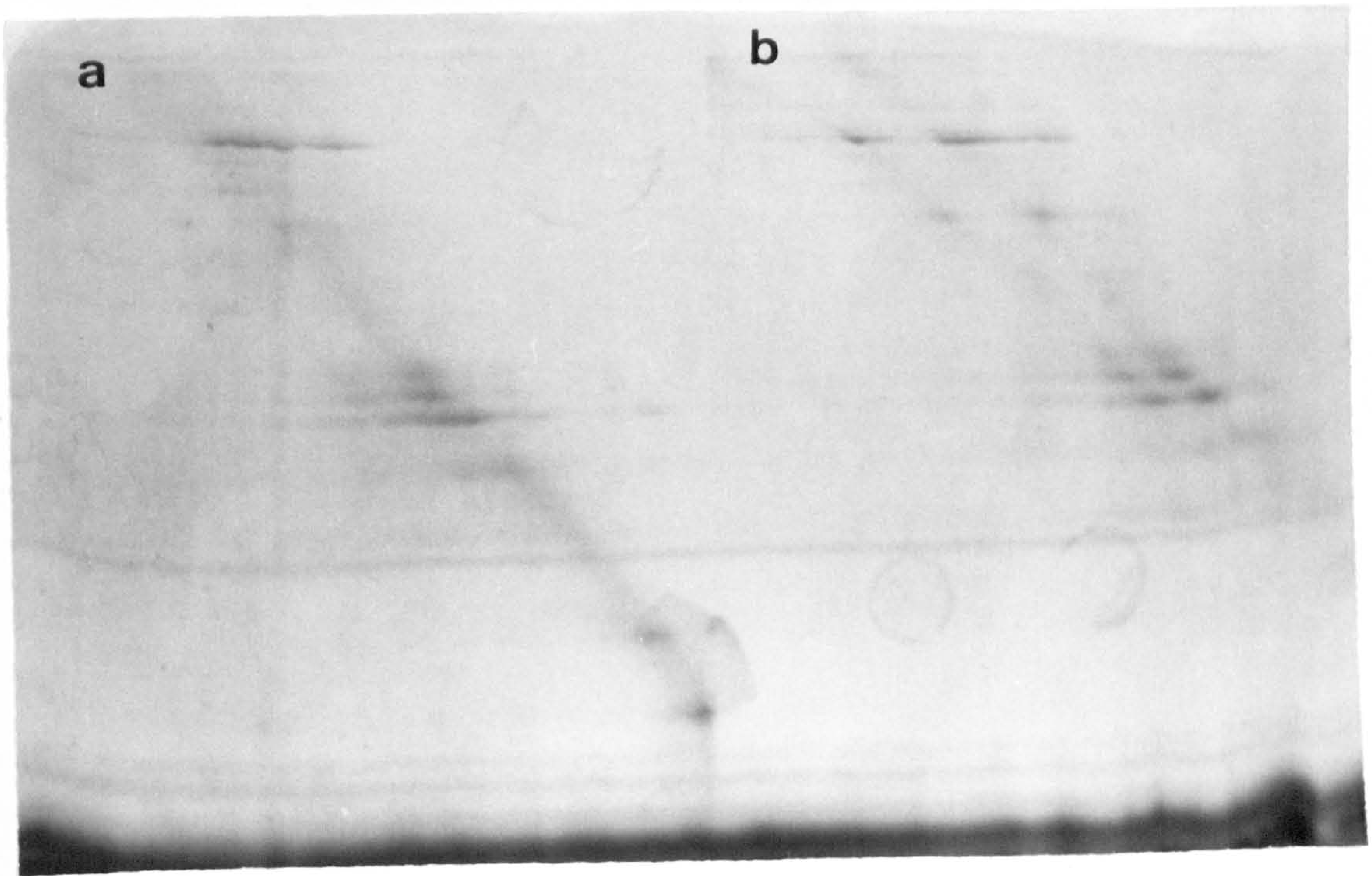


Fig. 6.12 Peptide Mapping of SDS-Washed Insoluble Endomysium from (a) Unconditioned and (b) Conditioned *Psoas Major* Muscle by 2-D SDS-Polyacrylamide Gel Electrophoresis.

CNBr digests of endomysial samples, dissolved in sample buffer at a concentration of 10 mg/ml, were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels applied to the top of the resolving gels. 400 µl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel and the system was incubated for 2 h whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

Because the results obtained from trypsin incubation with unwashed and urea washed perimysium were similar, it was concluded that denaturation of collagen did not occur by urea extraction of perimysium. Therefore, this was a suitable substrate for examination of conditioning effects on perimysial collagen and was used throughout the study.

The effect of pepsin on the perimysial substrate was slow at 4° C, as might be predicted, with a steady rise in the amount of solubilized collagen over a 24 h period. At this time 4.8 % of the original collagen had been solubilized (calculated from total insoluble and solubilized collagen) although damage to the residual material was not evident on one-dimensional SDS-polyacrylamide gels. A similar effect, in terms of the extent of collagen solubilized, was seen with the spleen cathepsin preparations (5.2 % of total original collagen solubilized) which closely mimicked the action of pepsin. However, the effect at 24 h was considerably greater than that observed *in situ* in studies of normal conditioning (see Chapter 3). In various muscles of differing quality it was found that an average of 3.4 % collagen was solubilized after extensive conditioning in beef (14 days at 4° C), a situation which closely matches the *in vitro* effect of cathepsin treatment noted here after only 5 h (3.4 % of original collagen solubilized). At 24 h, approximately 0.5 mg/ml collagen was released by pepsin and 0.8 mg/ml collagen was released by cathepsin. Concentrations of 5 to 6 mg/ml collagen in acidic solvents represent saturated solutions of collagen. The low concentrations of solubilized collagen obtained after 24 h incubation with pepsin and cathepsin reflect the intractable nature of the residual insoluble substrate. This is further highlighted by the fact that only 12 % and 18 % of total perimysial material was solubilized at this time by pepsin and cathepsin.

The changes in the residual insoluble perimysial collagen were investigated in this study by means of two-dimensional polyacrylamide gel electrophoresis. As in the previous study on conditioned perimysium (see Chapter 3) the CNBr peptide pattern obtained from insoluble perimysium after either pepsin or cathepsin incubation for 5 h showed little change when analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. After 24 h treatment with cathepsins, however, changes were apparent and seemed to centre on a general lessening of material with some indication of specific peptide damage. NEPHGE combined with SDS-polyacrylamide gel electrophoresis provided a more sensitive technique for comparing unconditioned insoluble perimysium with insoluble perimysium which had been incubated with the spleen cathepsin preparation for 24 h. This resulted in an altered peptide pattern, with the loss of some peptides and generation of others relative to the unconditioned (control) sample. The *in vitro* case was obviously extreme as the damage seen on one-dimensional analysis showed, but comparison with normal conditioned perimysium showed similar changes, i.e. there was direct evidence of

proteolytic damage to residual insoluble perimysium from conditioned muscle.

The alternative two-dimensional technique for comparing unconditioned with conditioned insoluble perimysium from *extensor capri radialus* revealed changes in the conditioned sample, evident as many additional peptides in the high molecular weight material, due to chymotrypsin digestion, as well as the appearance of a new peptide in the molecular weight range 40 000 - 50 000 in the conditioned sample and the loss of at least two other peptides due to conditioning. Similar results were obtained with a range of conditioned bovine muscles (see Figs. 6.9 a, b and 6.10 a, b).

2-D analysis of unconditioned and conditioned insoluble endomysium produced inferior results to those obtained with perimysium, because endomysial material proved more difficult to analyze by the 2-D protease system. The concentration of CNBr-digests loaded in the first dimension was increased from 5 mg/ml used for perimysial digests to 10 mg/ml for endomysial digests. Conditioning resulted in the appearance of a new peptide in the molecular weight range 40 000 - 50 000 and increased incidence of peptide material in the high molecular weight range.

The increased incidence of peptide material resulting from the secondary chymotryptic digestion of high molecular weight components in conditioned connective tissue fractions is an important finding. It has been shown that high molecular weight aggregates formed from the the C-terminal end of the collagen molecule are involved in stabilizing mature matrix collagen (Light & Bailey, 1980b, c). Also, previous work has indicated the similarity of cathepsins to pepsin in attacking matrix collagen at the non-helical terminal ends of the molecule (Etherington, 1980). Our data would suggest, therefore, that there was proteolytic damage to the high molecular weight aggregates present in the CNBr digest of the conditioned samples which allowed more extensive action by the chymotrypsin used before second dimension separation of the peptide material, thereby producing the increased number of peptides observed. Thus, it seems likely that a major cleavage site of cathepsins in perimysial collagen *in vivo* is by the route previously suggested, i.e. the terminal, non-helical sequences.

These results provide the first direct biochemical evidence of damage in insoluble perimysium after conditioning and support earlier physical studies which indicated that such damage might occur (Field et al., 1970; Robbins & Cohen, 1976; Kopp & Valin, 1980; Judge & Aberle, 1982).

Goll et al., (1964) proposed that differences in meat quality may be brought about by

variations in the structure of the collagen molecule. Kruggel et al., (1970) and Kruggel & Field (1971) supported this view and Mills et al., (1984) reported a decrease in the structural and thermal stability of collagen after conditioning, suggesting that these changes occurred within a few hours post-mortem. The integrity of collagen fibres after conditioning was investigated by examination of isolated intramuscular connective tissue using differential scanning calorimetry (Judge & Aberle,1982). A fall of 3 to 5° C in the thermal shrinkage temperature of bovine collagen after 24 h of conditioning and a fall of 7 to 8° C by 7 days post-slaughter was observed. Elsewhere a similar fall in shrinkage temperature was found for porcine muscle collagen at 24 h post-mortem (Field et al., 1970).

The study of post-mortem changes in meat connective tissue collagen has provided some evidence for the action of lysosomal proteases released during the conditioning process (Dutson & Lawrie, 1974; Wu et al.,1981). The main proteinase constituents of the cell lysosome, the cathepsins, operate most effectively under mildly acidic conditions, as exist in meat during the conditioning process. In model studies, the cathepsins have been shown to cleave bonds in the non-helical peptide domains of native collagen molecules resulting in the formation of soluble components at pH values above 5.0 (Etherington, 1987a).

Negligible solubilization of insoluble fibrous collagen occurs above pH 4.5 (Etherington, 1981) and until recently little information was available on any weakening action by these proteinases at the post-mortem pH of 5.5. Incubation of fresh muscle with a crude preparation of spleen cathepsins at pH 5.5 resulted in disruption of endomysial sheaths as evidenced by SEM (Robbins & Cohen, 1976). The effect of incubation of a muscle lysosomal lysate with isolated epimysial collagen fibres at pH 5.5 was a substantial reduction (by 50 %) in the isometric tension subsequently developed during heating and an increase in the thermolabile fraction of collagen (Kopp & Valin, 1980). More recently, Etherington (1987a) incubated insoluble bovine tendon collagen with a lysate of rat liver lysosomes at pH 5.5. The results showed little direct solubilization of the collagens by the enzymes at pH 5.5, although this is at variance with the DSC analysis findings of Judge & Aberle (1982) mentioned above.

Contribution of the collagen component to the texture of meat is a complex problem and involves a number of factors including total collagen content, collagen crosslinking, collagen type, collagen fibre size, ageing and glycosylation (see Light, 1987, for review). Evidence for weakening of this component during conditioning of meat has been contradictory, and little or no direct evidence revealing changes in the collagenous matrix

during conditioning is available. The data provided in this study indicate that subtle modifications occur in intramuscular collagen during conditioning which can be correlated with catheptic action on the endomysial and perimysial components of meat connective tissue and which probably lead to the physical weakening of the matrix observed in earlier studies.

CHAPTER 7

INVESTIGATION OF PRE-RIGOR LACTIC ACID INJECTION FOR PRODUCTION OF RAPID CONDITIONING EFFECTS IN BOVINE MEAT

7.1 INTRODUCTION

Following death of the animal, lactic acid is produced via anaerobic glycolysis causing a decrease in pH to 5.5 as discussed in Chapter 1 (section 1.5.1) and activation of acid proteases in the muscles. The best quality meat is obtained when the carcass is chilled slowly and then allowed to hang, which for beef may be 2 weeks or more at the chill temperature. Although ageing improves beef quality, the cost of holding carcasses for an extended period at chill temperatures can be very high. Therefore, the producer is seeking ways of reducing the conditioning time period without incurring a forfeit on quality.

The work outlined in this chapter investigates the potential of a new procedure for tenderizing meat. The procedure is based on the concept of marinading which is recognized in culinary circles as a means of imparting flavour to meat and improving tenderness (Gault, 1984) but alterations to the colour, flavour and odour of red meat are substantial, therefore rendering this method unsuitable for routine tenderization of beef. While marinading involves immersing meat in an acidic solution of vinegar, wine or fruit juice, which may result in uneven penetration of the marinading acid throughout the meat, the procedure employed in this study involved introduction, into pre-rigor muscle, of lactic acid solution, the acid naturally produced in muscle, post-slaughter. It was postulated that rapid introduction of lactic acid, by multiple needle injection, immediately following slaughter of the animal, may cause rapid tenderizing effects, by inhibiting rigor-mortis and/or by causing earlier activation of muscle cathepsins. The result would be a reduction in the conditioning time from 14 days, a parameter of economic interest to the meat industry.

Initially, a pilot study was conducted, to determine suitable concentrations of lactic acid for pre-rigor injection which would cause rapid pH decline, without the problem of adverse colour changes in the meat. Onset of rigor mortis was monitored, post-injection, by disappearance of muscle ATP levels.

The main study involved the investigation of lactic acid injection and subsequent conditioning in high, medium and poor quality muscles compared with untreated controls. Lactic acid treated and untreated muscles were conditioned for times ranging from 1 to 14

days. pH and ATP levels were assessed in all muscles following conditioning. Solubilization of perimysial material and perimysial collagen were monitored in all muscles following conditioning and results obtained from lactic acid treated muscles were compared with those obtained from untreated controls. Residual perimysial material remaining after conditioning in both sets of muscles (lactic acid treated and controls) was analyzed by 2-D electrophoresis to compare the extent of damage caused to the collagen component by lactic acid treatment with untreated controls.

7.2 MATERIALS AND METHODS

7.2.1 Treatment of Meat with Lactic Acid

Samples of fresh unconditioned meat (50 g) were injected to a final weight 10 % greater than original meat weight with lactic acid of varying concentrations by multiple injections. Throughout this study this same level of lactic acid addition was used. Colour (by visual assessment), pH and ATP of the injected meat were monitored over 24 h.

7.2.2 ATP Measurement of Meat

10 g meat was homogenized in 20 ml distilled water, for 20 sec at 20° C in a Waring Blendor. 0.5 ml of the homogenate was diluted 1:1 with trichloroacetic acid (TCA) (12 % w/v) and allowed to stand in an ice-bath for 5 min. The suspension was centrifuged at 9 000 g x 5 min, and ATP quantified in the supernatant using an ATP assay kit (Sigma Diagnostics, Poole, UK). This method employed a coupled-enzyme-assay based on the reactions described by Bucher (1947) as modified by Adams (1963). The reaction of 3 - phosphoglycerate with ATP, catalyzed by phosphoglycerate kinase was coupled with a dephosphorylation reaction that involved oxidation of NADH. Formation of NAD was quantified by measuring the change in absorbance at 340 nm.

7.2.2.1 ATP Assay

Into a pre-weighed 0.3 mg single determination NADH vial, the following were introduced in the order indicated.

1.0 ml	Buffered Solution of 3 - phosphoglyceric acid, 18 mmol/l magnesium ions and EDTA, to which chloroform was added as preservative.
1.5 ml	distilled water
0.5 ml	supernatant (from section 7.2.2).

The vial was capped and inverted several times to dissolve the NADH. The entire contents of the vial were decanted into a cuvette and initial absorbance (A) was recorded against water as reference at 340 nm. Into the cuvette 0.04 ml of a suspension in ammonium sulphate of glyceraldehyde 3 - phosphate dehydrogenase (rabbit muscle), 800 units/ml and 3 - phosphoglyceric phosphokinase (yeast), 450 units/ml was introduced and mixed by inversion of the cuvette several times. The absorbance was continually read and recorded using water as reference at 340 nm, until the minimum absorbance reading was reached (usually required less than 10 mins). This was recorded as final A.
Meat ATP was calculated from the following equation:

Meat ATP (μmol/100 ml) = $\Delta A \times 195 \times 2$

where:

ΔA = Initial A - Final A

The factor 195 was derived as follows:

$$195 = \frac{3.04 \times 100}{6.22 \times 0.25}$$

3.04 = volume of liquid in cuvette
100 = conversion of concentration per 1 ml to concentration per 100 ml
6.22 = millimolar absorptivity of NADH at 340 nm
0.25 = sample volume
2 = dilution factor

7.2.3 Preparation of Bovine Muscles

Six bovine animals of similar age and sex were slaughtered and the following muscles were dissected out from each side of the carcasses: *serratus ventralis* (poor quality), *gluteus medius* (medium quality) and *psoas major* (high quality). Two of each muscle types were vacuum packed immediately after slaughter and stored frozen; these were termed unconditioned muscles.

Of the ten muscles of each type remaining, five were vacuum packed immediately after slaughter and conditioned at chill temperature 1 to 3° C for 1, 3, 7, 10 and 14 days. All muscles were stored frozen following conditioning. 0.1 M lactic acid was introduced into the five remaining muscles of each type to a level of 10 % the original weight of muscles as described above, by multiple needle injection immediately after slaughter. The muscles

were vacuum packed and stored frozen after conditioning at chill temperature for times as described above.

7.2.4 Preparation of Perimysium

4 x 25 g samples of meat were taken from different locations within the muscle to represent the general aspects of the whole muscle. These were combined and the sample of meat (100 g) was homogenized in 200 ml 0.05 M $CaCl_2$ (ice-cooled) for 10 sec at full speed in a Waring Blendor. The homogenate was filtered through a graded copper gird (1 mm^2 perforations) and the material retained on the filter was re-homogenized in 100 ml 0.05 M $CaCl_2$ and re-filtered. This process was repeated twice, using 50 ml 0.05 M $CaCl_2$ each time. The retained material was subjected to three washes in 6 M urea, 0.05 M tris-Cl, pH 7.4 as outlined in Chapter 2 (section 2.4.2). Soluble and insoluble perimysium were dialyzed for 48 h against running water and against two changes of distilled water. All fractions were frozen following dialysis, and subsequently freeze-dried if necessary, e.g. for total dry weight estimations.

7.3 RESULTS

7.3.1 Treatment of Meat with Lactic Acid

7.3.1.1 Colour

Samples of fresh unconditioned meat treated with varying concentrations of lactic acid and held at 15° C, were monitored over time for colour changes (Table 7.1). All meat samples injected with 0.01 M and 0.1 M lactic acid retained the bright red meat colour similar to the control sample over a 24 h period. Meat samples injected with concentrations of 0.2 M lactic acid and higher were deemed unacceptable 1 h after injection as colour changes to purple, purple-red, brown and even green were noted.

7.3.1.2 pH

Samples of fresh unconditioned meat treated with varying concentrations of lactic acid and held at 15° C were monitored over time for pH. pH decreased in all samples over a 26 h period (Fig. 7.1). The slowest pH decline was in the control which decreased from an initial pH value of 7.10 to 5.51 at 26 h. The pH of meat samples injected with lactic acid dropped increasingly rapidly as the lactic acid concentration increased (Fig. 7.1), with 1 M lactic acid producing the greatest pH decline at 2 h after injection (pH 3.17). Also,

Table 7.1 Meat Colour Change over Time after Lactic Acid Injection

[Lactic Acid] (M)	Time Elapsed after Injection (h) 0	1	2	3	4	8	24
1	+	-	-	-	-	-	-
0.8	+	-	-	-	-	-	-
0.6	+	-	-	-	-	-	-
0.4	+	-	-	-	-	-	-
0.2	+	-	-	-	-	-	-
0.1	+	+	+	+	+	+	+
0.01	+	+	+	+	+	+	+
0	+	+	+	+	+	+	+

+ denotes acceptable colour - bright red

- denotes unacceptable colour - dark red - purple - green

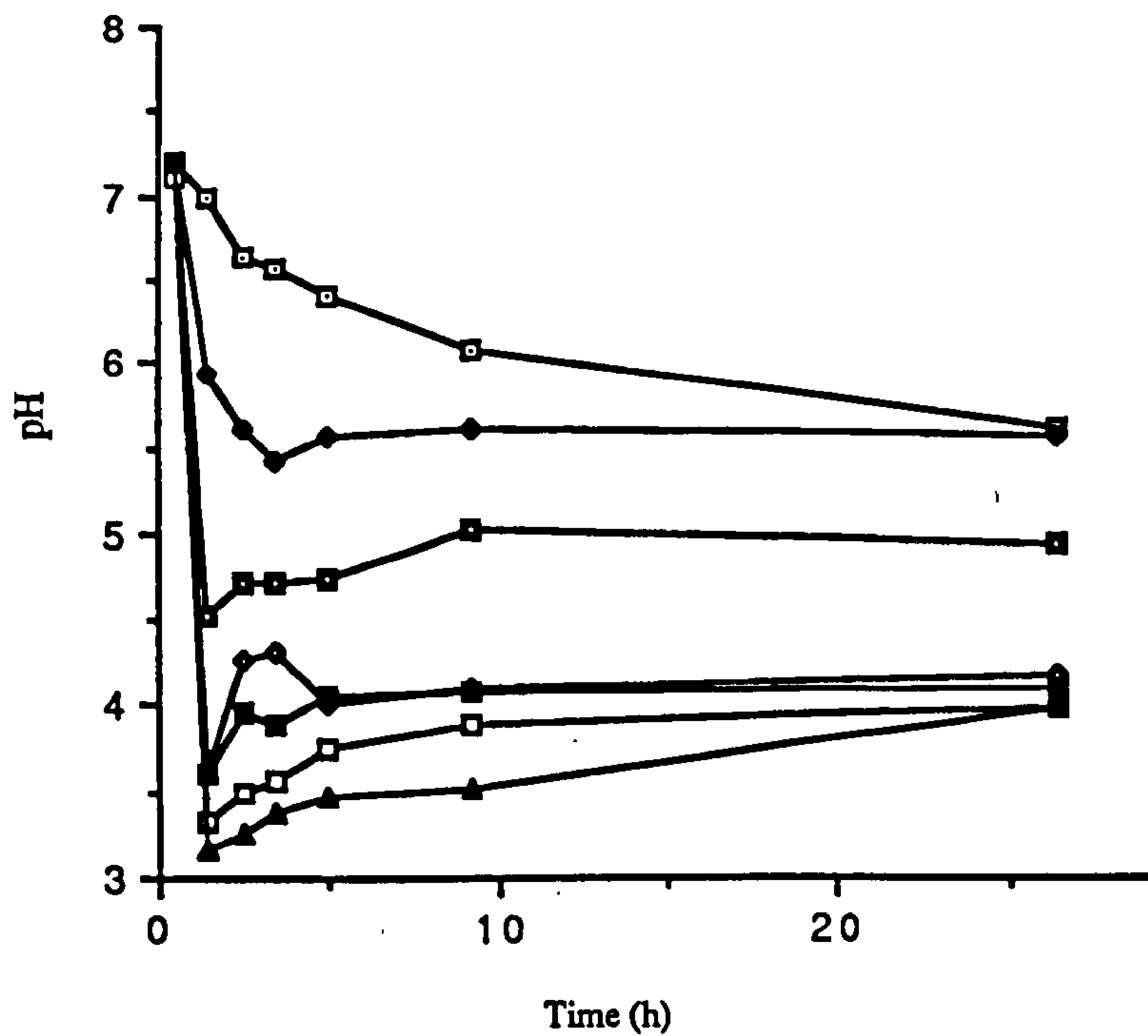


Fig. 7.1 Post-Mortem pH Decline of Lactic Acid Treated Meat Samples

Post-mortem pH decline of meat samples, injected with varying concentrations of lactic acid, to a final weight 10 % greater than original meat weight, was measured with a meat pH electrode.

▲ - 1 M lactic acid
□ - 0.8 M lactic acid
■ - 0.6 M lactic acid
◇ - 0.4 M lactic acid
◼ - 0.2 M lactic acid
◆ - 0.1 M lactic acid
⊡ - untreated control

concentrations of 0.2 to 1 M resulted in very rapid pH decreases followed by slight increases back to an equilibrium level which was always less than pH 5.0. The 0.1 M solution, however, gave a smooth but rapid decrease in pH and resulted in a similar pHu to the control.

7.3.1.3 ATP

The onset of rigor-mortis following 0.1 M lactic acid injection was monitored by disappearance of ATP. The rate of ATP decline was slightly more rapid over the first 8.75 h following lactic acid injection than the untreated control (Fig. 7.2). From 8.75 h to 24 h following 0.1 M lactic acid injection the rate of ATP decline was similar to the untreated control. Both the 0.1 M lactic acid treated and the untreated control had initial ATP levels of 121 μmol/100 g meat which declined over a 24 h period for both samples to a final ATP level of 3.9 μmol/100 g meat (Fig. 7.2).

7.3.2 Treatment of Bovine Muscles with 0.1 M Lactic Acid

7.3.2.1 pH

The pH values of frozen unconditioned, conditioned and lactic acid treated muscles are presented in Table 7.2. The pH of the unconditioned muscles was higher than that of all three muscle types treated from 1 to 14 days. However, the pH values of all muscles treated without and with 0.1 M lactic acid and conditioned from 1 to 14 days were similar. The average pH value of all *psoas major* and *gluteus medius* samples conditioned from 1 to 14 days (with and without lactic acid treatment), were similar at pH 5.5, whereas for *serratus ventralis* the average pH value of all conditioned muscles was pH 5.6.

7.3.2.2 ATP

The ATP levels of frozen unconditioned, conditioned and lactic acid treated muscles are shown in Table 7.3 For all three muscles examined, the unconditioned sample had a greater ATP level than conditioned and lactic acid treated samples. On average, the ATP level of all three muscles, conditioned normally for 1 day and subsequently frozen, dropped to 21 % of the average unconditioned frozen value for all three muscles examined, after which the ATP level did not change significantly. The average ATP level of the three unconditioned frozen muscles was only 14 % of the ATP value obtained from fresh unconditioned muscle (see section 7.3.1.3).

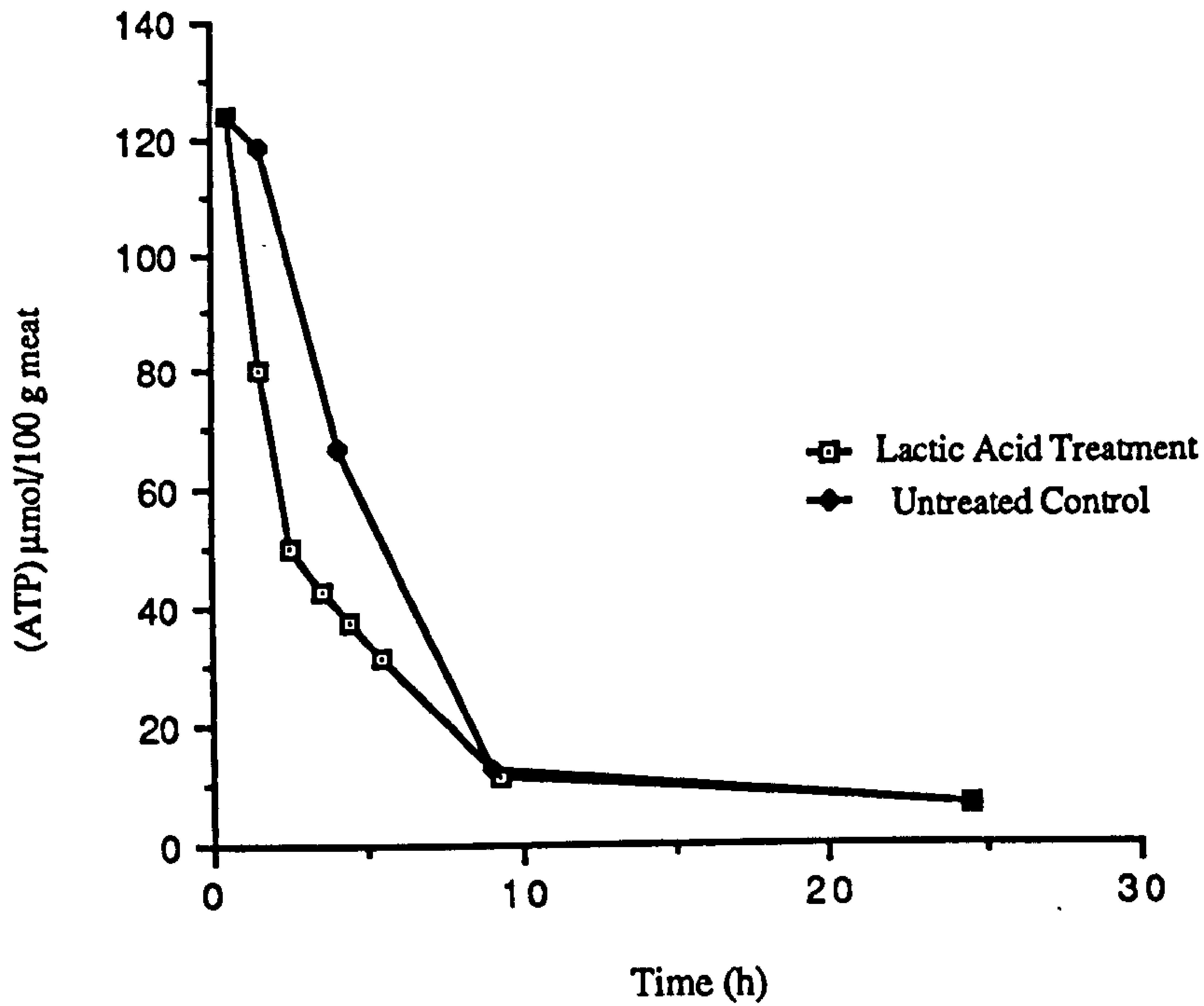


Fig. 7.2 Post-Mortem ATP Decline of Untreated and 0.1 M Lactic Acid Treated Meat Samples

Post-mortem ATP decline of meat samples, untreated and injected with 0.1 M lactic acid, to a final weight 10 % greater than original meat weight, was measured using an ATP assay kit (Sigma Diagnostics, Poole, UK).

Table 7.2 Meat pH Values following Lactic Acid Treatment and Conditioning

Muscle *	Unconditioned	Conditioned (1 day)	0.1M lactic acid - conditioned (1 day)	Conditioned (3 days)	0.1M lactic acid - conditioned (3 days)	Conditioned (7 days)	0.1M lactic acid - conditioned (7 days)	Conditioned (10 days)	0.1M lactic acid - conditioned (10 days)	Conditioned (14 days)	0.1M lactic acid - conditioned (14 days)
Psoas major	5.78	5.54	5.46	5.48	5.47	5.50	5.52	5.70	5.48	5.50	5.50
Gluteus Medius	5.72	5.53	5.43	5.54	5.40	5.53	5.45	5.50	5.50	5.50	5.50
Serratus Ventralis	5.78	5.63	5.57	5.64	5.61	5.65	5.65	5.75	5.60	5.60	5.60

* pH values were determined after thawing of frozen muscles

Table 7.3 Meat ATP levels (µmol/100 g meat) following Lactic Acid Treatment and Conditioning

Muscle *	Unconditioned	Conditioned (1 day)	0.1M lactic acid - conditioned (1 day)	Conditioned (3 days)	0.1M lactic acid - conditioned (3 days)	Conditioned (7 days)	0.1M lactic acid - conditioned (7 days)	Conditioned (10 days)	0.1M lactic acid - conditioned (10 days)	Conditioned (14 days)	0.1M lactic acid - conditioned (14 days)
Psoas major	10.14	3.51	3.40	3.90	3.51	3.65	3.45	3.80	3.55	3.90	3.60
Gluteus Medius	31.20	4.29	2.73	5.07	5.07	4.50	4.15	3.50	2.98	2.73	2.80
Serratus Ventralis	9.75	3.12	1.17	4.29	3.51	3.95	3.55	3.20	3.50	3.27	3.50

* ATP levels were determined after thawing of frozen muscles

7.3.3 Yields and Collagen Content of Perimysial Fractions Obtained from Unconditioned, Conditioned and Lactic Acid Treated Bovine Muscles.

Fig. 7.3 shows the time course of solubilization of perimysial material from *psoas major*, *gluteus medius* and *serratus ventralis* bovine muscles, conditioned for times ranging from 1 to 14 days. The yields of soluble perimysial material obtained from *psoas major* were less than those obtained from *gluteus medius* and *serratus ventralis* after similar periods of conditioning. Similarly, the yields of soluble perimysial material obtained from *gluteus medius* were less than those obtained from *serratus ventralis* after similar conditioning periods, except after 14 days of conditioning, when the yield of soluble perimysial material obtained from *gluteus medius* was greater than that obtained from *serratus ventralis*. The yields of soluble perimysial material obtained from *psoas major* muscle were not greater than 60 % of the yields obtained from *gluteus medius* muscle at equivalent periods of conditioning investigated, while the yields of soluble perimysial material obtained from *psoas major* were less than 50 % of the yields obtained from *serratus ventralis* for all conditioning periods shown, except after 14 days of conditioning when this values reached 57 %. The yields of soluble perimysial material obtained from *gluteus medius* after 1 and 3 days of conditioning were less than 50 % of the values obtained for *serratus ventralis*, however, at later periods of conditioning investigated, the yields from both muscles tended towards a similar value.

The yields of soluble perimysial material monitored over 14 days of conditioning, obtained from *psoas major* and *gluteus medius* muscles indicated an increase due to conditioning (Fig. 7.3). The correlation coefficients obtained for amount of perimysial material solubilized and time of conditioning was $r = 0.73$ for *psoas major* and $r = 0.97$ for *gluteus medius* muscles. However, the yields of soluble perimysial material obtained from *serratus ventralis* muscle indicated no such increase due to conditioning (Fig. 7.3).

Fig. 7.4 shows the time course of solubilization of perimysial material from *psoas major*, *gluteus medius* and *serratus ventralis* bovine muscles, injected with 0.1 M lactic acid and conditioned for times ranging from 1 to 14 days. The trend in the yields of soluble perimysial material obtained from these muscles was similar to that observed due to conditioning alone (Fig. 7.3), that is, without lactic acid treatment. The yields of soluble perimysial material obtained from *psoas major* were less than those obtained from *gluteus medius* and *serratus ventralis* muscles after similar periods of conditioning. Similarly, the yields of soluble perimysial material obtained from *gluteus medius* were less than those obtained from *serratus ventralis* after similar conditioning periods, except after 14 days of conditioning, when the yield of soluble perimysial material obtained from *gluteus medius*

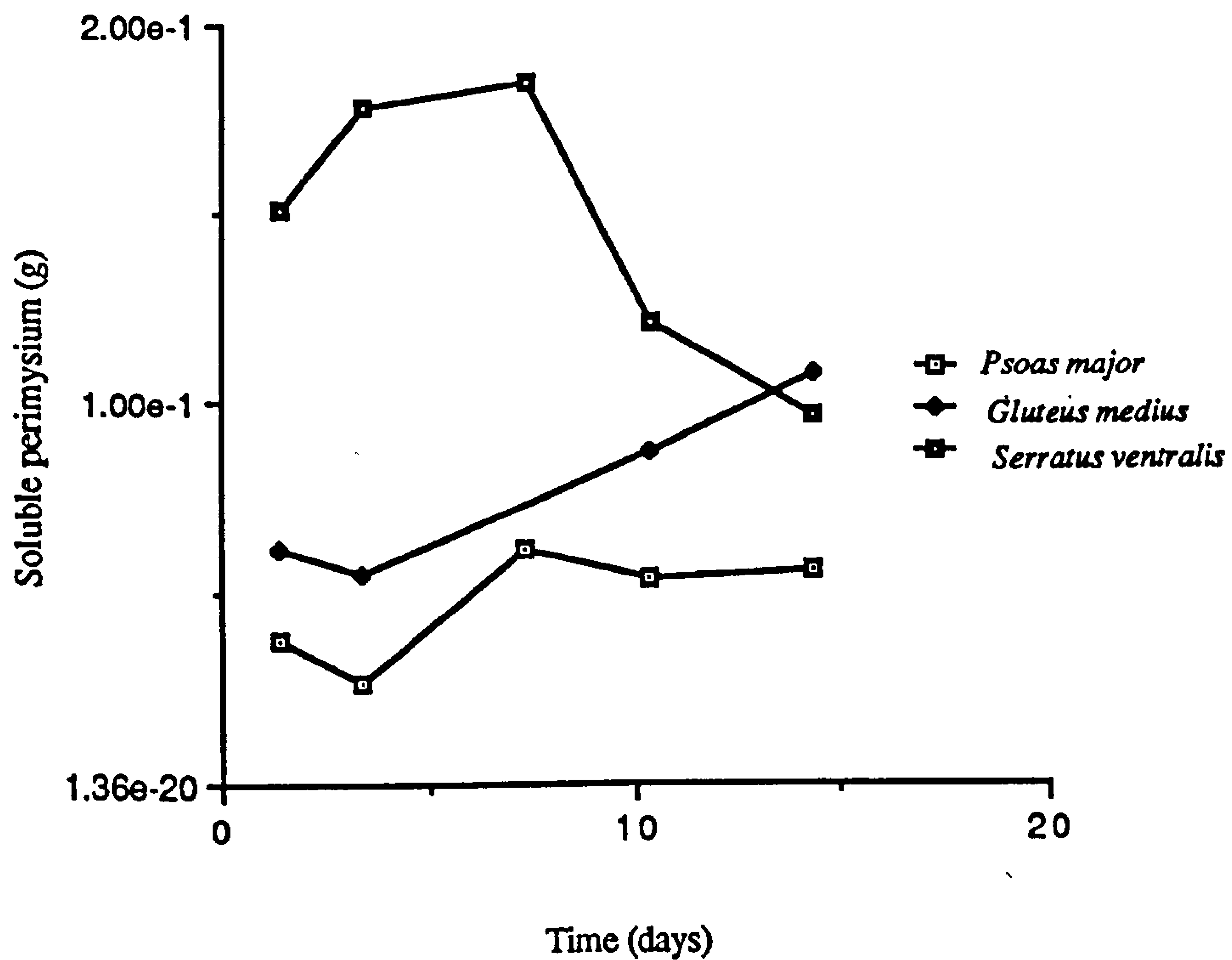


Fig. 7.3 Solubilization of Perimysial Material from Bovine Muscles, Conditioned from 1 to 14 Days

Yields of solubilized perimysial material, extracted with 6 M urea, 0.05 M tris-Cl, pH 7.4, from *psoas major*, *gluteus medius* and *serratus ventralis* were quantified from 100 g meat over 14 days of conditioning.

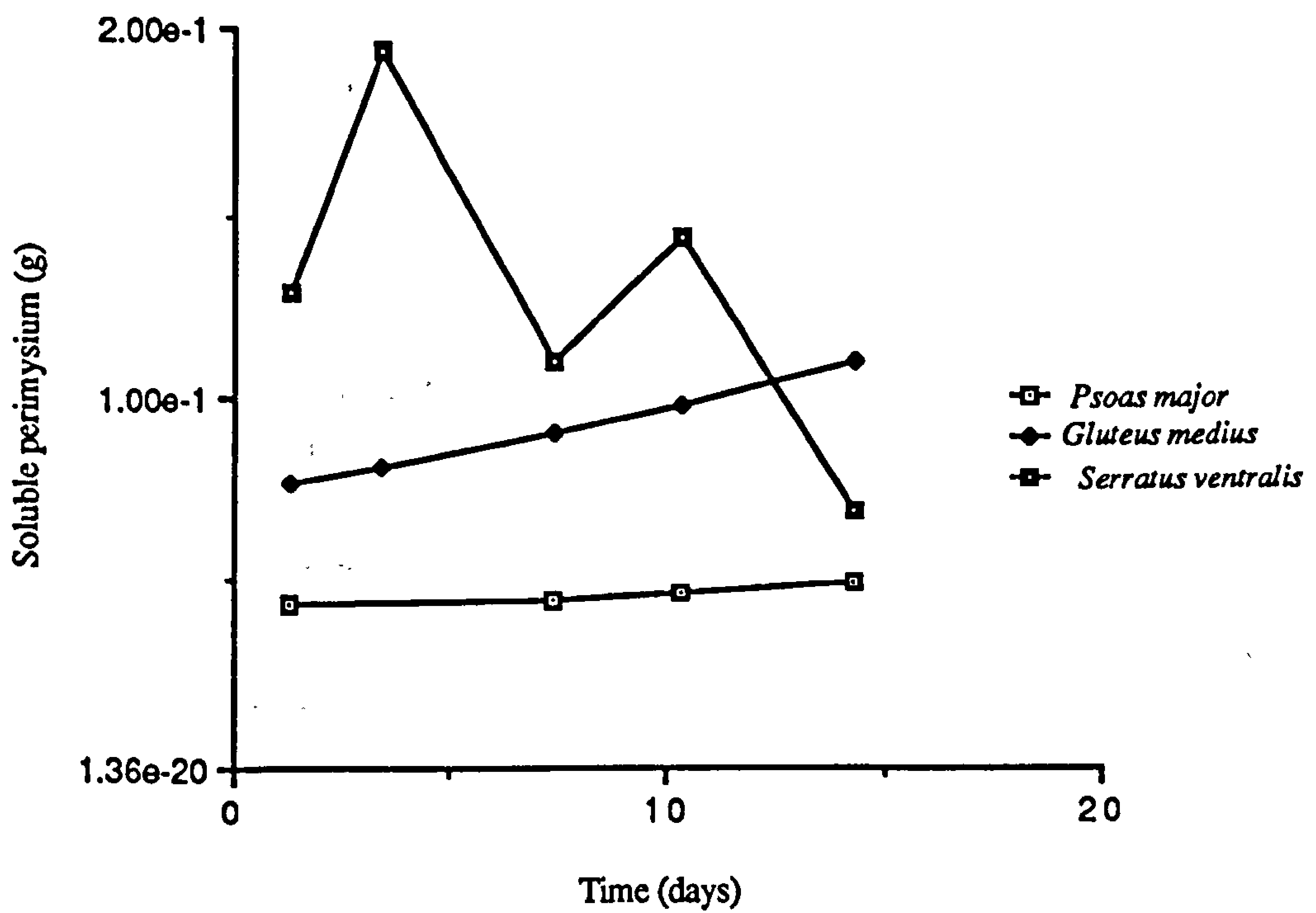


Fig. 7.4 Solubilization of Perimysial Material from Lactic Acid Treated Bovine Muscles, Conditioned from 1 to 14 Days

Yields of solubilized perimysial material, extracted with 6 M urea, 0.05 M tris-Cl, pH 7.4, from 0.1 M lactic acid injected meat to a final weight 10 % greater than original meat weight, were quantified from 100 g *psoas major* , *gluteus medius* and *serratus ventralis* , over 14 days of conditioning.

muscle was greater than that obtained from *serratus ventralis*, a result identical to that obtained by conditioning of these muscles alone. The yields of soluble perimysial material obtained from *psoas major* muscle after 1 day of conditioning was 67 % of that obtained from *gluteus medius* muscle, however at all other conditioning time periods investigated, this value represented less than 50 %. The yields of soluble perimysial material obtained from *psoas major* was less than 50 % of the yields obtained from *serratus ventralis*, except after 14 days of conditioning when this value represented 71 %. The yields of soluble perimysial material obtained from *gluteus medius* muscle, following treatment with 0.1 M lactic acid, showed similar trends, following conditioning, to those obtained by conditioning alone. Following 1 and 3 days of conditioning after lactic acid treatment, the yield of soluble perimysial material obtained from *gluteus medius* were 58 % and 41 % respectively of the values obtained from *serratus ventralis*, while at later periods of conditioning the yields for both muscles tended towards similar values, until at the final conditioning period investigated, the yield of soluble perimysial material obtained from *gluteus medius* was greater than that obtained from *serratus ventralis*.

The yields of soluble perimysial material obtained after lactic acid treatment and conditioning from *psoas major* and *gluteus medius* muscles indicated an increase due to conditioning (Fig. 7.4). Good correlations between amount of solubilized perimysial material and time of conditioning after lactic acid treatment was obtained ($r = 0.94$) for *psoas major*, and for *gluteus medius* ($r = 1.0$). The yields of solubilized perimysial material obtained from lactic acid treated *serratus ventralis* muscle indicated no such increase due to conditioning (Fig. 7.4), a result similar to that obtained by conditioning of *serratus ventralis* alone (Fig. 7.3).

Table 7.4 shows the yields of solubilized perimysial material, expressed as a percentage of total dry weight (soluble + insoluble perimysium) obtained from the set of three unconditioned bovine muscles and compared with the same set of three muscles, conditioned for times ranging from 1 to 14 days, and injected with 0.1 M lactic acid and conditioned for times ranging from 1 to 14 days. No significant differences were observed between the percentage solubilized perimysium obtained from conditioned muscles, when compared with their lactic acid treated counterparts.

Figs. 7.5, 7.6 and 7.7 shows the time course of solubilization of collagen from *psoas major*, *gluteus medius* and *serratus ventralis* bovine muscles respectively, due to conditioning and treatment with lactic acid prior to conditioning. Conditioning with and without prior treatment with lactic acid resulted in increased solubilization of perimysial collagen from all three muscles examined. Injection of lactic acid before conditioning

Table 7.4 Yields of Solubilized Perimysial Material Expressed as % of Total Dry Weight obtained from Unconditioned, Conditioned and Lactic Acid Treated Bovine Muscles.

Time of Conditioning (days)	*Psoas Major*		*Gluteus Medius*		*Serratus Ventralis*	
	Untreated	Lactic Acid/ Conditioned	Untreated	Lactic Acid/ Conditioned	Untreated	Lactic Acid/ Conditioned
0	19	-	24	-	20	-
1	16	20	14	18	18	20
3	16	25	15	17	23	18
7	23	20	25	25	24	18
10	17	23	17	17	15	21
14	22	16	27	14	17	16

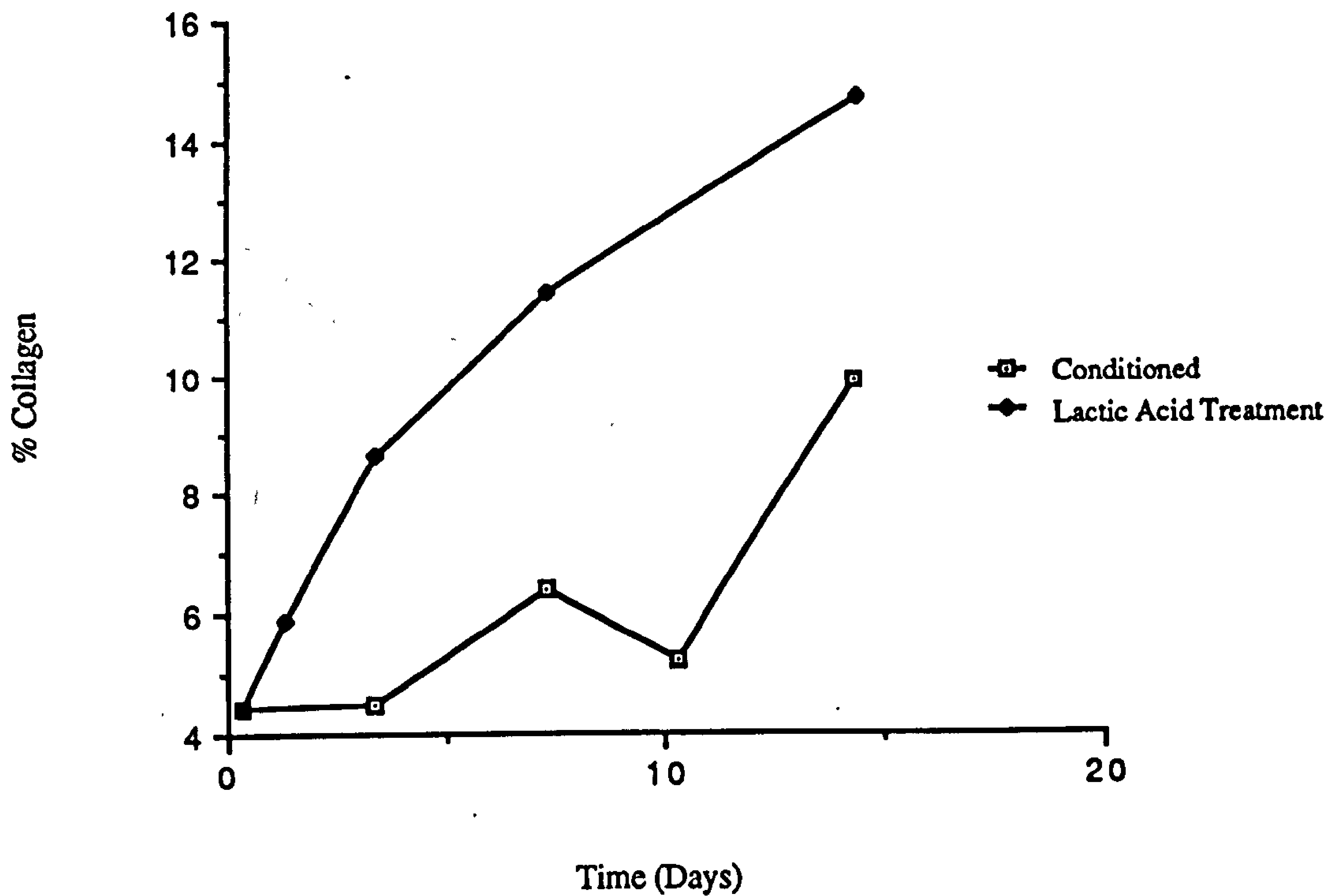


Fig. 7.5 Solubilization of Perimysial Collagen from Untreated and Lactic Acid Treated *Psoas Major* Muscle, Conditioned from 1 to 14 Days

Hydroxyproline was quantified in soluble perimysial fractions, extracted with 6 M urea, 0.05 M tris-Cl, pH 7.4, by HPLC, using a conversion factor of 7.14 to convert hydroxyproline content to collagen content, assuming an average hydroxyproline content of 14 % in meat collagens

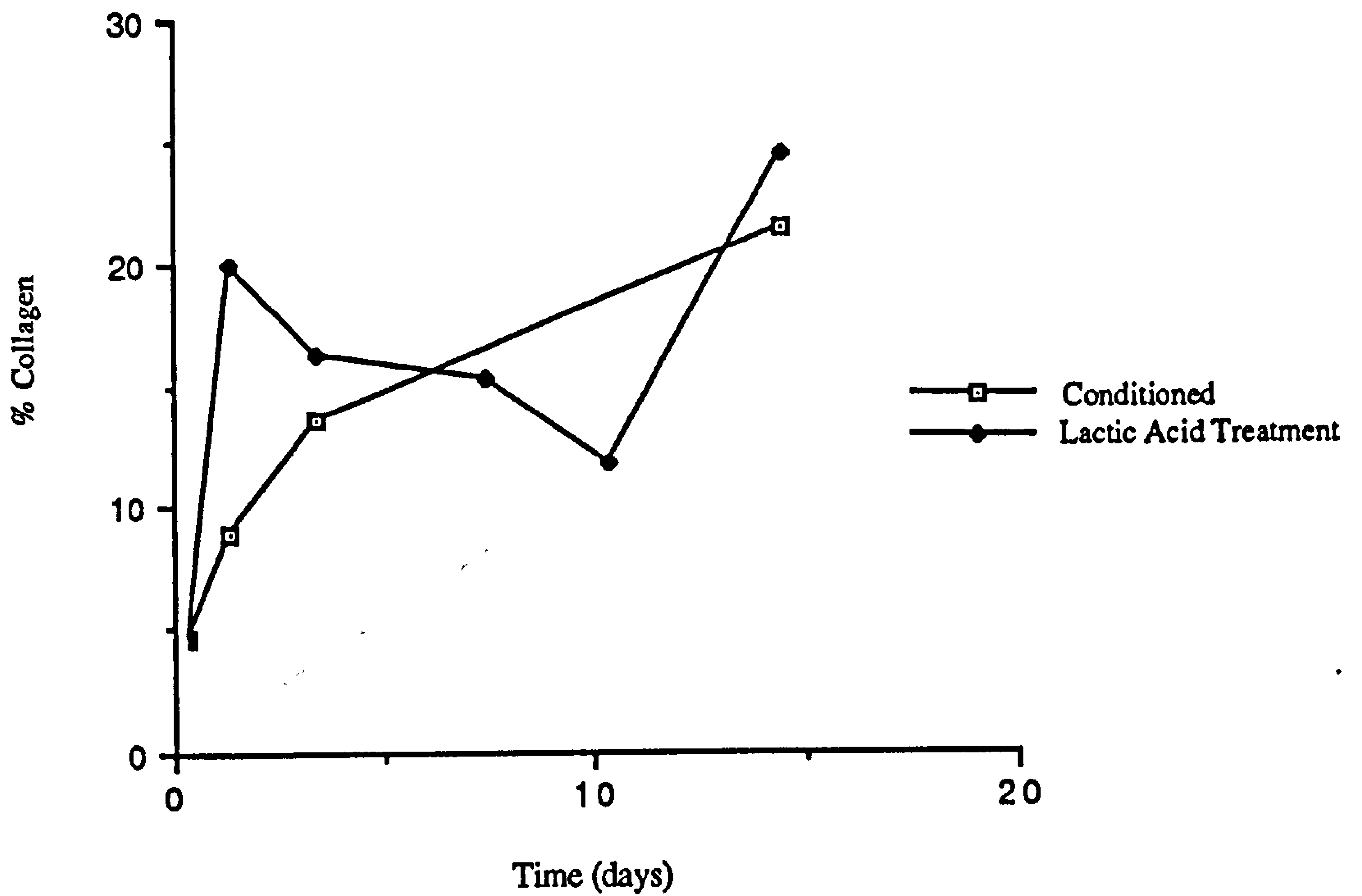


Fig. 7.6 Solubilization of Perimysial Collagen from Untreated and Lactic Acid Treated *Gluteus Medius* Muscle, Conditioned from 1 to 14 Days

Hydroxyproline was quantified in soluble perimysial fractions, extracted using 6 M urea, 0.05 M tris-Cl, pH 7.4, by HPLC, using a conversion factor of 7.14 to convert hydroxyproline content to collagen content, assuming an average hydroxyproline content of 14 % in meat collagens.

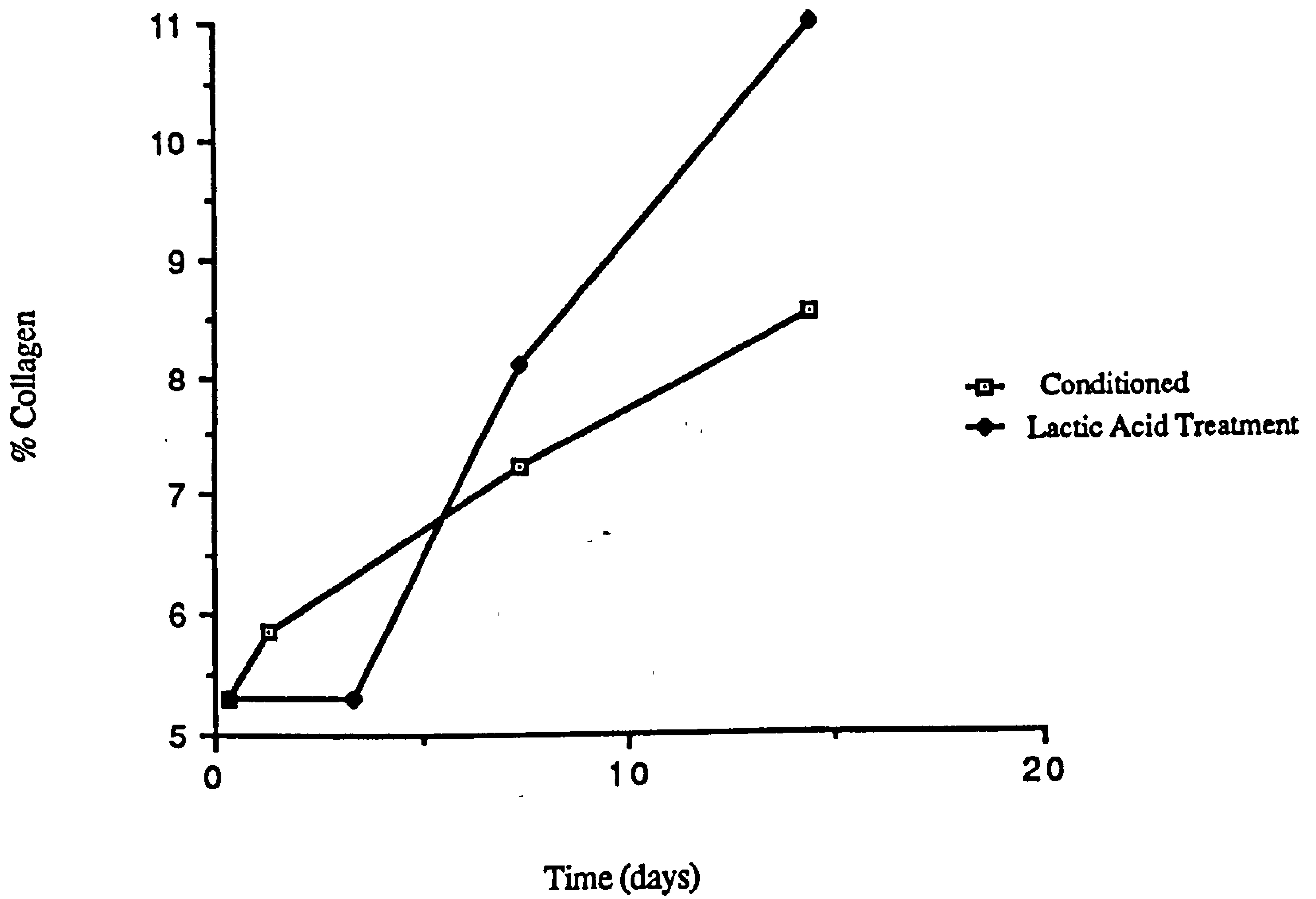


Fig. 7.7 Solubilization of Perimysial Collagen from Untreated and Lactic Acid Treated *Serratus Ventralis* Muscle, Conditioned from 1 to 14 Days

Hydroxyproline was quantified in soluble perimysial fractions, extracted by 6 M urea, 0.05 M tris-Cl, pH 7.4, by HPLC, using a conversion factor of 7.14 to convert hydroxyproline content to collagen content, assuming an average hydroxyproline content of 14 % in meat collagens.

resulted in increases in the percentage collagen in the soluble perimysial fractions obtained from all three muscles, relative to the untreated controls.

There were good correlations between the percentage collagen in the soluble perimysial fractions obtained from the 3 muscles examined, due to conditioning and treatment with lactic acid prior to conditioning. The correlation coefficients obtained for percentage collagen in the soluble perimysial fractions with time obtained from conditioned and lactic acid treated *psoas major* muscles were $r = 0.84$ and $r = 0.97$, respectively. Statistical analysis using 2 way ANOVA indicated that the percentage collagen in the soluble perimysial fractions obtained from *psoas major* due to conditioning were significantly lower ($p < 0.05$) than the values obtained from lactic acid treated muscles. The correlation coefficients obtained for percentage collagen in the soluble perimysial fractions with time obtained from conditioned and lactic acid treated *gluteus medius* muscles were $r = 0.94$ and $r = 0.52$ respectively. Statistical analysis using 2 way ANOVA indicated that the percentage collagen in the soluble perimysial fractions obtained from *gluteus medius* due to conditioning were significantly lower ($p < 0.05$) than the values obtained from lactic acid treated muscles. The correlation coefficients obtained for percentage collagen in the soluble perimysial fractions with time obtained from conditioned and lactic acid treated *serratus ventralis* muscles were $r = 0.99$ and $r = 0.98$ respectively. Statistical analysis using 2 way ANOVA again indicated that the percentage collagen in the soluble perimysial fractions obtained from *serratus ventralis* due to conditioning were significantly lower ($p < 0.05$) than the values obtained from lactic acid treated muscles.

7.3.4 One-Dimensional SDS-Polyacrylamide Gel Electrophoretic Analysis of Insoluble Perimysial Preparations

Insoluble perimysial fractions obtained from *psoas major, gluteus medius* and *serratus ventralis* unconditioned bovine muscles were analyzed and compared with the same set of 3 muscles, conditioned for times ranging from 1 to 14 days, and injected with 0.1 M lactic acid and conditioned for times ranging from 1 to 14 days, after CNBr digestion, by one-dimensional SDS-polyacrylamide gel electrophoresis.

Figs. 7.8 a-e, 7.9 a-e and 7.10 a-e show the CNBr peptide maps obtained from insoluble perimysia from unconditioned *psoas major, gluteus medius* and *serratus ventralis* muscles, respectively, compared with those obtained from muscles conditioned for times ranging from 1 to 14 days, and injected with 0.1 M lactic acid and conditioned for times ranging from 1 to 14 days.

Fig. 7.8 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr-Peptides of Urea Washed Insoluble Perimysium from *Psoas Major.*

6 M urea, 0.05 M tris-Cl, pH 7.4 washed insoluble perimysium from *psoas major* was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2.

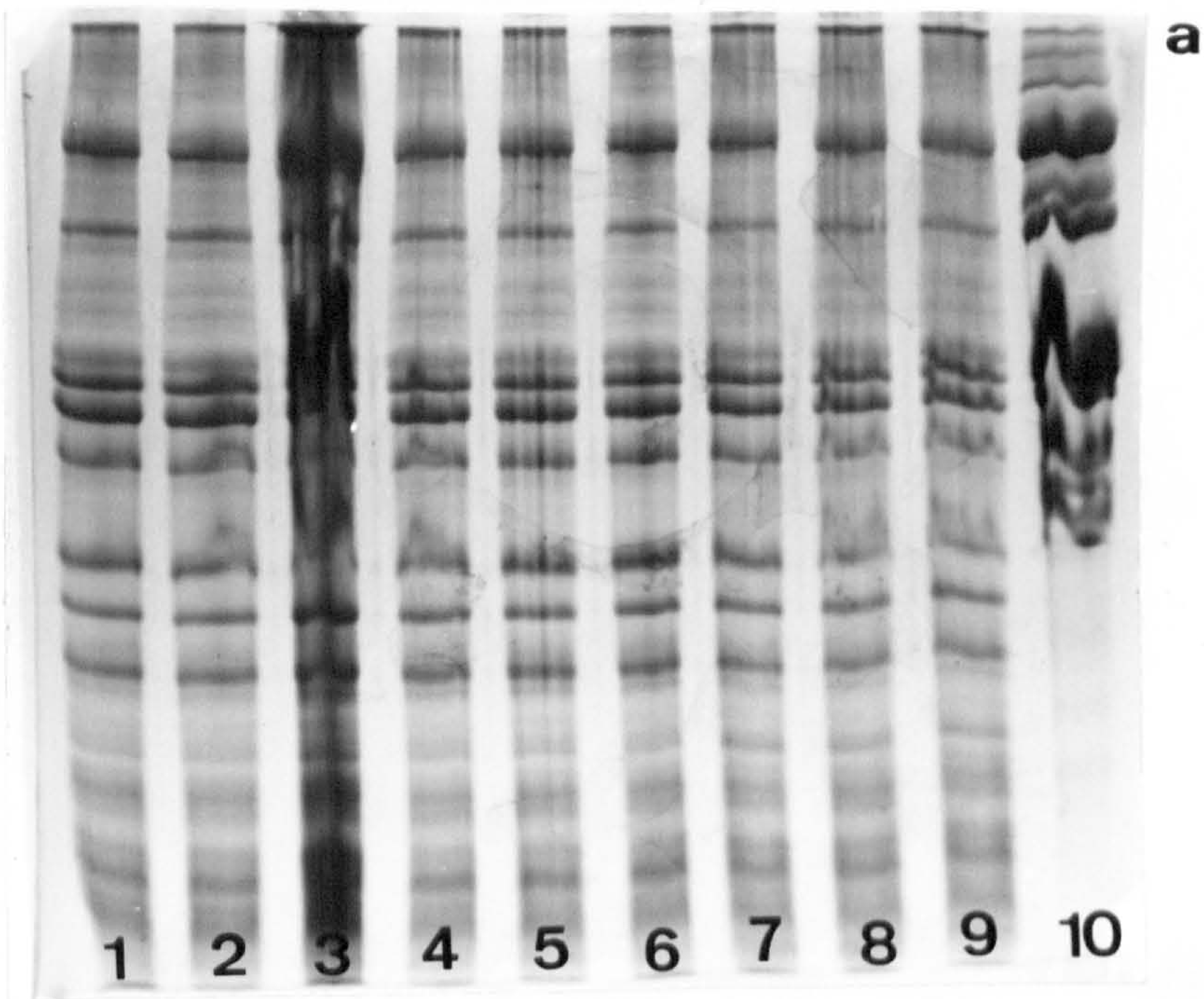
Fig. 7.8a: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *psoas major.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *psoas major*, conditioned for 1 day. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *psoas major*, treated with lactic acid and conditioned for 1 day. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.8b: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *psoas major.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *psoas major*, conditioned for 3 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *psoas major*, treated with lactic acid and conditioned for 3 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.8c: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *psoas major.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *psoas major*, conditioned for 7 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *psoas major*, treated with lactic acid and conditioned for 7 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.8d: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *psoas major.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *psoas major*, conditioned for 10 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *psoas major*, treated with lactic acid and conditioned for 10 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.8e: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *psoas major.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *psoas major*, conditioned for 14 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *psoas major*, treated with lactic acid and conditioned for 14 days. Track (10) is rat tail tendon collagen type I digest standard.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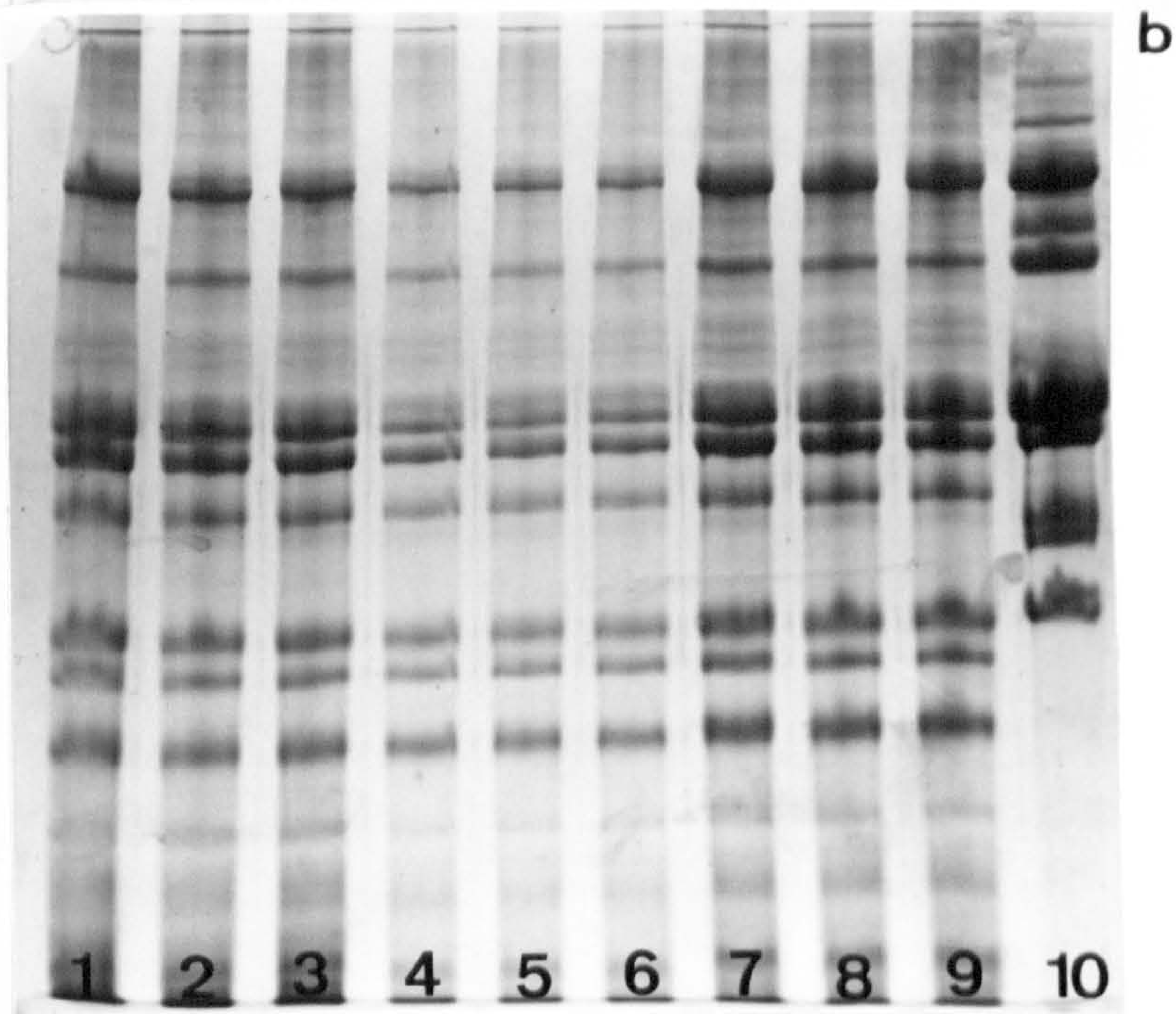
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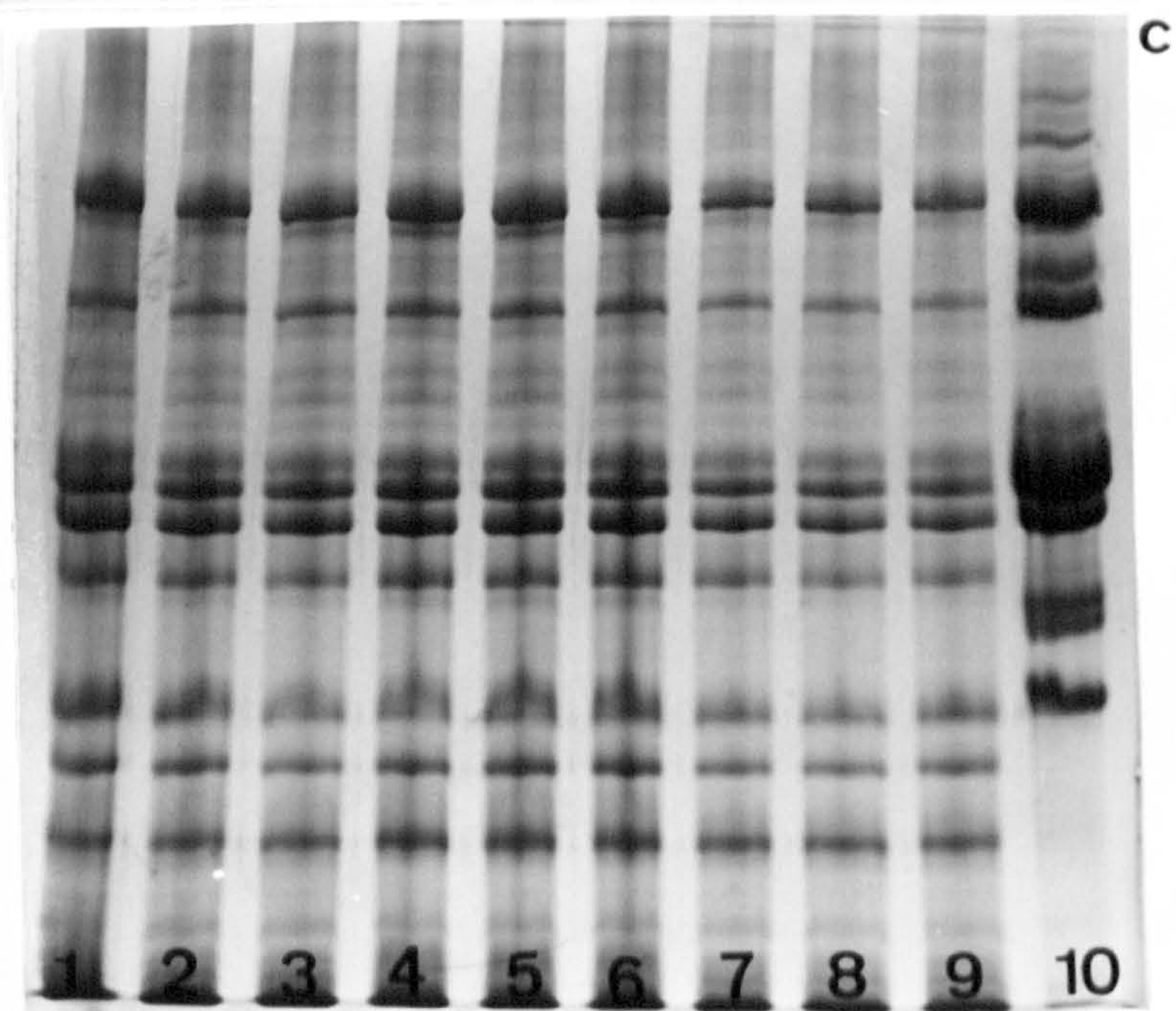
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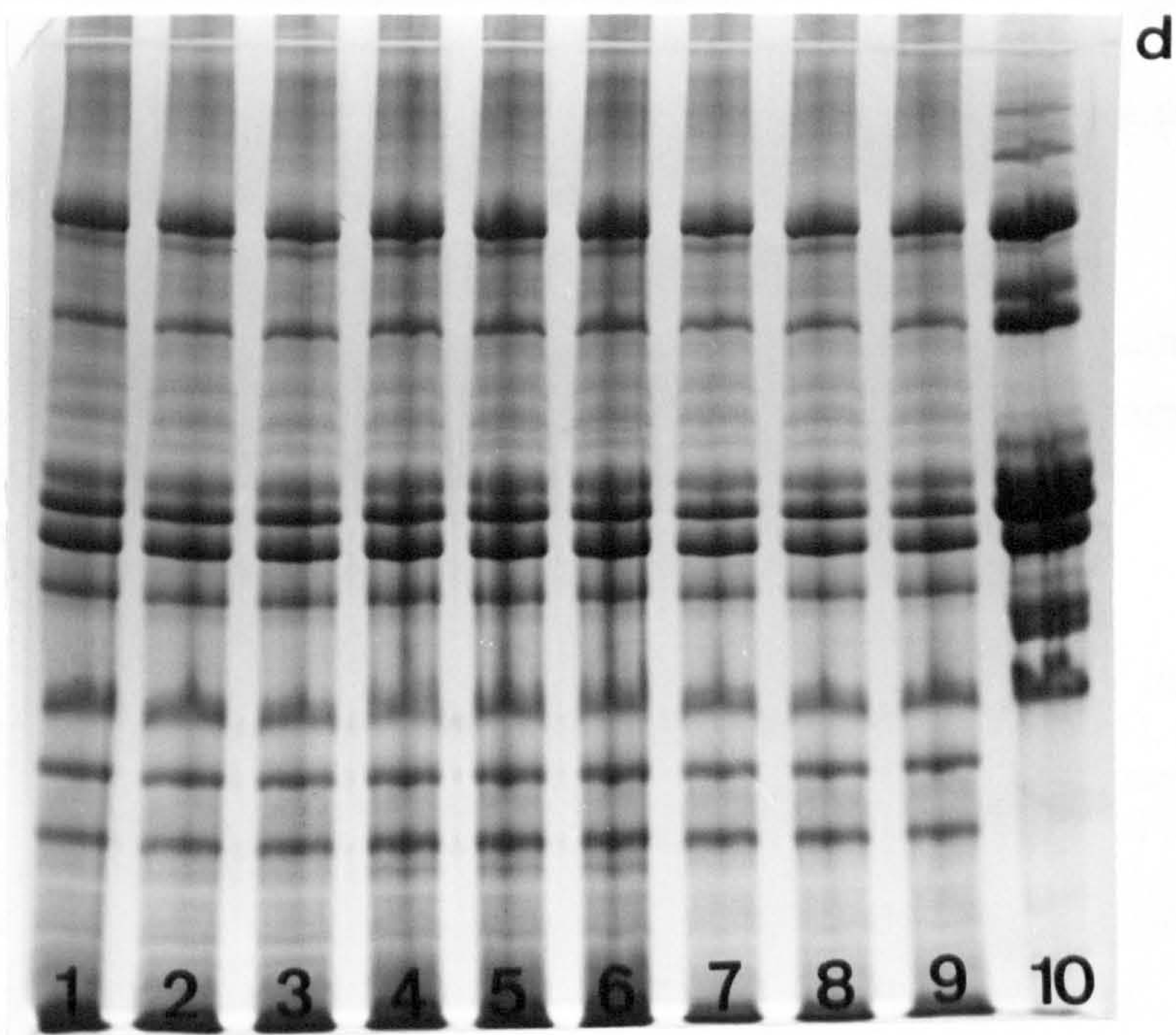
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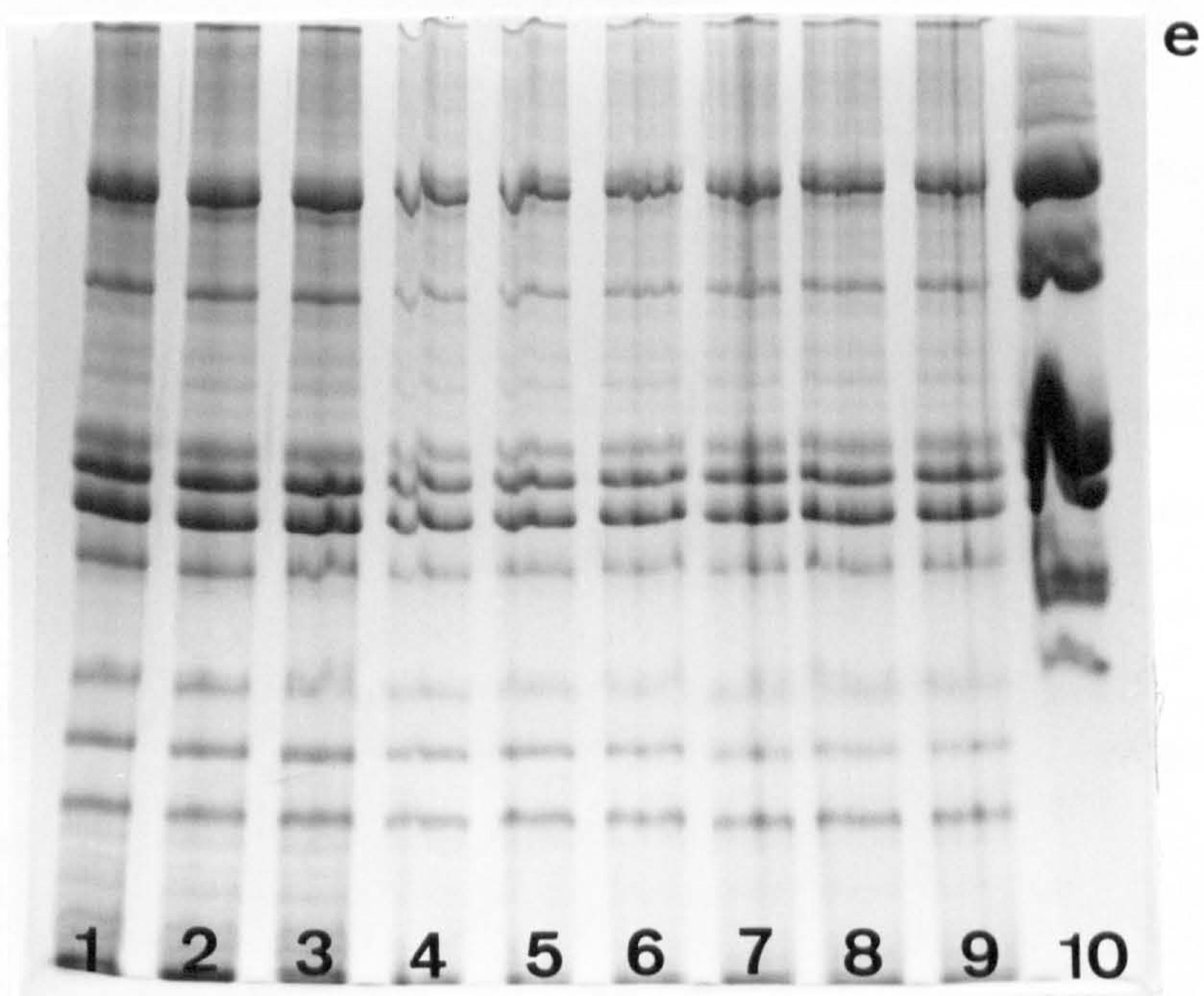
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Fig. 7.9 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr-Peptides of Urea-Washed Insoluble Perimysium from *Gluteus Medius.*

6 M urea, 0.05 M tris-Cl, pH 7.4 washed insoluble perimysium from *gluteus medius* was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2.

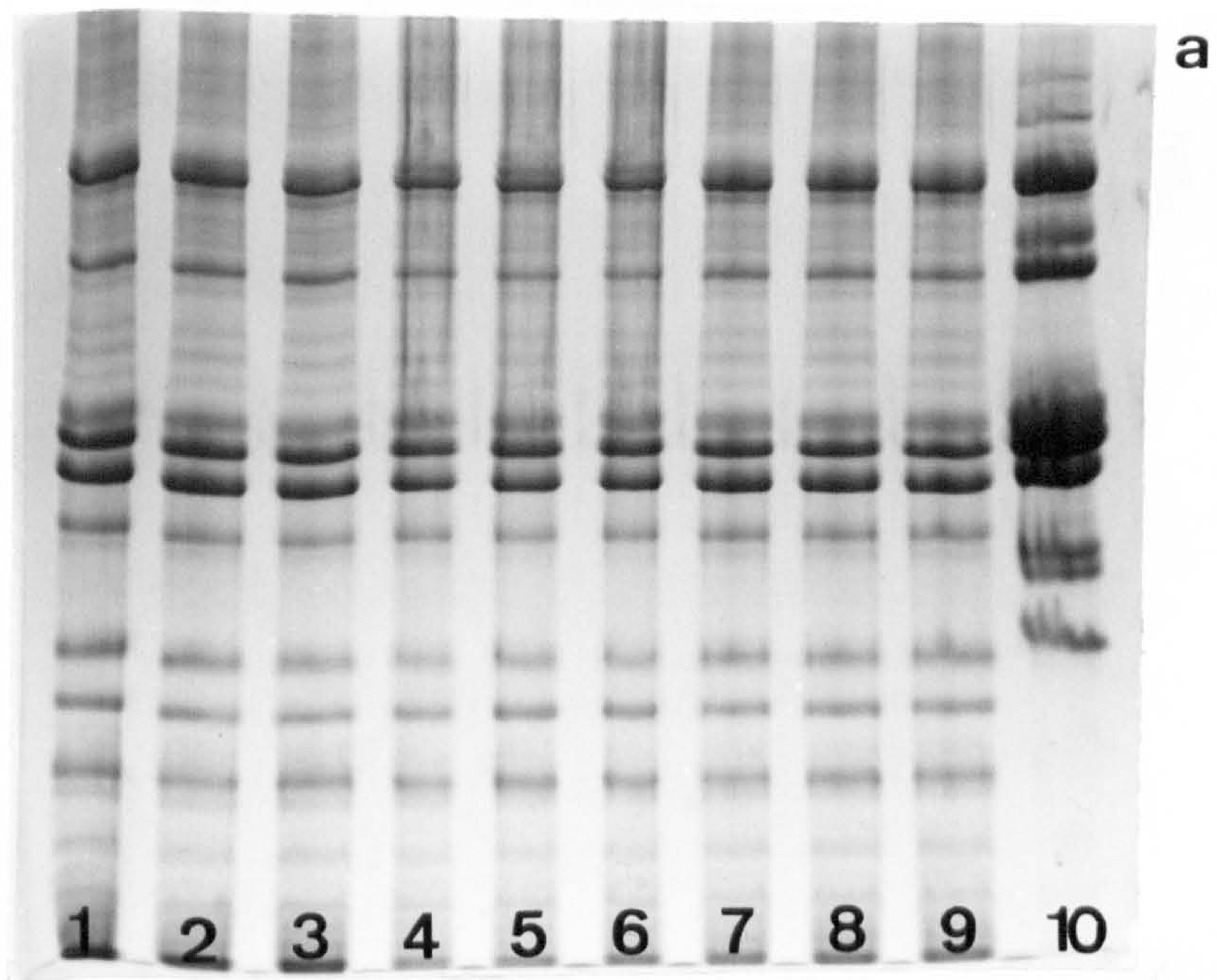
Fig. 7.9a: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *gluteus medius.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *gluteus medius*, conditioned for 1 day. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *gluteus medius*, treated with lactic acid and conditioned for 1 day. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.9b: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *gluteus medius.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *gluteus medius*, conditioned for 3 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *gluteus mediuss*, treated with lactic acid and conditioned for 3 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.9c: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *gluteus medius.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *gluteus medius*, conditioned for 7 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *gluteus mediuss*, treated with lactic acid and conditioned for 7 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.9d: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *gluteus medius.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *gluteus medius*, conditioned for 10 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *gluteus mediuss*, treated with lactic acid and conditioned for 10 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.9e: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *gluteus medius.* Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *gluteus medius*, conditioned for 14 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *gluteus medius*, treated with lactic acid and conditioned for 14 days. Track (10) is rat tail tendon collagen type I digest standard.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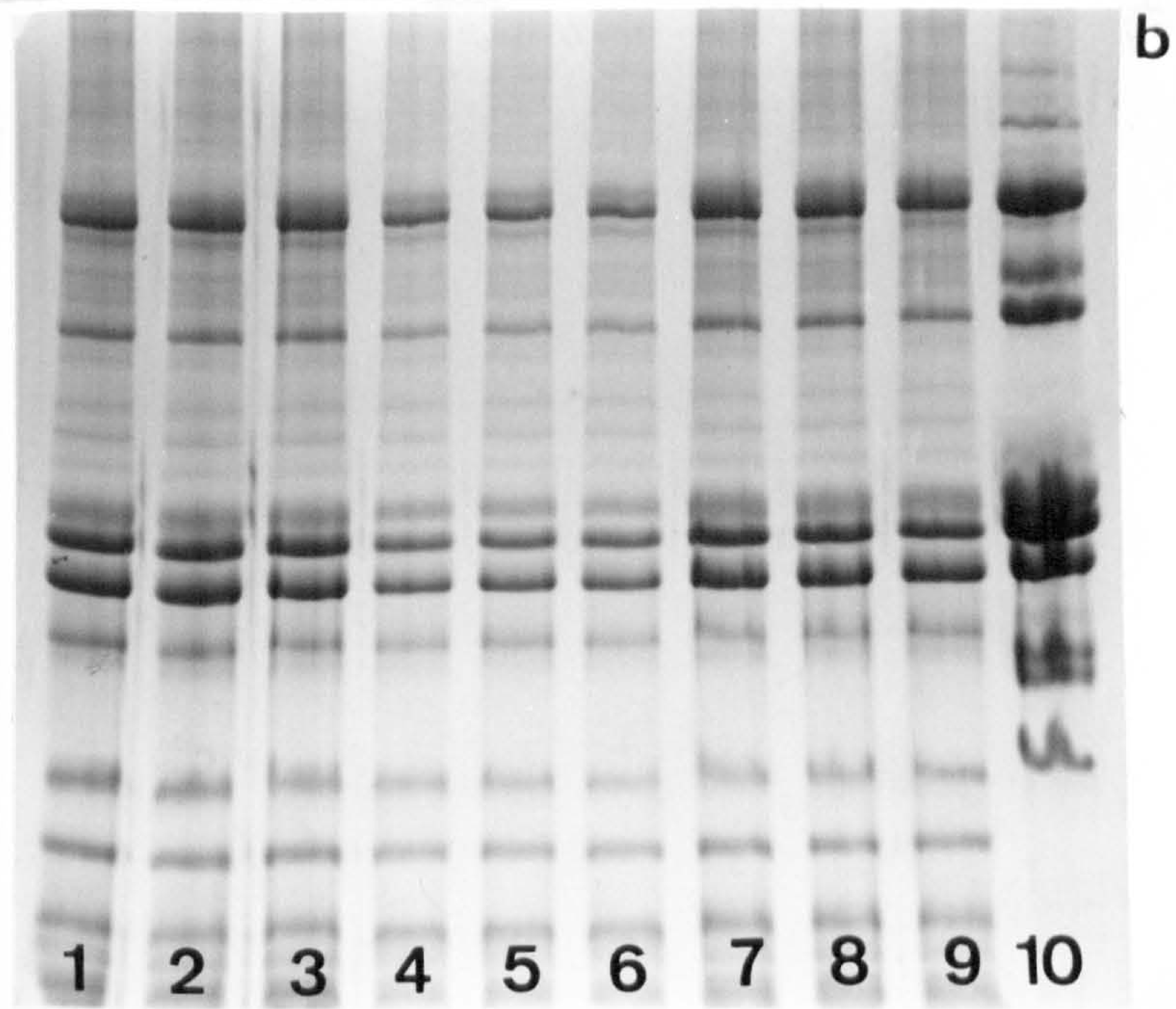
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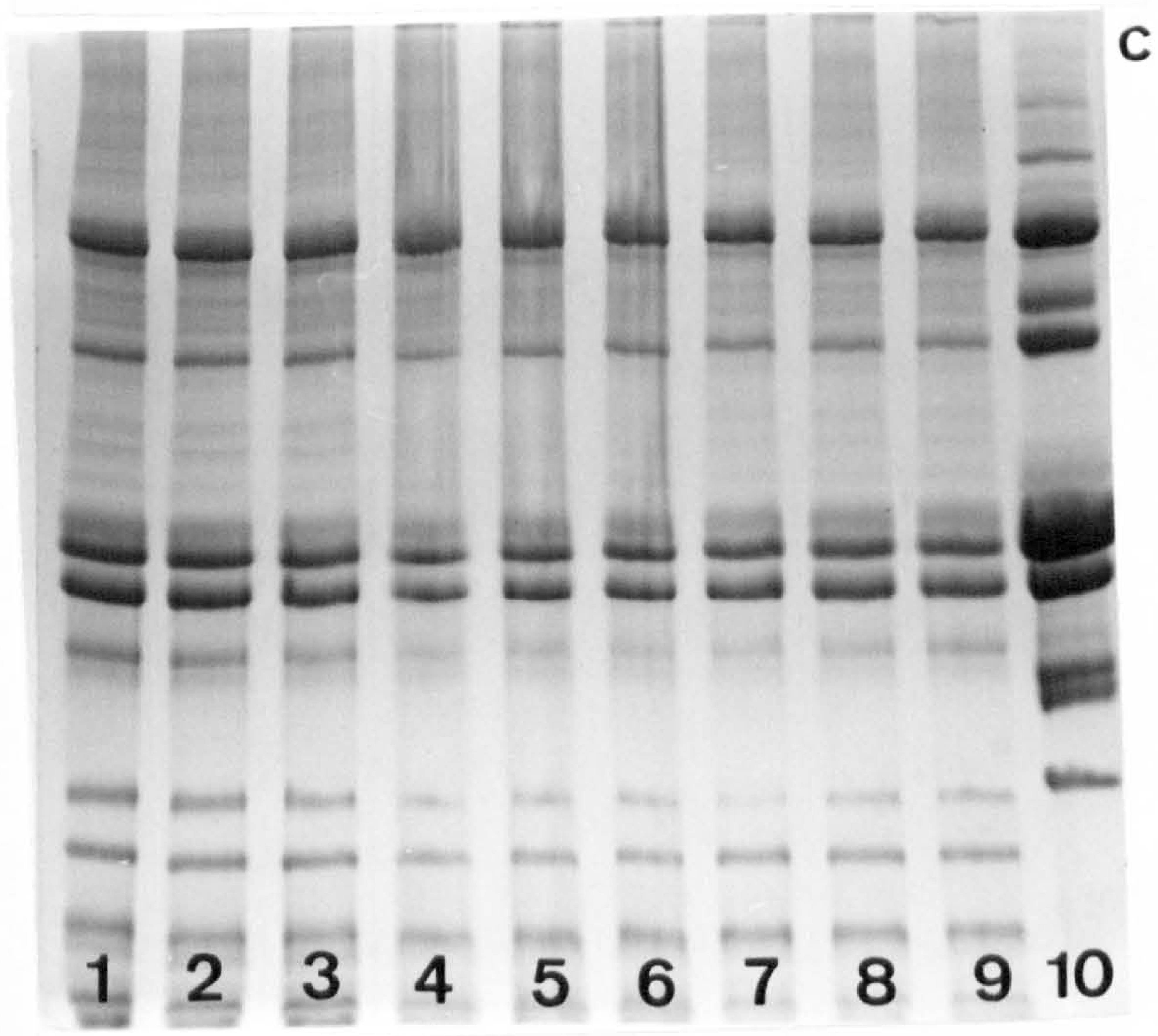
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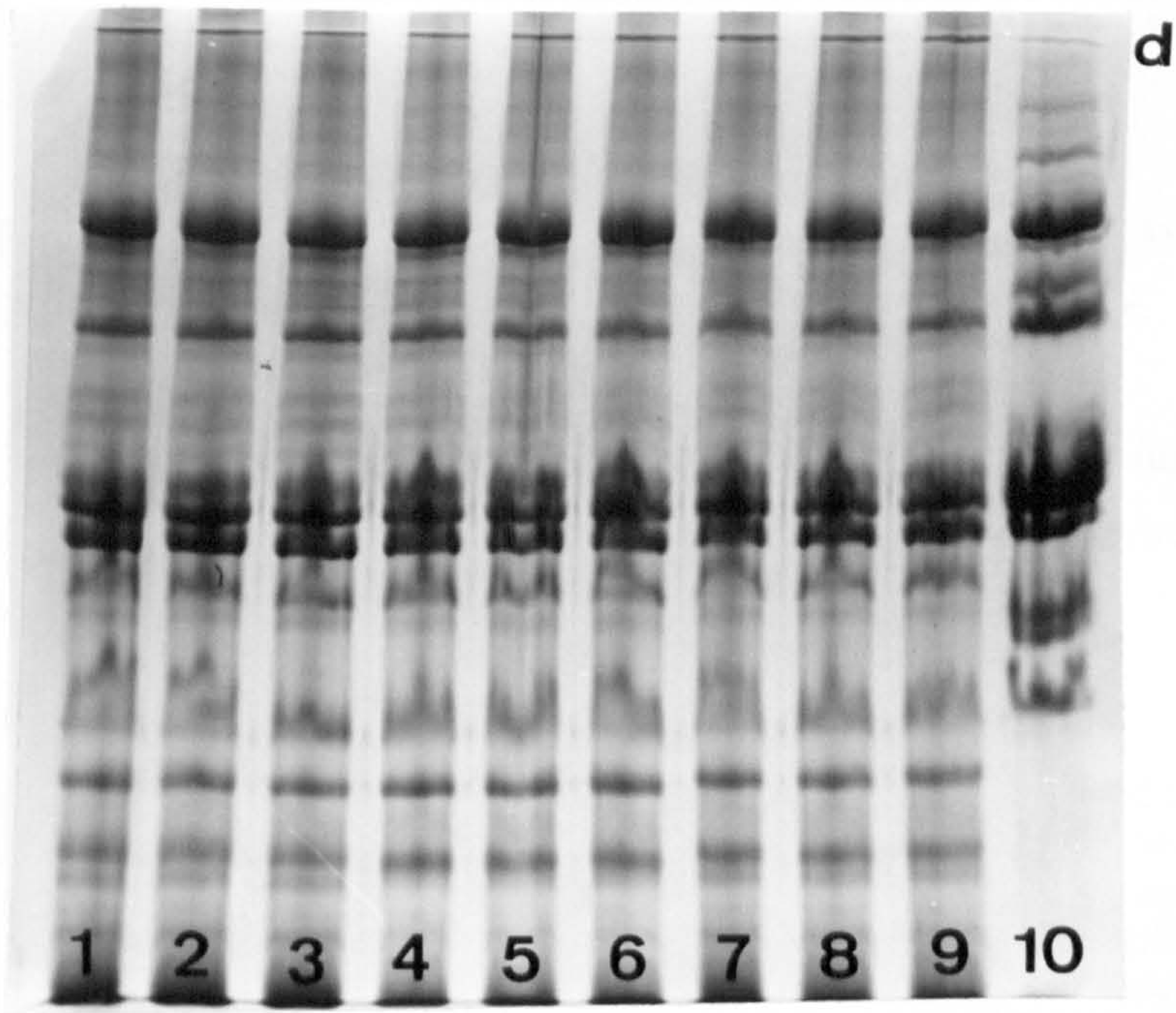
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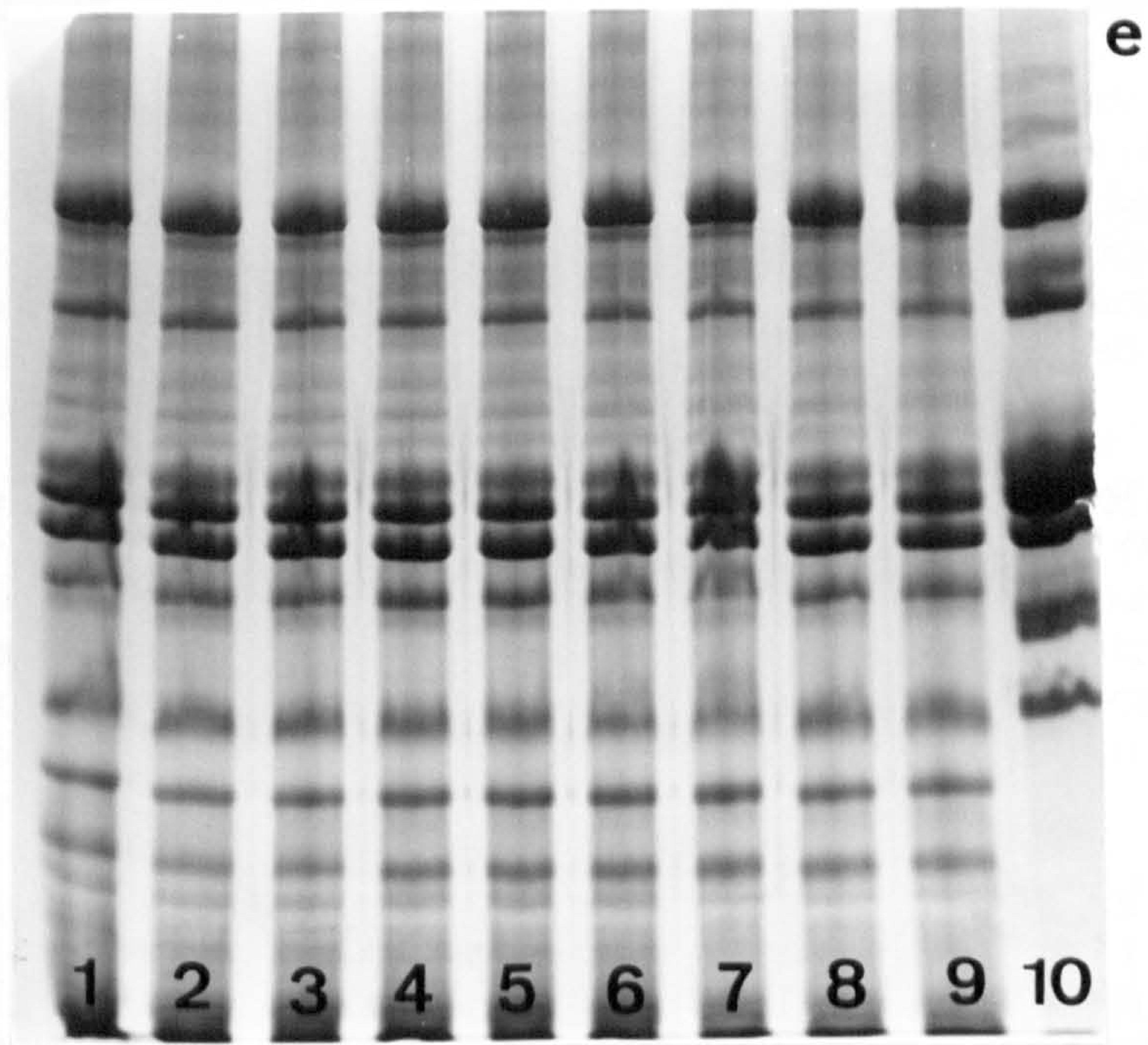
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Fig. 7.10 SDS-Polyacrylamide Gel Electrophoretic Analysis of CNBr-Peptides of Urea-Washed Insoluble Perimysium from *Serratus Ventralis.*

6 M urea, 0.05 M tris-Cl, pH 7.4 washed insoluble perimysium from *serratus ventralis* was digested with CNBr and analyzed by SDS-polyacrylamide gel electrophoresis as described in Chapter 2.

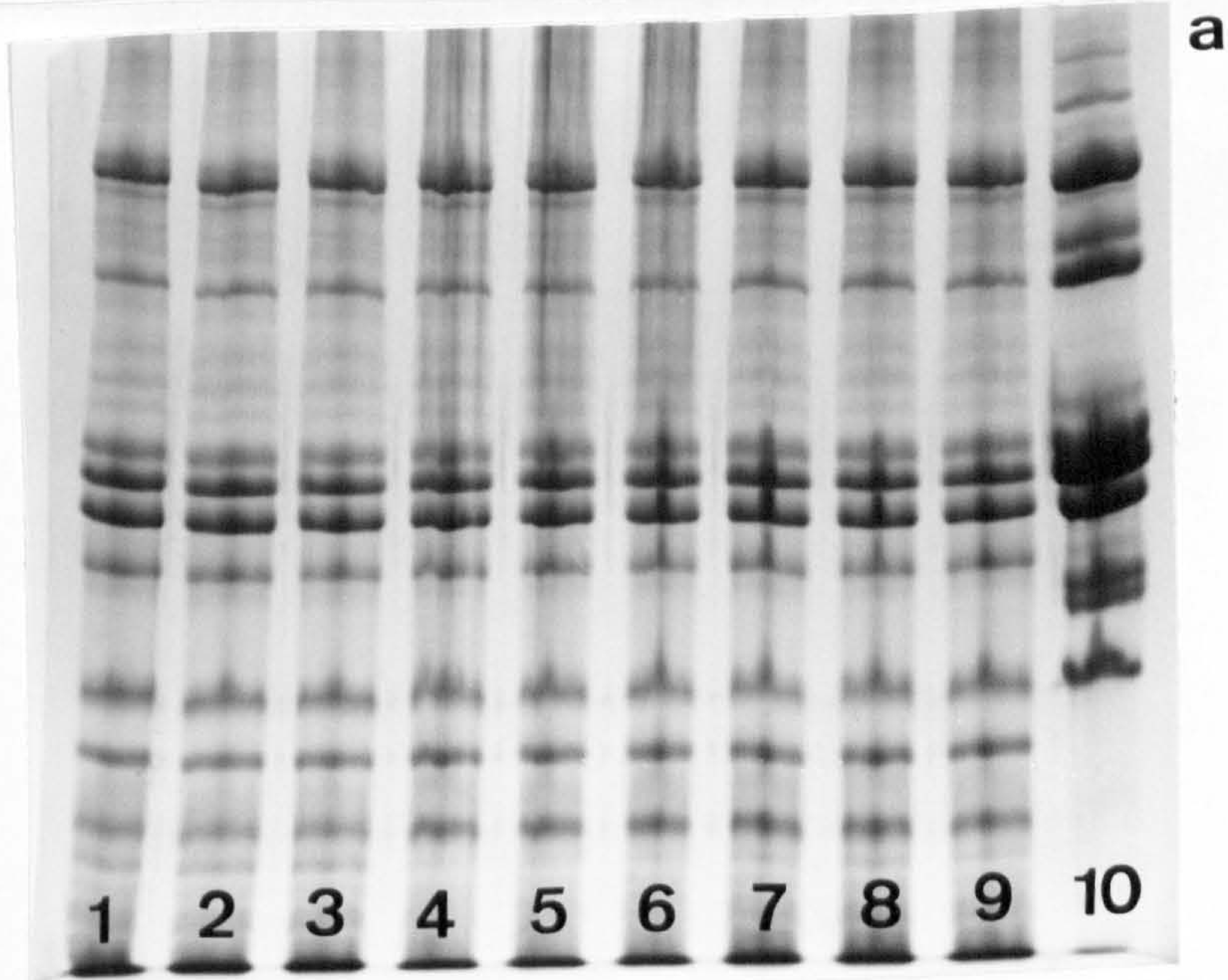
Fig. 7.10a: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *serratus ventralis*. Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, conditioned for 1 day. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, treated with lactic acid and conditioned for 1 day. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.10b: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *serratus ventralis*. Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, conditioned for 3 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, treated with lactic acid and conditioned for 3 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.10c: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *serratus ventralis*. Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, conditioned for 7 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, treated with lactic acid and conditioned for 7 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.10d: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *serratus ventralis*. Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, conditioned for 10 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, treated with lactic acid and conditioned for 10 days. Track (10) is rat tail tendon collagen type I digest standard.

Fig. 7.10e: Tracks (1), (2) and (3) are CNBr peptide maps of insoluble perimysium from unconditioned *serratus ventralis*. Tracks (4), (5) and (6) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, conditioned for 14 days. Tracks (7), (8) and (9) are CNBr peptide maps of insoluble perimysium from *serratus ventralis*, treated with lactic acid and conditioned for 14 days. Track (10) is rat tail tendon collagen type I digest standard.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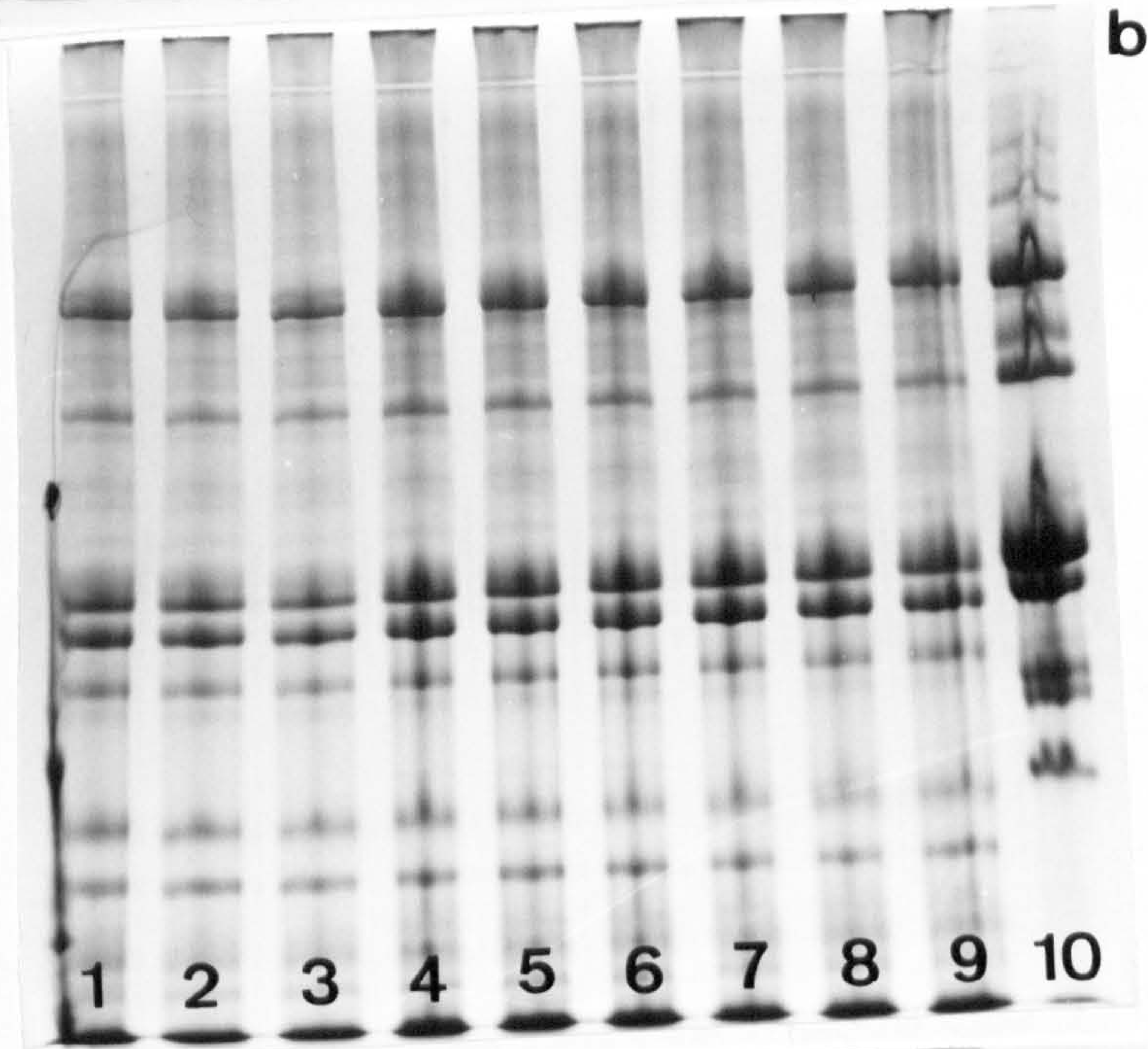
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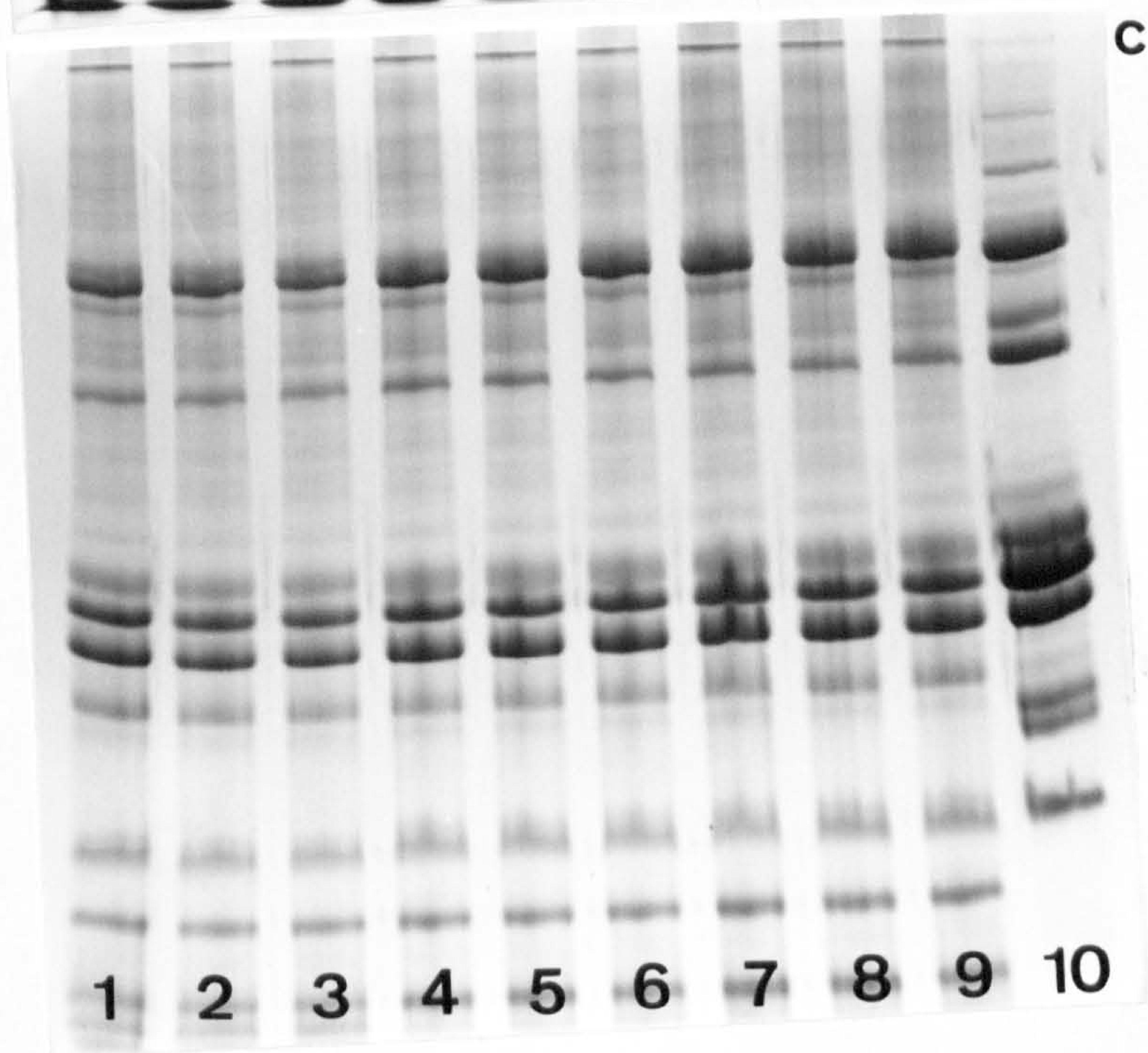
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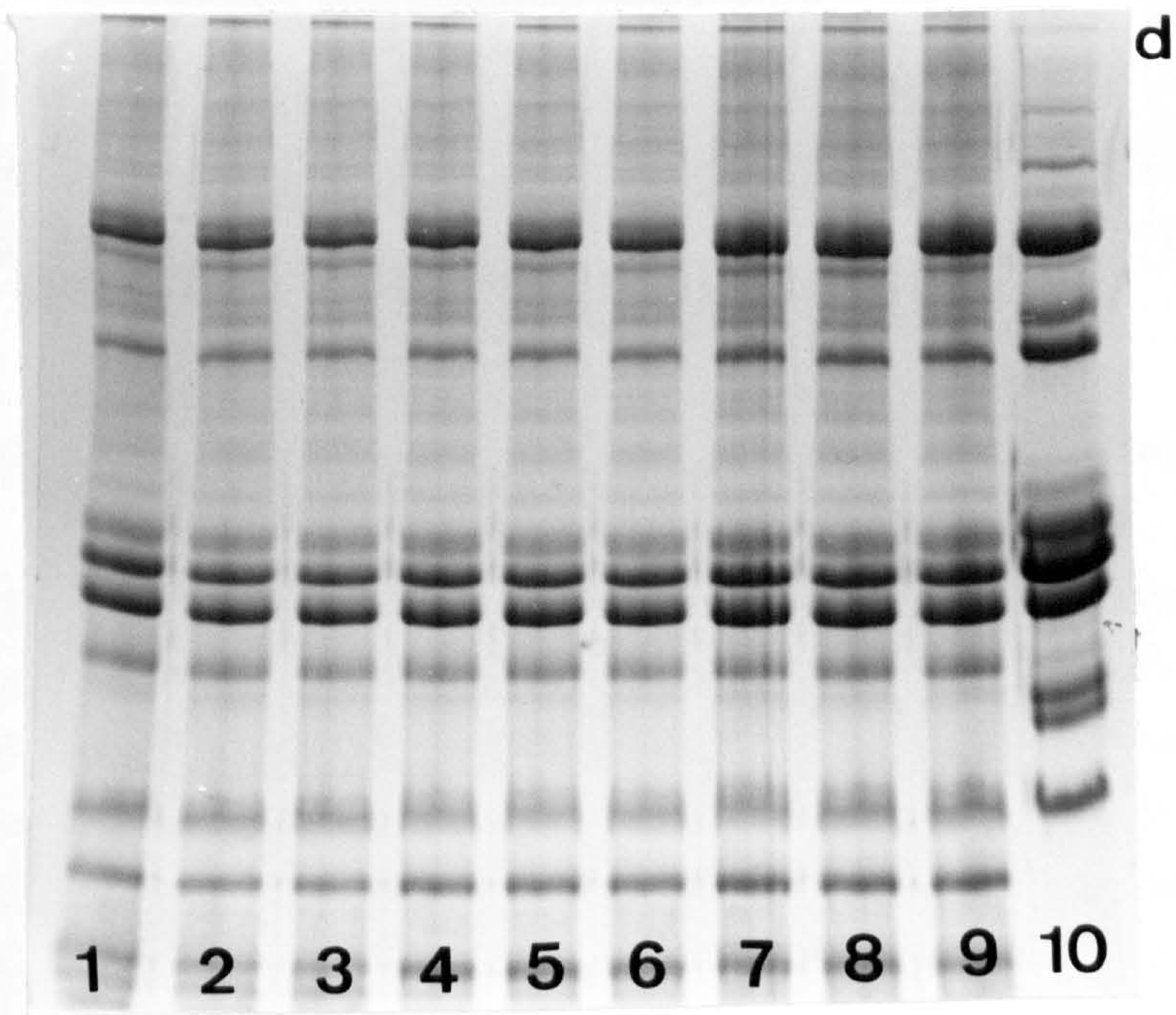
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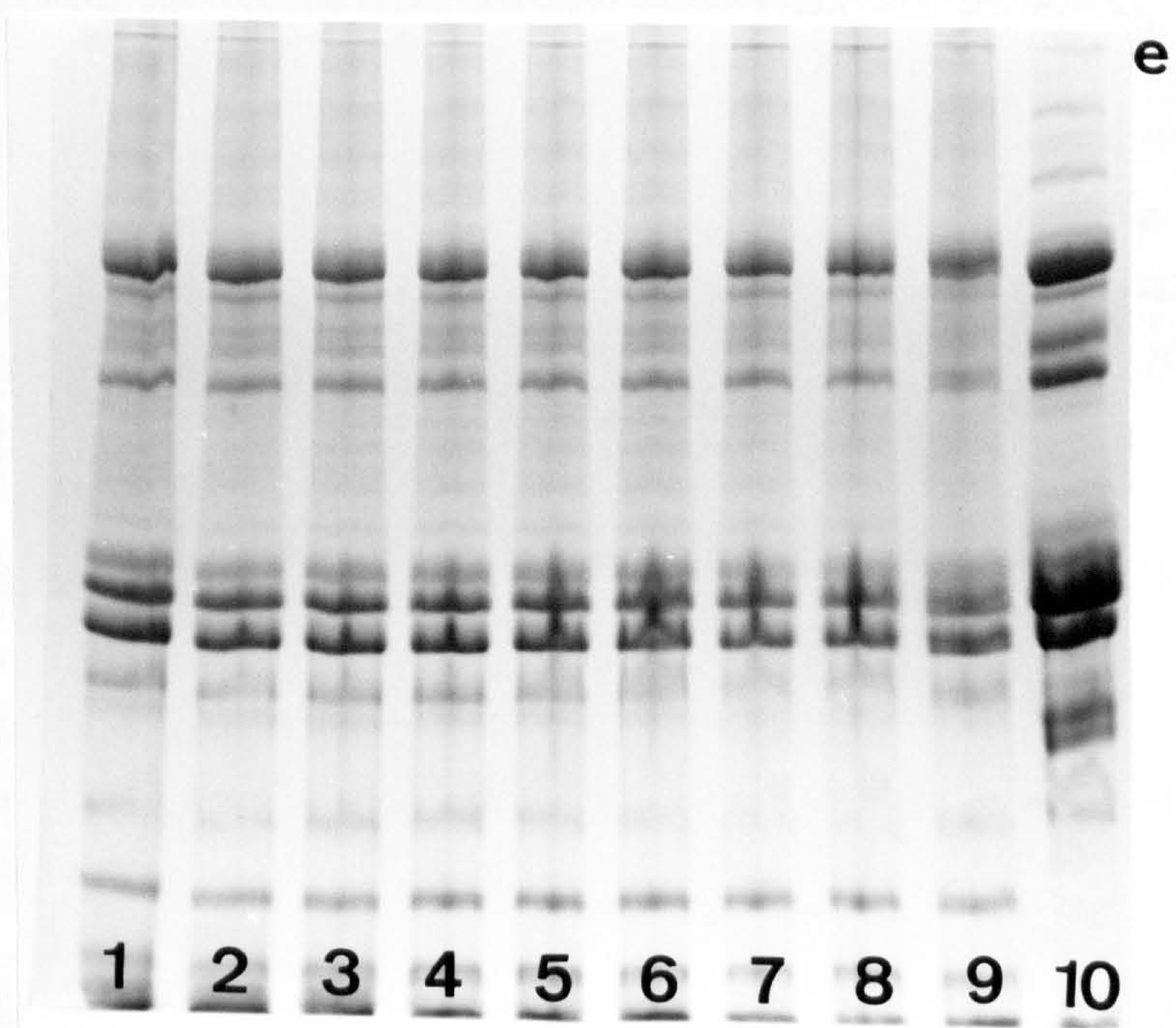
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No evidence of damage, due to conditioning or lactic acid treatments, to the main peptide components observed, from types I and III collagen, was visible on the peptide maps obtained.

7.3.5 2-D Gel Electrophoretic Analysis of Insoluble Perimysial Preparations

Figs 7.11, 7.12 and 7.13 show the results of 2-D analysis of insoluble perimysium obtained from *psoas major, gluteus medius* and *serratus ventralis* muscles respectively conditioned for 3 days (left) and treated with lactic acid prior to conditioning for 3 days (right). These results are typical of the gels obtained for comparison of perimysium obtained from conditioned muscles with that obtained from muscles injected with lactic acid before conditioning for all times of conditioning examined. It can be seen that lactic acid treatment of all 3 muscles prior to conditioning for 3 days, resulted in alterations of the perimysial peptide maps obtained when compared with untreated samples. Comparison of the two results from *serratus ventralis* (Fig 7.13) revealed increased incidence of peptides resulting from chymotryptic digestion of the high molecular weight material in the lactic acid treated sample which were absent from the untreated sample. Lactic acid treatment of *gluteus medius* and *psoas major* muscles prior to conditioning appeared to play a more subtle role in alteration of the perimysial peptide maps obtained. However, loss of at least 1 peptide (highlighted with an arrow) in the lactic acid treated perimysium from both muscles was evident.

7.4 DISCUSSION

The pH value of post-mortem muscle drops from pH 7.3 to about 5.5, 6 to 24 h after slaughter, primarily because of the formation of lactic acid from glycogen via the glycolytic pathway in the intracellular space (Pearson, 1971).

The rapid decrease in pH, brought about by pre-slaughter injection of lactic acid would favour early activation of muscle cathepsins and possibly enhanced collagen degradation in the muscle connective tissue, during the subsequent conditioning period. The work presented in this chapter investigated this concept with respect to perimysial collagen. In the first instance, preliminary work was carried out in order to discover a suitable concentration of lactic acid which would rapidly bring meat pH to 5.5 or lower, without altering meat colour.

With a normal ultimate pHu of about 5.5, bovine meat has an open structure and is bright red in colour. Myoglobin is mainly responsible for meat colour, accounting for about

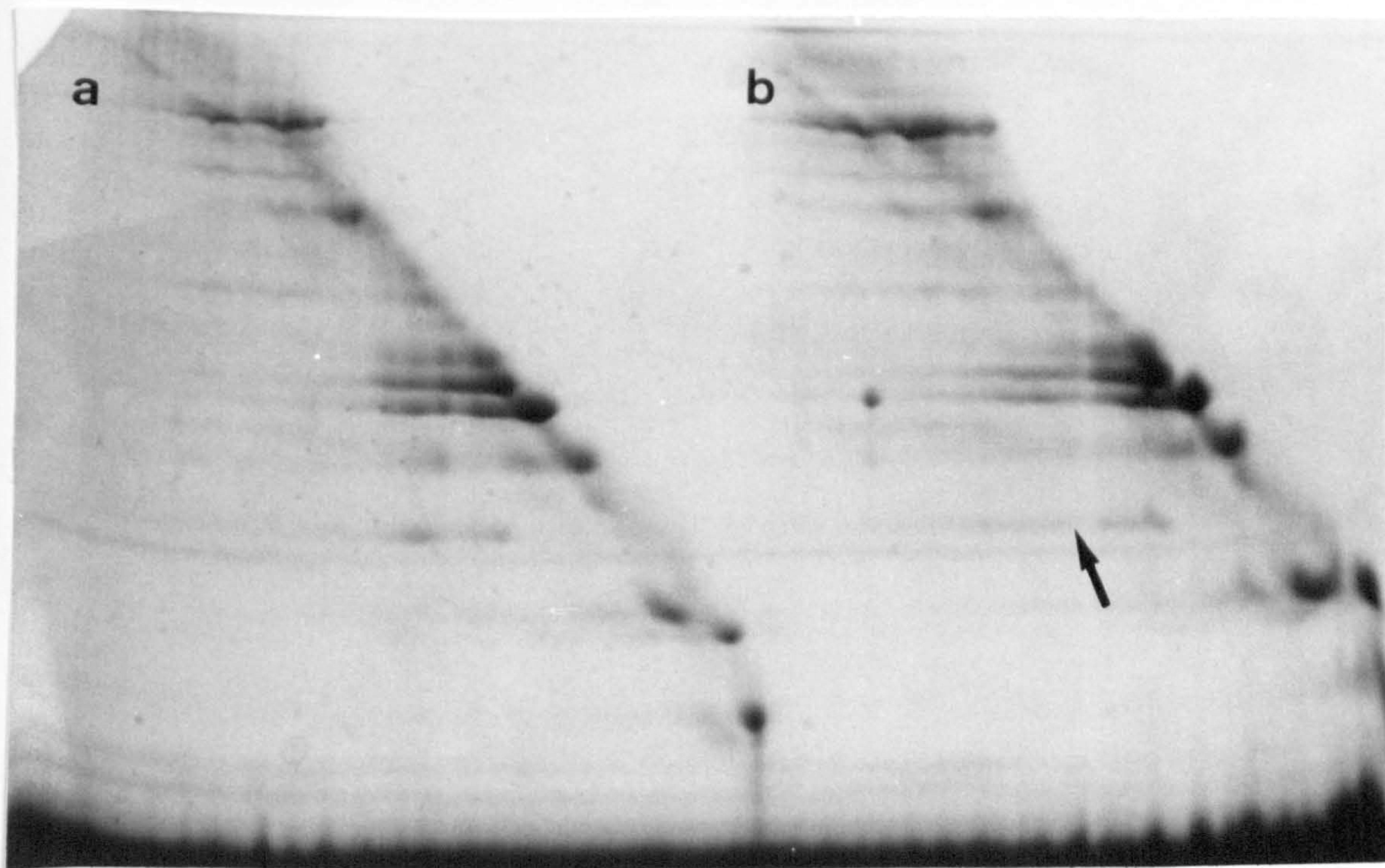


Fig. 7.11 2-D Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium from (a) *Psoas Major*, Conditioned for 3 Days and (b) *Psoas Major*, Lactic Acid Treated and Conditioned for 3 Days

CNBr digest of perimysial samples, dissolved in gel sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels. 400 μl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

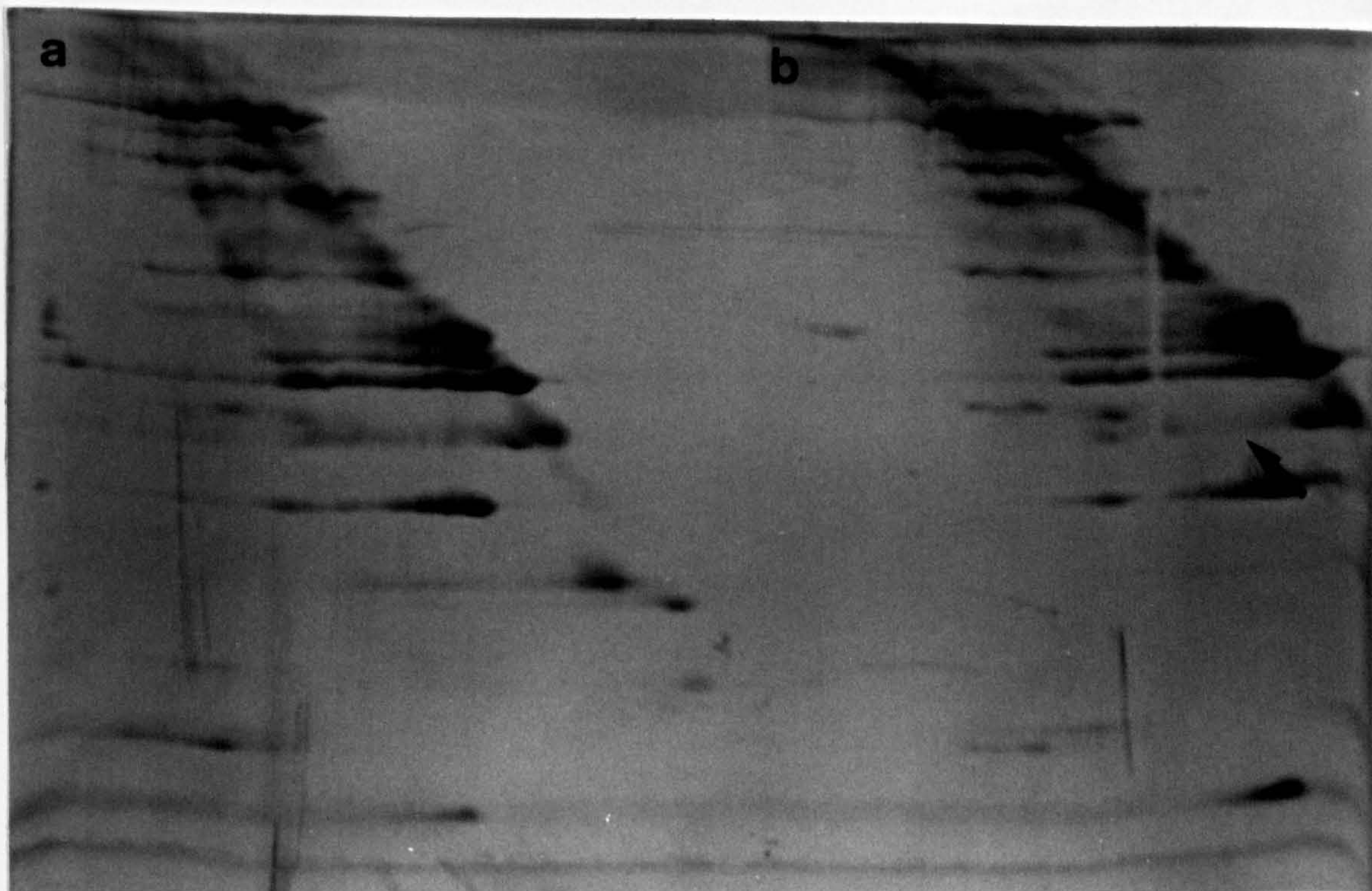


Fig. 7.12 2-D Peptide Mapping of 6 M Urea-Washed Insoluble Perimysium from (a) *Gluteus Medius*, Conditioned for 3 Days, and (b) *Gluteus Medius*, Lactic Acid Treated and Conditioned for 3 Days

CNBr digest of perimysial samples, dissolved in gel sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels. 400 μl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

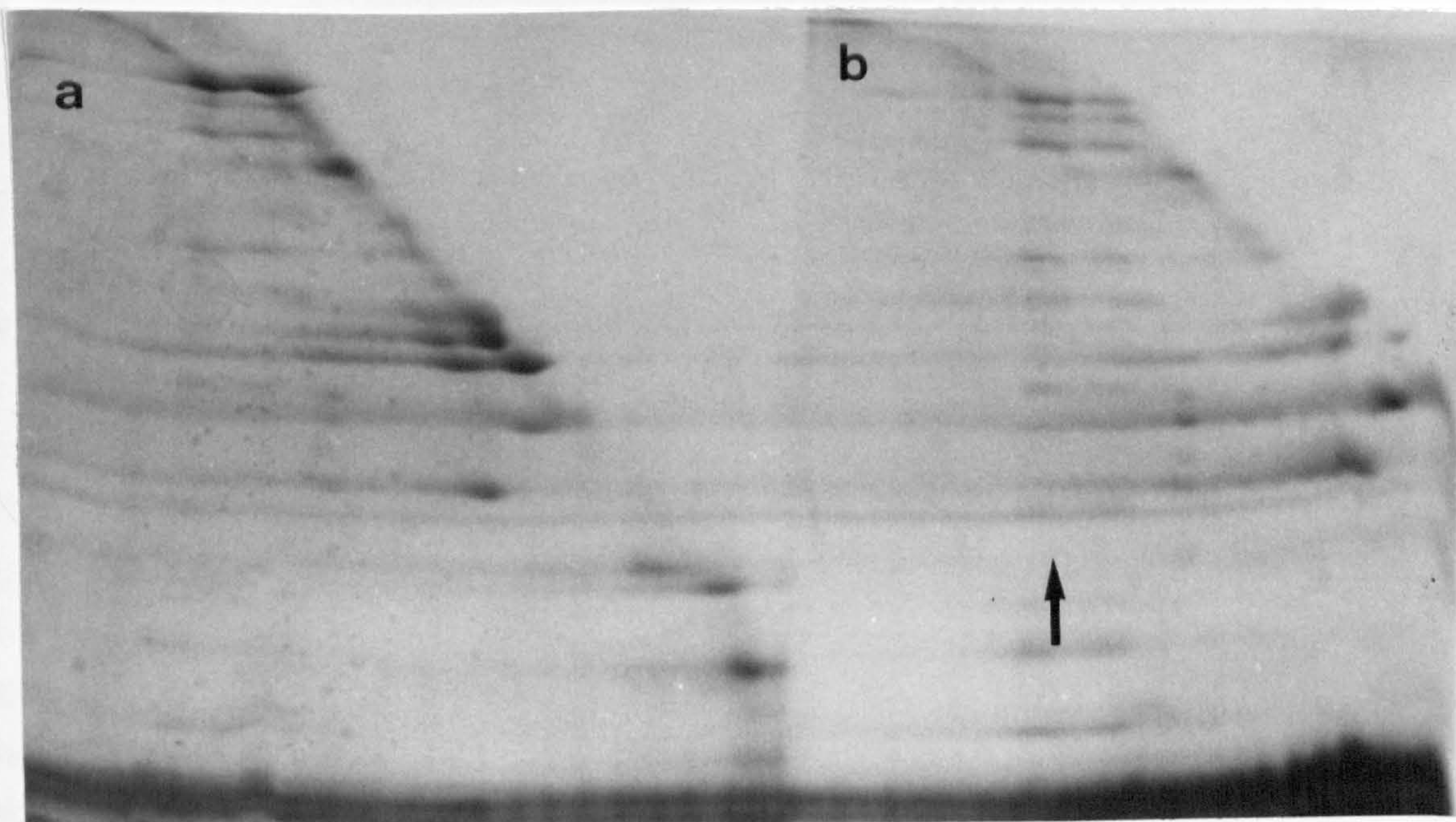


Fig. 7.13 2-D Peptide Mapping of 6 M UreaWashed Insoluble Perimysium from (a) *Serratus Ventralis*, Conditioned for 3 Days and (b) *Serratus Ventralis*, Lactic Acid Treated and Conditioned for 3 Days

CNBr digest of perimysial samples, dissolved in gel sample buffer at a concentration of 5 mg/ml were resolved in the first dimension in 10 % (w/v) polyacrylamide tube gels. Tube gels were set in 7.5 % (w/v) acrylamide stacking gels. 400 μl of chymotrypsin solution (3.33 mg/ml, 200 units/ml) in gel sample buffer was applied to the top of the gel and the system was incubated for 2 h, whilst proteolysis occurred in the first dimension gel. Second dimension electrophoresis was carried out in 15 % (w/v) acrylamide slabs using conditions as described in Chapter 2.

90 % of the total pigment in beef muscle (see Tarrant, 1982 for review). Haemoglobin of red blood cells is another pigment in meat but, because animals are bled out at slaughter, is of less significance in final meat colour. Myoglobin consists of a protein monomer (globin) and a single, iron-containing haem group. The oxidation state and type of ligand (i.e. oxygen, water, nitric oxide) bound to the iron centre of the haem group determines the colour of myoglobin under most conditions. Deoxymyoglobin, the native meat pigment in which iron is in the ferrous (Fe^{2+}) state, exists under anoxic conditions such as occur in the interior of meat cuts. This form of myoglobin is purplish-red and is responsible for the corresponding colour in the interior of freshly cut beef. Upon exposure to the atmosphere, myoglobin rapidly becomes oxygenated to oxymyoglobin, which is bright red in colour and responsible for the familiar 'bloom' of fresh meats. For the preservation of fresh meat colour, conditions favouring the pigmentation balance towards the reduced form of myoglobin must be maintained. Metmyoglobin, the oxidized form of myoglobin in which iron is in the ferric (Fe^{3+}) state, is a brown derivative and is undesirable in fresh meat. The colour of fresh meat is determined by the relative proportions of the three states of myoglobin present at the cut surface of the meat. Consumer discrimination against discoloured meat increases with increase in metmyoglobin content. Denaturation of myoglobin by heat and pH enhances autooxidation and is responsible for the colour fading observed in PSE pork.

Fresh, unconditioned meat colour was unaltered following injection of 0.1 M lactic acid to a level of 10 % the original weight of muscles and lower concentrations and was considered acceptable in the present study. However, pre-rigor injection of 0.2 M lactic acid and higher concentrations, led to formation of highly unacceptable meat colour, and these concentrations were deemed unsuitable for the purposes of this study. 0.1 M lactic acid was the highest concentration employed in the study, which caused no adverse colour changes, but which caused rapid pH decline to a minimum of pH 5.33 only 3 h after injection after which it increased again and plateaued at 8.75 h reaching a pH value of 5.5. pH decline in the untreated control progressed constantly from an initial value of 7.1 to pH 5.5 over the 26 h period studied. Following injection of concentrations of 0.2 M lactic acid to 1 M lactic acid, the pH values dropped initially over the first hour to the minimum values obtained. In all of these cases, the pH values increased again, and plateaued at 8.75 h, except for the meat sample injected with 1.0 M lactic acid. The pH values of this meat sample were still increasing 26 h after injection.

The synthesis of ATP through the TCA-cycle ceases after death due to termination of the blood supply. ATP levels are maintained for a time, however, by the breakdown of CP and glycogen via the Embden-Meyerhof pathway (see Chapter 1). When the level of ATP

can no longer be maintained from CP and glycogen stores, due either to the amount being reduced to a critical level or to a decrease in the activity of the enzymes associated with glycolysis, the muscles start to contract and rigor mortis develops (Bendall, 1973). The rate of development of rigor mortis, monitored by disappearance of ATP from fresh unconditioned muscle, was not significantly altered due to prior injection with lactic acid. The ATP level dropped to 3 % of the initial value in lactic acid treated and untreated control samples, 24 h after slaughter.

There was a small increase in pH of the set of bovine muscles, injected with lactic acid prior to conditioning, following conditioning for 14 days. However, no such increase in pH was observed for those muscles which were conditioned normally for 14 days. The small increase in pH observed here on conditioning of lactic acid treated bovine muscles, has been observed previously for meat held above the freezing point (Lawrie, 1985). The pH rise is more marked if the temperature of holding is high, and is greater in pork than beef. The average pH values obtained from all good quality muscles, *psoas major* and *gluteus medius*, conditioned with and without prior lactic acid treatment, were 5.5, while the average pH values obtained was 5.6 for the poorer quality muscle, *serratus ventralis*. This result correlates with that of Bouton et al., (1957) who observed a curvilinear relationship between tenderness and pH value, showing that minimum tenderness occurred at a pH value around 6.0 and increased linearily on either side of this value.

The ATP levels of all frozen bovine muscles were significantly reduced compared to the unfrozen unconditioned sample examined. Fresh unconditioned muscle sample had an ATP level of 121 μmol/100g meat while the average ATP level of the frozen *psoas major, gluteus medius* and *serratus ventralis* unconditioned muscles was only 14 % of this value. The reason for the difference in ATP levels recorded in fresh pre-rigor muscle and muscle frozen in the pre-rigor state is due to thaw-rigor as discussed in Chapter 1 (section 1.5.2.2). On subsequent thawing of pre-rigor frozen meat an exceedingly rapid rate of ATP breakdown and onset of rigor-mortis occurs. On average, the ATP level of all 3 muscles dropped to 21 % of the frozen unconditioned value following conditioning for 1 day and subsequent freezing after which the ATP level of the frozen, conditioned and lactic acid treated muscles did not change significantly. The average ATP level of all 3 muscles, following conditioning for 1 day and subsequent freezing was only 3 % of the ATP level of the fresh (unfrozen) meat sample while this value represented 2 % of all 3 lactic acid treated muscles, conditioned for 1 day and subsequently frozen. It was therefore concluded that rigor-mortis had developed in all muscles, conditioned for 1 day and longer with and without lactic acid treatment prior to conditioning.

The yields of solubilized perimysial material obtained from conditioned and lactic acid treated *psoas major* and *gluteus medius* muscles increased from 1 to 14 days due to conditioning. However, the yields of solubilized perimysial material obtained from *serratus ventralis* muscles, conditioned normally and with prior lactic acid treatments, indicated no such increases due to conditioning. In fact, in both cases, the total yields of solubilized perimysial material obtained from *serratus ventralis* muscles indicated a decrease due to conditioning. The reason for this is unclear, but it may have resulted from variations in the procedure of perimysial preparation, which involved sampling of meat samples from 4 different locations within the muscles, which were subsequently combined. The high quantities of embedded tendon material in *serratus ventralis* muscles may have contaminated the selected meat samples, distorting the results.

The percentage collagen in the solubilized perimysial fractions obtained from conditioned and lactic acid treated *psoas major, gluteus medius* and *serratus ventralis* muscles increased with time of conditioning. As stated above, the yield of solubilized perimysial material obtained from *serratus ventralis* was found to decrease on conditioning yet the percentage collagen solubilized in the perimysial fractions increased, suggesting that yields of non-collagenous material caused the discrepencies observed above for solubilized perimysial material. However, the percentage collagen solubilized from muscles treated with lactic acid prior to conditioning were significantly higher ($p < 0.05$) for all three muscles examined than the untreated controls. This indicates that introduction of lactic acid to muscle before conditioning has a role to play in collagen solubilization during the subsequent conditioning period. It was found that the percentage collagen in the solubilized perimysial fractions obtained from lactic acid treated *psoas major*, at 3 to 4 days of conditioning was equivalent to that reached after 14 days of normal conditioning, while similar comparisons for *gluteus medius* and *serratus ventralis* revealed that 12 to 13 days and 6 to 7 days respectively, of conditioning after lactic acid treatment was required to attain levels of solubilized perimysial collagen equivalent to that attained by 14 days of normal conditioning. The earlier attainment of the conditioning effects on solubilized perimysial collagen, normally observed after 14 days, suggests that injected lactic acid has a role to play in producing rapid conditioning effects, which may have resulted from earlier activation of muscle cathepsins, due to muscle pre-rigor introduction of lactic acid.

Comparison of insoluble perimysial fractions obtained from unconditioned, conditioned and lactic acid treated *psoas major, gluteus medius* and *serratus ventralis* muscles indicated no significant alterations in the peptide spectrum obtained by one-dimensional SDS-polyacrylamide gel electrophoresis of the CNBr peptides. In respect of perimysial samples obtained from muscles conditioned normally from 1 to 14 days, this was to be

expected (similar results are presented in Chapter 3). However, in the case of perimysial samples derived from lactic acid treated muscles prior to conditioning, it might be expected that there would have been some visible damage to insoluble collagen due to 'clips' or incomplete cleavages by early activation of the acid proteases of meat, due to introduction of lactic acid to the pre-rigor muscles. However, this was not evident from one-dimensional SDS-polyacrylamide gel electrophoresis, and suggested that the damage incurred to the insoluble material must be minor.

2-D analysis of insoluble perimysium obtained from *psoas major, gluteus medius* and *serratus ventralis* muscles, conditioned and lactic acid treated prior to conditioning for 3 days indicated a role for lactic acid treatment on perimysial collagen breakdown, during subsequent conditioning. The degree of collagen breakdown varied, with the major differences observed in the poor quality *serratus ventralis* muscle. Normal conditioning for 3 days resulted in low incidence of chymotrypsin digested peptides from the high molecular weight material, as evidenced in perimysium obtained from muscles conditioned for 14 days (see Chapter 6). However, with lactic acid treatment of *serratus ventralis*, prior to conditioning for 3 days, the perimysial peptide map obtained showed similarities with that obtained from perimysium from *extensor capri radialus* conditioned for 14 days (see Chapter 6), evident as increased incidence of peptides resulting from chymotrypsin digestion of the high molecular weight material. These results suggest a faster conditioning effect due to lactic acid treatment of muscles prior to conditioning, evident as an earlier disruption of the perimysial collagen matrix from the 14 days of conditioning normally required to 3 days with lactic acid treatment. It was noted that the effects of lactic acid treatment were more evident in the poorer quality muscle, *serratus ventralis* which contained a higher collagen content than in either of the two higher quality muscles examined. This may suggest a role for lactic acid treatment for up-grading poorer quality muscles. However, verification of such a conclusion necessitated taste panel assessment of the lactic acid treated and untreated meat, which is presented in Chapter 8.

CHAPTER 8

SENSORY ANALYSIS OF THE EFFECTS OF PRE-RIGOR LACTIC ACID TREATMENT ON BOVINE MUSCLES

8.1 INTRODUCTION

The work outlined in Chapter 7 provided biochemical assessment of the effects of pre-rigor lactic acid injection and subsequent conditioning of poor, medium and high quality muscle. The results indicated a positive effect from lactic acid injection prior to subsequent conditioning on collagen breakdown. This suggested a role for pre-rigor lactic acid injection of muscle prior to conditioning in the tenderization of meat. However, such a conclusion could only be reached by taste panel assessment of the lactic acid treated and untreated meats.

This chapter is concerned with taste panel assessment of *psoas major, gluteus medius* and *serratus ventralis* muscles, conditioned normally and lactic acid treated prior to conditioning, for 1 to 14 days. The purpose of the sensory investigations was to determine the effects of lactic acid injection prior to conditioning on the subsequent quality of the meat in order to identify the benefits, if any, gained by this treatment.

A taste panel of 10 judges, male and female, were selected, ranging in age from 20 to 50 years. The sensory investigation began with a familiarization session where the procedures to be adopted were discussed and the quality characteristics of the meat, selected for the study were defined. The familiarization session was designed not only to make panellists conversant with the procedures but in addition, to emphasize that personal preferences should be disregarded as they could interfere with their objective decision making, and so that all taste panellists would gain substantially uniform understanding over the whole sensory evaluation process.

The quality attributes of the meat, selected for assessment in the subsequent study were aroma, juiciness, moistness after chewing, resistance to first bite, ease of chewing, tenderness, meat colour and overall acceptability of the product.

The method chosen involved descriptive analysis with scaling of the acceptability of aspects of the product described above. Anchor words were inserted at each end of the 14 cm horizontal line and the panellist had to indicate his/her judgement by making a vertical line at the appropriate point of the horizontal line. The left handed side

represented very good acceptability whereas the right-handed side represented negative acceptance of the product. The raw data, that is, the arbitrary score for each attribute, were produced through measurement of the horizontal line to the vertical mark made by the panellist, and statistical analysis was carried out on the results obtained.

8.2 MATERIALS AND METHODS

8.2.1 Preparation of Bovine Muscles

Serratus ventralis, gluteus medius and *psoas major*, representing poor, medium and high quality muscles, respectively, were unconditioned, conditioned normally and treated prior to conditioning with 0.1 M lactic acid (to a level of 10 % of the original weight of the muscle) as described in Chapter 7 (section 7.2.3). All muscles were stored frozen following conditioning.

8.2.2 Preparation of Meat for Cooking

The three cuts of beef used in this study were fillet steak (from *psoas major*), rump steak (from *gluteus medius*) and chuck steak (from *serratus ventralis*). The frozen muscles were allowed to thaw slowly prior to use. Slices, approximately 3 cm thick, were cut, against the grain, from the upper middle section of the prepared *psoas major* muscle, skin and excess fat were removed, yielding fillet steak. Slices, approximately 2 cm thick, were cut, against the grain, across the whole prepared *gluteus medius* muscle, skin and excess fat were removed, yielding rump steak. Slices, approximately 2.5 cm were cut, against the grain from the prepared *serratus ventralis* muscle, skin, excess tendon and fat were removed, yielding chuck steak, which was then cut into 2.5 cm cubes.

8.2.3 Cooking of Prepared Steaks

Prepared fillet and rump steaks were grilled, at high temperatures for short times, while chuck steak was braised. All cooking conditions were standardized in an effort to minimize inconsistencies of the judges' scores.

8.2.3.1 Method of Grilling

The method of grilling chosen employed a top-heat electric grill. This was lit in sufficient time for it to reach maximum heat by the time grilling was to commence. The prepared fillet and rump steaks were brushed with oil prior to grilling. The grilling plates were

brushed with oil to prevent the meat from sticking to them and the prepared steaks were placed directly on the plates. The fillet and rump steaks were grilled quickly until coloured on top, after which they were turned and cooked on the other side until well coloured. The rack was then lowered and cooking was allowed to continue until the meat was cooked. The total grilling time was 2.5 to 3 minutes on either side, yielding 'medium' steaks.

8.2.3.2 Method of Braising

The prepared cubes from chuck steak were shallow fried until brown. This enhanced flavour and visual appearance of the product. These were then removed from heat and placed in a braising pan. Convenience beef stock was added to the pan, which was then covered and placed in a moderate convection oven at 140 to 150° C for 3 h.

8.2.4 Selection and Training of Judges

Prospective judges were staff members of the Dorset Institute, all of whom were unpaid volunteers. Requests for volunteers were made during a short introductory talk about the project and the sensory experiments. Handouts explaining the commitment and work involved were dispersed together with a questionnaire to be completed by volunteers, which provided information on the age, sex, smoking habits, food dislikes, relevant medical conditions (ulcers, sinus problems, allergies and use of dentures) and the time availability of the volunteers. Selected panellists were initially subjected to a preliminary session (lasting 45 min), where they were familiarized with the procedures of the main taste panel programme to be undertaken. Introduction to scoring methods, questionnaires and terms commonly used in sensory analysis were discussed. Samples of fillet steak were available to the panellists and important sensory attributes of the meat were identified. Each panel member independently examined samples and discussed their findings as a group. Language difficulties and disputed points were resolved and ideas were exchanged. A sensory profile of the perceived characteristics of the product was established and a questionnaire was formed which is shown in Fig. 8.1.

8.2.5 Taste Panel Procedure

The duration of each taste panel session was approximately 45 min, conducted at the same time each day. Questionnaires and water were available to each judge. All cooked meats were served immediately after cooking. Rump and fillet steaks were cut into 2.5 and 3 cm cubes following cooking and two cubes of meat were allocated to each judge. Chuck

Acceptability

Place a vertical line across the horizontal line at the point representing your opinion.

appetizing aroma	unappetizing aroma
initially juicy	initially dry
moist after chewing	dry after chewing
not resistant to first bite	resistant to first bite
very easy to chew	very difficult to chew
very tender	very tough
expected meat colour	unacceptable meat colour
like extremely	dislike extremely

Fig. 8.1 The Score Sheet Used in the Sensory Experiment

steak was served unaltered following cooking.

11 different samples of meat were tested per day, derived from the various treatments of the muscles (unconditioned, conditioned normally and lactic acid treated prior to conditioning for 1, 3, 7, 10 and 14 days). Triplicate testings of the meat samples, derived from each muscle, were carried out over 3 days per week, such that the entire taste panel programme lasted 9 days, conducted over 3 weeks.

8.2.6 Methods of Analysis

Before statistical evaluation, all judgements were transformed into scores by placing a ruler against the 14 cm scoring line, scores were measured to the nearest mm. Each judge scored each sample on an unstructured scale anchored at either end by the quality attributes described in section 8.1 and Fig. 8.1. Low scores indicated an extremely acceptable product, while high scores indicated an unacceptable product. The mean scores of the 3 replicates for each attribute were considered prior to two-way analysis of variance with repeated observations (MANOVA) by the SPSSX system, using the individual data scores as input. Initially, evaluation of individual judges performance and the taster/treatment interaction were considered.

8.3 RESULTS

8.3.1 Analysis

Statistical analysis using two-way analysis of variances with repeated observations indicated significant differences ($p < 0.05$) were found between tasters for every sensory attribute for all 3 muscles indicating inconsistencies of the judges scores. The interaction between judges and treatment of the meats was found not to be significant ($p > 0.05$), which for a given meat treatment considers differences between judges, and this interaction was therefore eliminated. The correlation coefficients between the sensory attributes used in this study were determined for *psoas major* (Table 8.1), *gluteus medius* (Table 8.2) and *serratus ventralis* (Table 8.3). As expected, high correlation coefficients were found to exist between the following attributes for all 3 muscles: initial juiciness and moistness after chewing, resistance to first bite and ease of chewing, resistance to first bite and tenderness, ease of chewing and tenderness, ease of chewing and overall acceptability and tenderness and overall acceptability. Odour and colour of the meats were poorly correlated with all other attributes studied.

Table 8.1 Correlation Coefficients Between Sensory Attributes for *Psoas Major*

Sensory Attribute*	1	2	3	4	5	6	7
1	-	-	-	-	-	-	-
2	0.15	-	-	-	-	-	-
3	0.12	0.68	-	-	-	-	-
4	0.03	0.32	0.38	-	-	-	-
5	0.05	0.41	0.52	0.72	-	-	-
6	0.10	0.48	0.60	0.68	0.89	-	-
7	0.27	0.16	0.31	0.25	0.30	0.39	-
8	0.18	0.47	0.58	0.42	0.68	0.71	0.42

* Sensory Attribute

1 Odour
2 Initial Juiciness
3 Moistness After Chewing
4 Resistance to First Bite
5 Ease of Chewing
6 Tenderness
7 Colour
8 Overall Acceptability

Table 8.2 Correlation Coefficients Between Sensory Attributes for *Gluteus Medius*

Sensory Attribute*	1	2	3	4	5	6	7
1	-	-	-	-	-	-	-
2	0.07	-	-	-	-	-	-
3	0.22	0.63	-	-	-	-	-
4	0.24	0.16	0.38	-	-	-	-
5	0.27	0.28	0.39	0.76	-	-	-
6	0.37	0.29	0.40	0.75	0.92	-	-
7	0.32	0.31	0.28	0.14	0.31	0.27	-
8	0.38	0.19	0.35	0.55	0.64	0.65	0.26

* Sensory Attribute

1 Odour
2 Initial Juiciness
3 Moistness After Chewing
4 Resistance to First Bite
5 Ease of Chewing
6 Tenderness
7 Colour
8 Overall Acceptability

Table 8.3 Correlation Coefficients Between Sensory Attributes for *Serratus Ventralis*

Sensory Attribute*	1	2	3	4	5	6	7
1	-	-	-	-	-	-	-
2	0.02	-	-	-	-	-	-
3	-0.09	0.77	-	-	-	-	-
4	-0.12	0.25	0.30	-	-	-	-
5	-0.18	0.27	0.40	0.71	-	-	-
6	-0.13	0.32	0.44	0.70	0.93	-	-
7	0.35	0.21	0.24	0.02	0.27	0.35	-
8	0.08	0.25	0.34	0.58	0.68	0.74	0.30

* Sensory Attribute

1 Odour
2 Initial Juiciness
3 Moistness After Chewing
4 Resistance to First Bite
5 Ease of Chewing
6 Tenderness
7 Colour
8 Overall Acceptability

In the following section, the 8 sensory attributes considered in this study are examined separately for all 3 muscles.

Initially, the mean scores of the 3 replicates for each attribute are considered. Subsequently further tests which were carried out for comparison of meat conditioned normally with meat injected with lactic and prior to conditioning are presented.

8.3.2 Odour

The sensory attribute of odour of the meat as mentioned previously, was described as being either an appetizing or unappetizing aroma. It should be noted as mentioned previously, that low scores indicated an extremely acceptable product, while high scores indicated an unacceptable product. The mean scores for all attributes of cooked *psoas major*, *gluteus medius* and *serratus ventralis* studied are presented in Appendix 1. The final column in these tables and in the subsequent ones shows the mean scores for the 11 meat samples of each judge. The coefficients of variation (CV) of the judges scores for odour which indicate variability of the scores, were 24 % for *psoas major*, 33 % for *gluteus medius* and 36 % for *serratus ventralis*. The high variability observed for this and subsequent sensory attributes reflect difficulty of consistent assessment of sensory attributes by individuals. The data presented in Appendix 1 for each attribute is summarized in graphical form. Each point on these graphs represents the mean scores of all judges for sensory attributes of unconditioned, conditioned and lactic acid treated meat samples from 1 to 14 days. The mean scores obtained for odour of all 3 muscles indicated similar trends due to conditioning and lactic acid treatment (Fig. 8.2a, b and c). The odour of *psoas major* was perceived to be slightly higher by conditioning up to 3 days and by lactic acid treatment up to 7 days, and thereafter was perceived to decrease continually up to 14 days for both treatments (Fig. 8.2a). The odour of the lactic acid treated sample was favoured over the conditioned sample at 14 days. The odour of *gluteus medius* was perceived to be lower at 1 day of conditioning than the lactic acid treated sample (Fig. 8.2b). From 3 to 14 days of treatment the odour of the conditioned sample was favoured over the lactic acid treated sample. The mean scores for odour of *serratus ventralis* overlapped at 2 points (Fig. 8.2c) and this attribute was favoured in the conditioned sample over the lactic acid treated sample at 14 days.

8.3.3 Initial Juiciness

The CV of the judges scores for initial juiciness were 14 % for *psoas major*, 19 % for *gluteus medius* and 31 % for *serratus ventralis*. Initial juiciness of *psoas major* did not

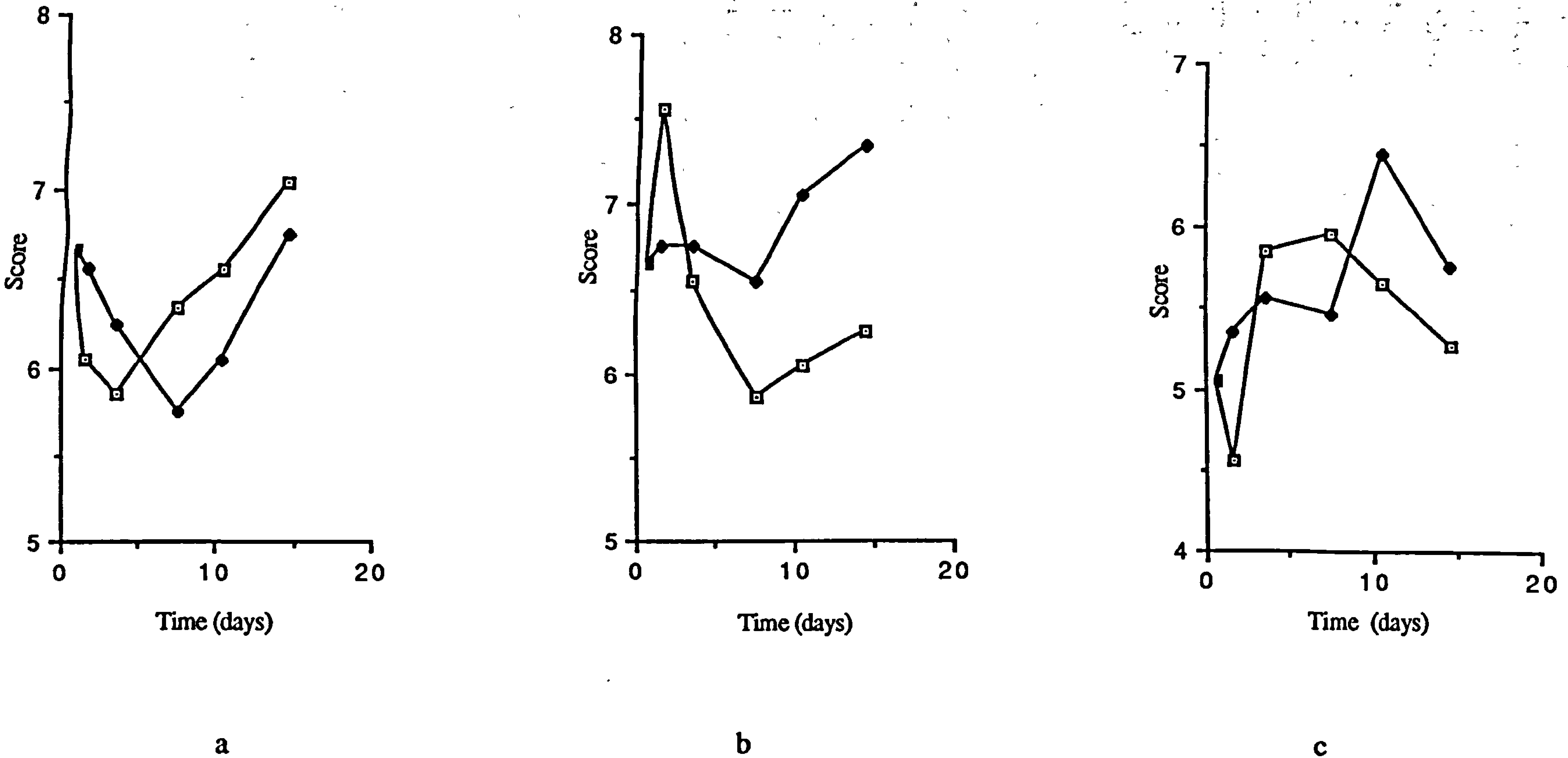


Fig. 8.2 Judges Mean Scores for Odour of Conditioned (□) and Lactic Acid Treated (♦) Bovine Muscles (mean of 8 judges, for *psoas major* (a), mean of 10 judges for *gluteus medius* (b), and mean of 9 judges for *serratus ventralis* (c) and 3 replications).

appear to be considerably affected by lactic acid treatment when compared to the conditioned sample (Fig. 8.3a). However, this attribute was considered most favourable in the unconditioned sample and least favourable after 1 day of conditioning and 3 days of lactic acid treatment, after which it improved again due to both treatments. After 14 days, the conditioned *psoas major* was considered slightly more favourable than the lactic acid treated sample. The mean scores for initial juiciness of *gluteus medius* overlapped at 3 points (Fig. 8.3b) and was perceived to be very similar after 14 days of conditioning and lactic acid treatment. Initial juiciness of *serratus ventralis* appeared to be unaffected by lactic acid treatment, when compared to the conditioned sample (Fig. 8.3c). This attribute was perceived to be least favourable at 7 days of lactic acid treatment and conditioning, after which it improved up to 14 days of treatment. The lactic acid treated sample was perceived to be more favourable than the conditioned sample at 14 days.

8.3.4 Moistness after Chewing

The CV of the judges score for moistness after chewing were 11 % for *psoas major*, 19 % for *gluteus medius* and 16 % for *serratus ventralis*. This sensory attribute was negatively affected by lactic acid treatment and conditioning of *psoas major* (Fig. 8.4a) from 1 to 14 days, and was favoured in the conditioned sample over the lactic acid treated sample at 14 days. The mean scores for moistness after chewing of *gluteus medius* overlapped at 3 points (Fig. 8.4b) and was favoured in the conditioned samples over the lactic acid treated sample at 14 days. Lactic acid treatment of *serratus ventralis* did not appear to affect moistness after chewing considerably when compared to the conditioned sample (Fig. 8.4c) and at 14 days of conditioning appeared equal in both samples.

8.3.5 Resistance to First Bite

The CV of the judges score for resistance to first bite were 14 % for *psoas major*, 16 % for *gluteus medius* and 36 % for *serratus ventralis*. The high variability observed for *serratus ventralis* may reflect the variable quantities of connective tissue of this muscle. At 1, 3 and 7 days, resistance to first bite of *psoas major* was perceived to be higher in lactic acid treated than conditioned samples (Fig. 8.5a). However, after 10 days of treatment the lactic acid treated sample was favoured over the conditioned sample, while after 14 days of treatment, the conditioned sample was favoured over the lactic acid treated sample. The pattern of resistance to first bite of *gluteus medius* was similar to *psoas major* over 14 days of conditioning and lactic acid treatment (Fig. 8.5b) in that up to 7 days of treatment, the

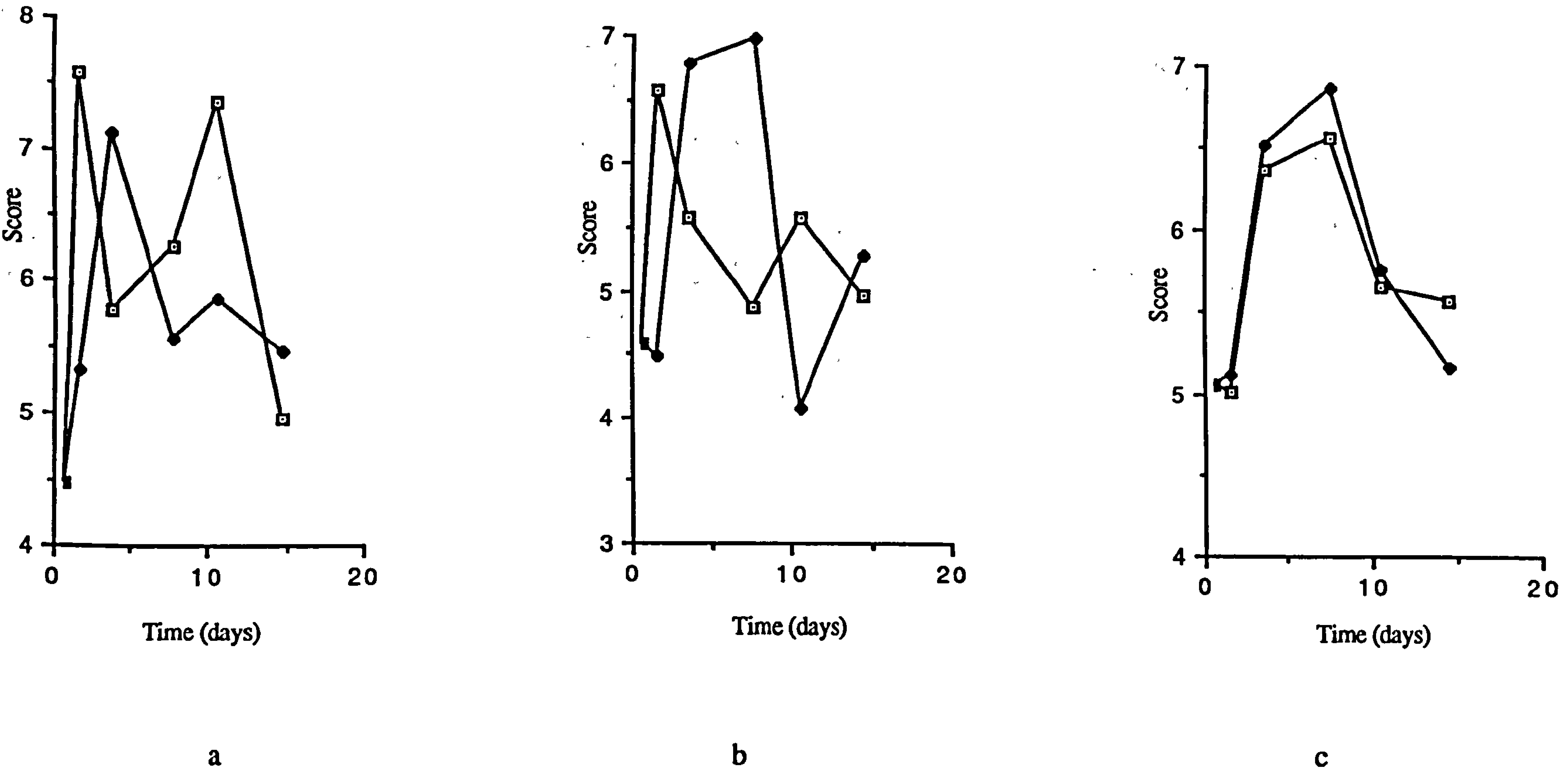


Fig. 8.3 Judges Mean Scores for Initial Juiciness of Conditioned (□) and Lactic Acid Treated (♦) Bovine Muscles (mean of 8 judges, for *psoas major* (a), mean of 10 judges for *gluteus medius* (b), and mean of 9 judges for *serratus ventralis* (c) and 3 replications).

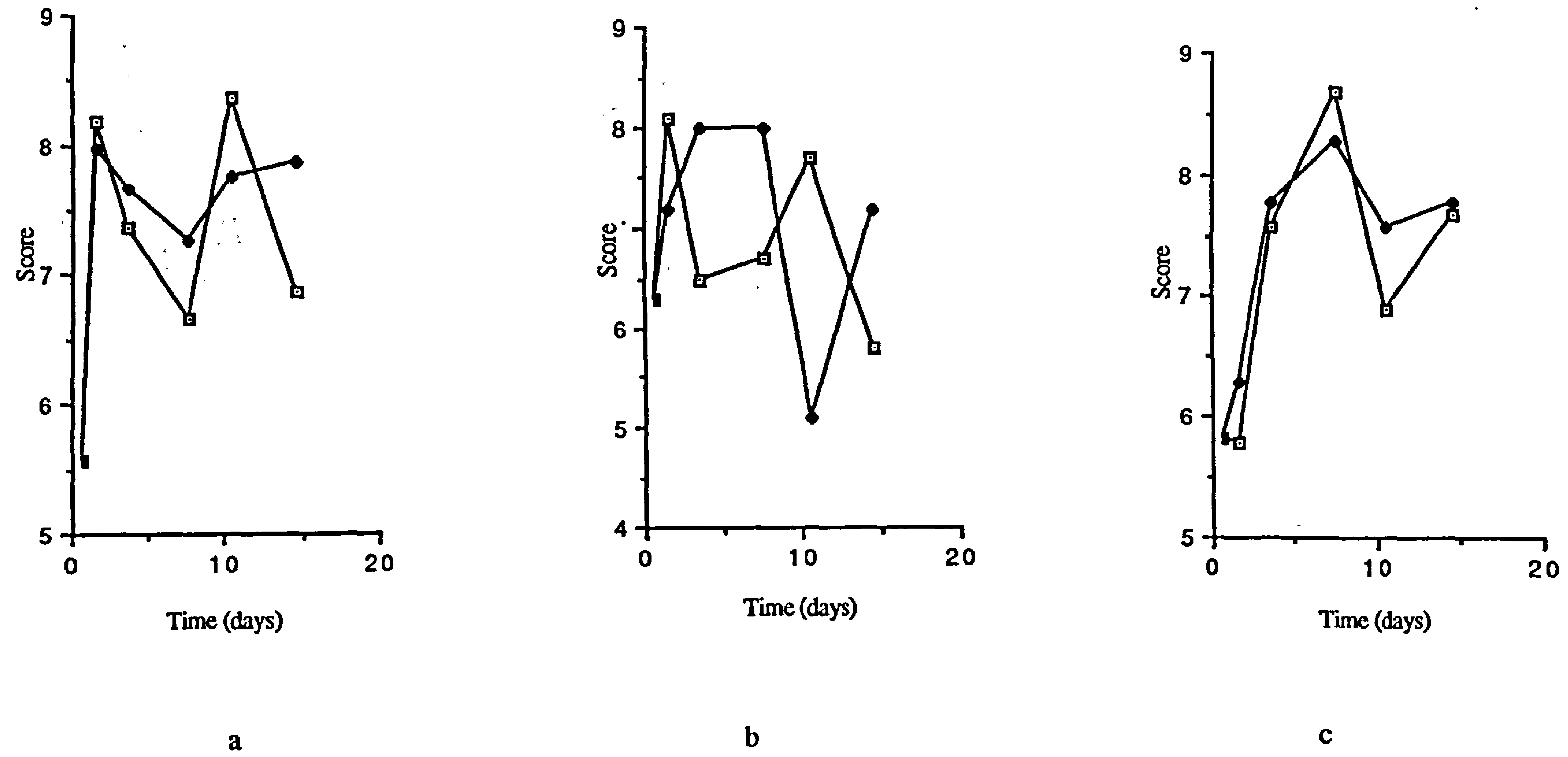


Fig. 8.4 Judges Mean Scores for Moistness After Chewing of Conditioned (⊡) and Lactic Acid Treated (♦) Bovine Muscles (mean of 8 judges, for *psoas major* (a), mean of 10 judges for *gluteus medius* (b), and mean of 9 judges for *serratus ventralis* (c) and 3 replications).

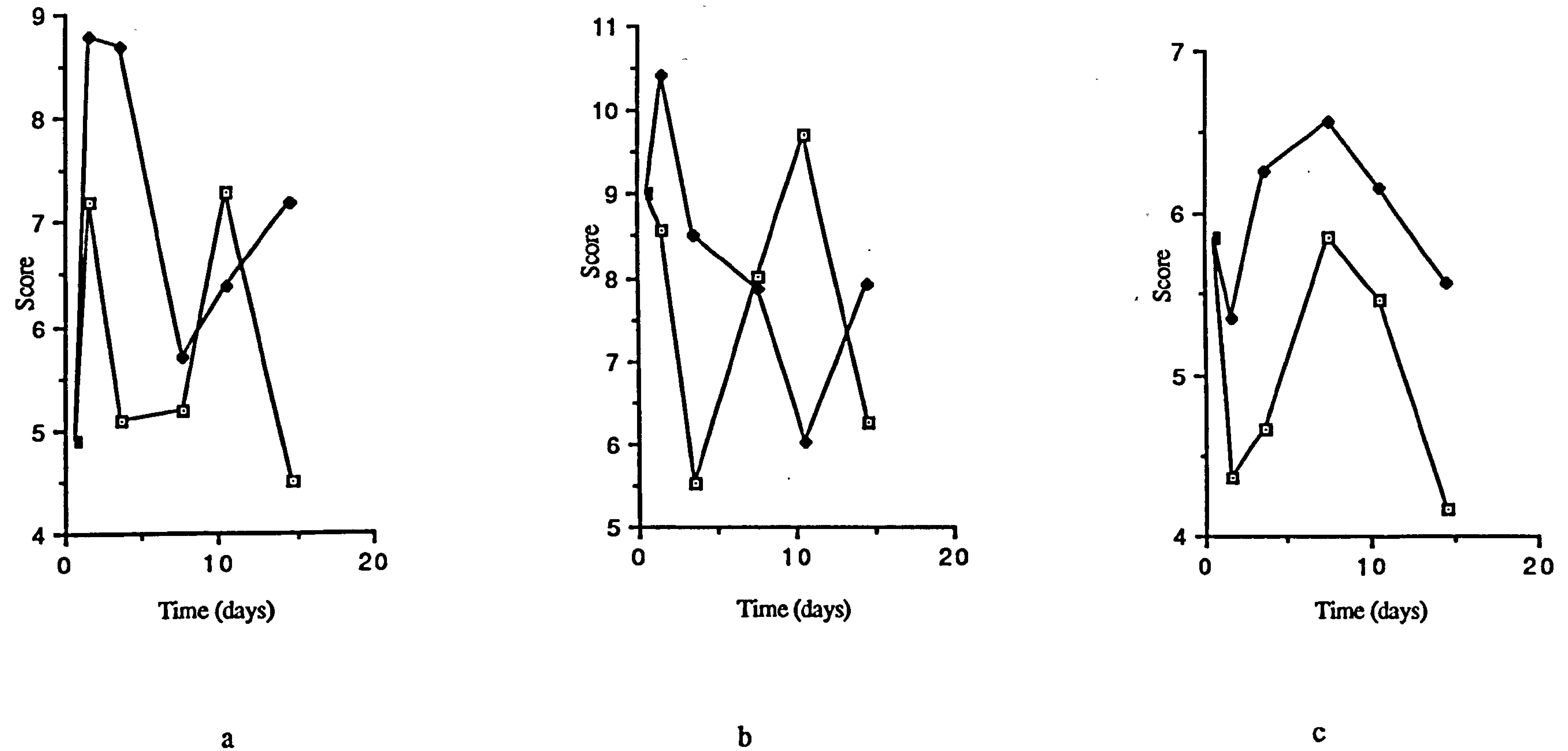


Fig. 8.5 Judges Mean Scores for Resistance to First Bite of Conditioned (⊡) and Lactic Acid Treated (♦) Bovine Muscles (mean of 8 judges, for *psoas major* (a), mean of 10 judges for *gluteus medius* (b), and mean of 9 judges for *serratus ventralis* (c) and 3 replications).

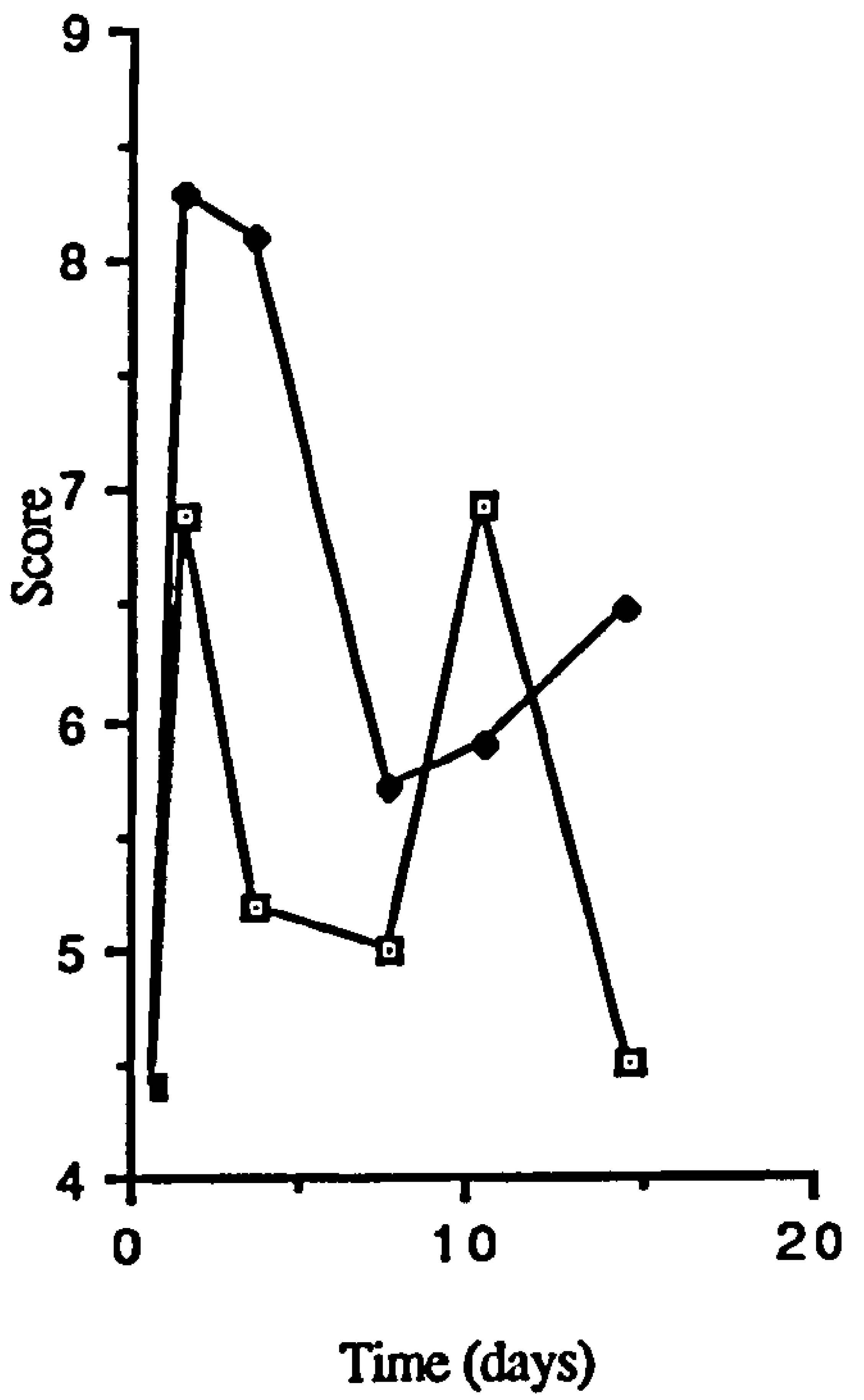
conditioned sample was favoured over lactic acid treated samples. At 7 days, no difference was perceived between the 2 treatments. After 10 days of treatment, resistance to first bite of the lactic acid treated sample was perceived to be considerably lower than in the conditioned sample. However, at 14 days of treatment the conditioned sample was perceived to be higher than the lactic acid treated sample. The results of the judges score for resistance to first bite of *serratus ventralis* indicated that the conditioned samples were favoured over lactic acid treated samples for all treatment times (Figs. 8.5c).

8.3.6 Ease of Chewing

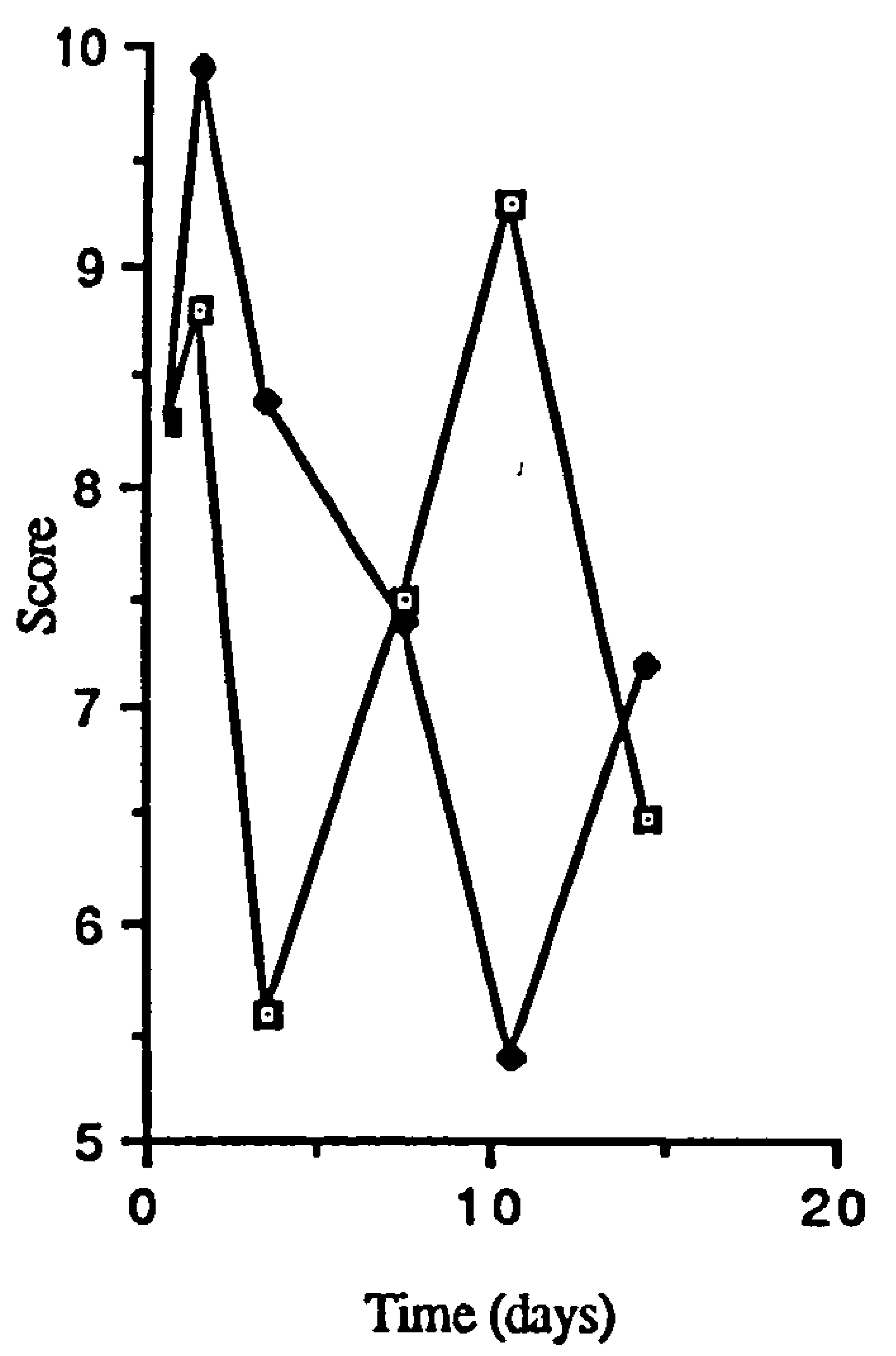
The CV of the judges score for ease of chewing were 15 % for *psoas major*, 13 % for *gluteus medius* and 29 % for *serratus ventralis*. Again the CV obtained for *serratus ventralis* was observed to be much higher than for either of the other 2 muscles studied, reflecting the variable nature of this muscle. Chewiness of *psoas major* was perceived to be low in the unconditioned sample and increased with lactic acid treatment for 14 days and conditioning for 10 days relative to the unconditioned sample (Fig. 8.6a). For all times of treatment studied except at 10 days, the conditioned sample was favoured over the lactic acid treated sample. Chewiness of *gluteus medius* generally indicated an improvement from conditioning and lactic acid treatment over 14 days of treatment (Fig. 8.6b). However, a negative effect was observed for treatment of samples for 1 day by conditioning and lactic acid. At 10 days, the lactic acid treated sample was considerably favoured over the conditioned sample, while at 14 days of treatment the conditioned sample was perceived to be higher than the lactic acid treated sample. The degree of chewiness of *serratus ventralis* was perceived to be higher in the lactic acid treated sample than conditioned samples for all times of treatment studied (Fig. 8.6c). At 14 days of treatment the conditioned sample appeared to be highly favoured over the lactic acid treated sample with respect to chewiness.

8.3.7 Tenderness

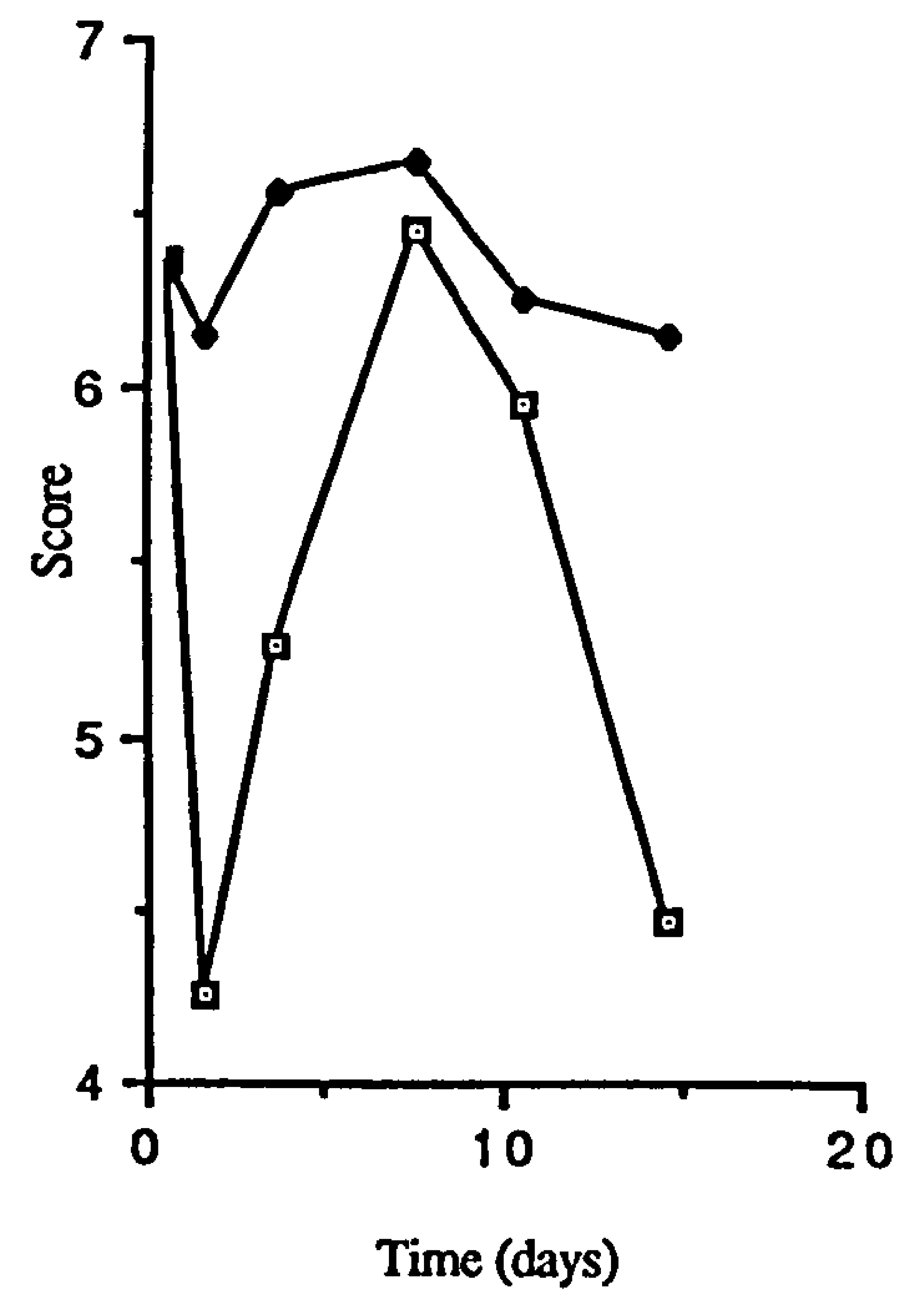
The CV of the judges score for tenderness were 15 % for *psoas major*, 11 % for *gluteus medius* and 28 % for *serratus ventralis*. Because of the relationship observed between resistance to first bite, ease of chewing and tenderness, the high degree of variability observed for the first 2 attributes in *serratus ventralis*, as expected, influenced tenderness. Unconditioned *psoas major* was perceived to be more tender than conditioned and lactic acid treated samples (Fig. 8.7a). In all, except 10 day treated samples, conditioned *psoas major* was favoured over lactic acid treated samples. Comparison of the graphs obtained for resistance to first bite (Fig. 8.5a) and ease of chewing (Fig. 8.6a) with tenderness



a



b



c

Fig. 8.6 Judges Mean Scores for Ease of Chewing of Conditioned (□) and Lactic Acid Treated (♦) Bovine Muscles (mean of 8 judges, for *psoas major* (a), mean of 10 judges for *gluteus medius* (b), and mean of 9 judges for *serratus ventralis* (c) and 3 replications).

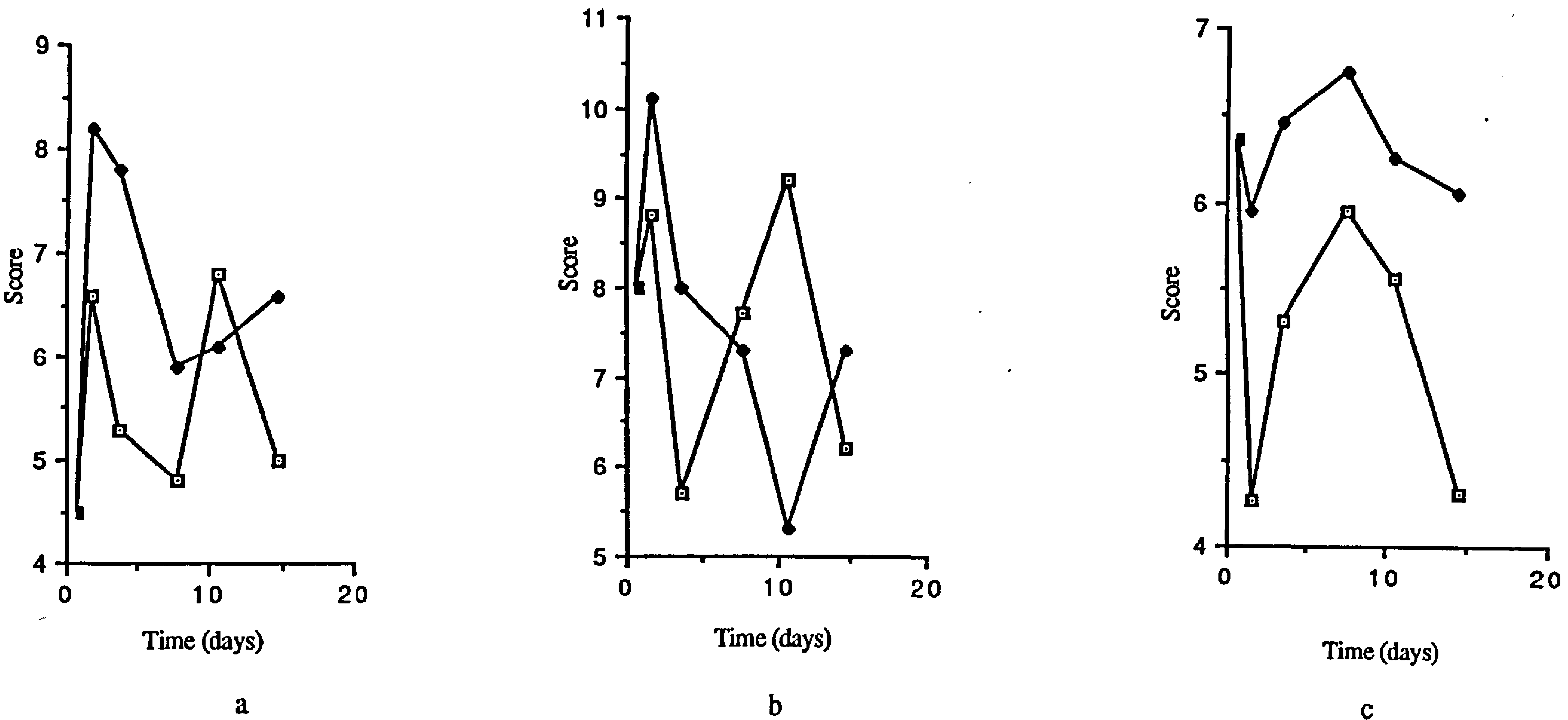


Fig. 8.7 Judges Mean Scores for Tenderness of Conditioned (⊡) and Lactic Acid Treated (♦) Bovine Muscles (mean of 8 judges, for *psoas major* (a), mean of 10 judges for *gluteus medius* (b), and mean of 9 judges for *serratus ventralis* (c) and 3 replications).

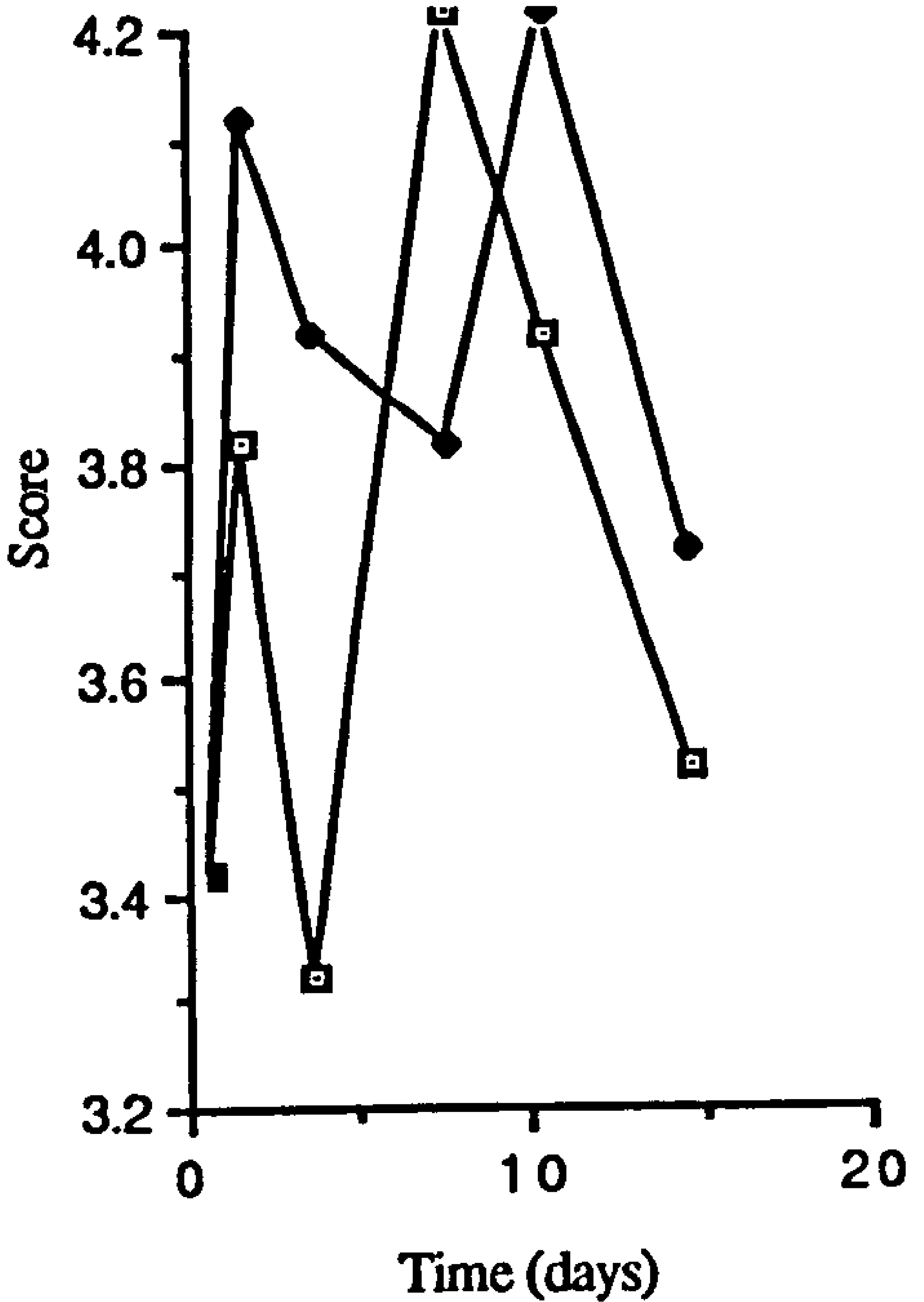
(Fig. 8.7a) of *psoas major* revealed a similar trend in all 3 attributes over 14 days of treatment, reflecting the relationship between these. Tenderness of *gluteus medius* generally increased over 14 days of treatment (Fig. 8.7b). However, a negative tenderizing effect was observed for treatment of *gluteus medius* for 1 day by lactic acid and conditioning. At 10 days the lactic acid treated sample was considered the most tender of all treatments while at 14 days the conditioned sample was again favoured over the lactic acid treated sample. Comparison of the results obtained for resistance to first bite (Fig. 8.5b) and ease of chewing (Fig. 8.6b) with tenderness (Fig. 8.7b) of *gluteus medius* again revealed very similar trends in all 3 attributes. Tenderness of *serratus ventralis* (Fig. 8.7c) was favoured in conditioned samples over lactic acid treated samples for all treatment times studied. Samples treated for 1 day were perceived to be relatively tender, with the conditioned sample indicating considerable tenderness over the lactic acid treated sample. At 14 days of treatment, the conditioned sample was perceived to be more tender than the lactic acid treated sample (Fig. 8.7c). Comparison of the graphs obtained for resistance to first bite (Fig. 8.5c) and ease of chewing (Fig. 8.6c) with tenderness (Fig. 8.7c) of *serratus ventralis* revealed a similar trend due to conditioning and lactic acid treatment with respect to all 3 attributes, reflecting the interaction of these 3 attributes.

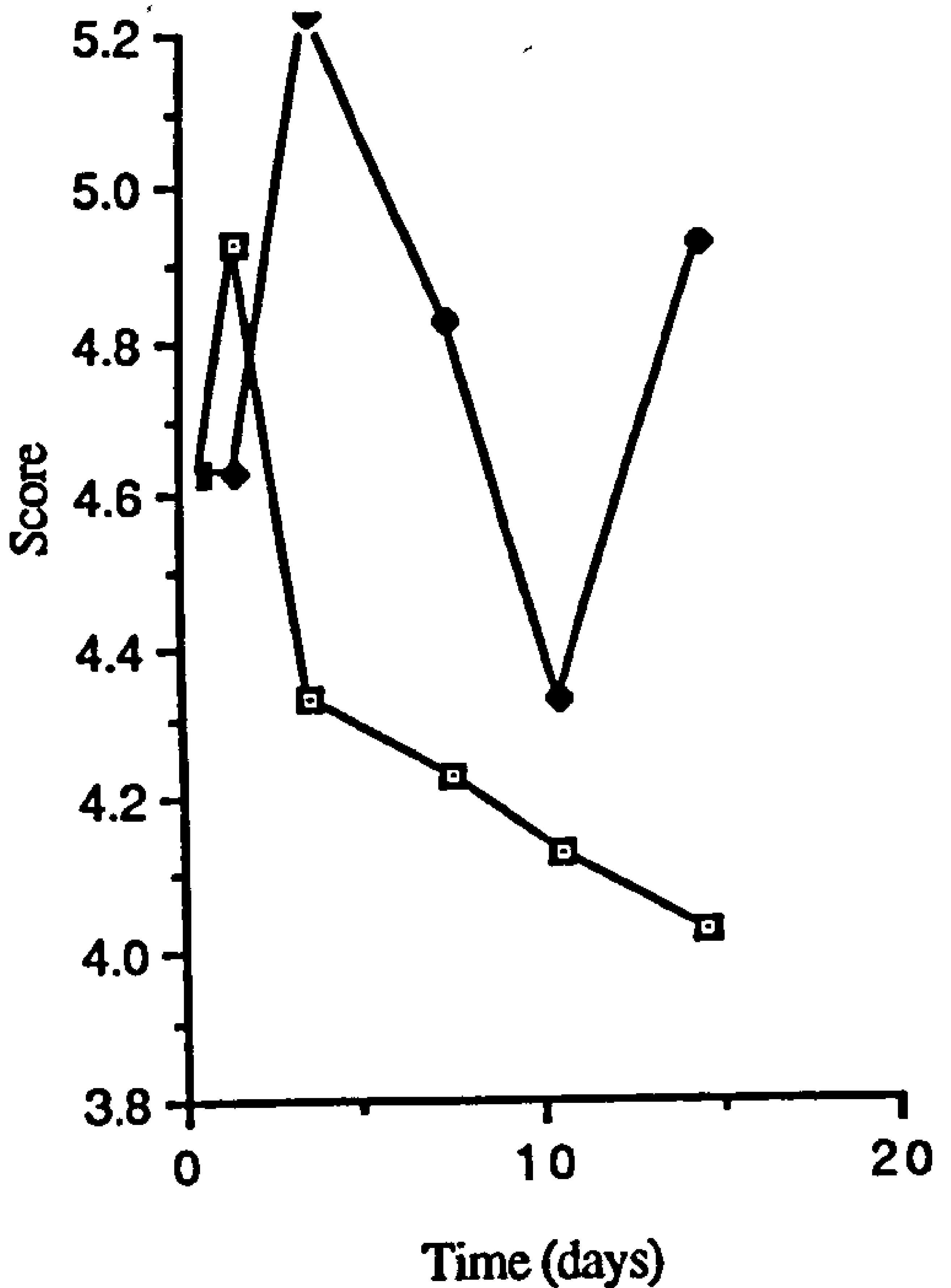
8.3.8 Colour

The CV of the judges scores for meat colour were 34 % for *psoas major*, 41 % for *gluteus medius* and 35 % for *serratus ventralis*. Assessed colour of lactic acid treated *psoas major* appeared less favourable at 1 and 3 days of lactic acid treatment than the conditioned samples Fig. 8.8a, while at 7 days of conditioning the lactic acid treated sample was favoured over the conditioned sample. Meat samples, conditioned for 10 and 14 days were favoured over the lactic acid treated samples for these times. Meat colour of *gluteus medius* was favoured in the lactic acid treated samples over the conditioned samples at 1 day of treatment (Fig. 8.8b). At all other times of treatment studied, however, colour of conditioned samples was favoured over lactic acid treated samples. Meat colour of *serratus ventralis*, conditioned for 1 day was favoured over the lactic acid treated sample. For all other times of treatment studied, lactic acid and conditioned samples showed similar meat colour (Fig. 8.8c).

8.3.9 Overall Acceptability

The CV of the judges scores for overall acceptability were 16 % for *psoas major*, 13 % for *gluteus medius* and 21 % for *serratus ventralis*. As this attribute attempts to encompass all 7 preceding attributes, it was to be expected that the CV of overall acceptability of *serratus*





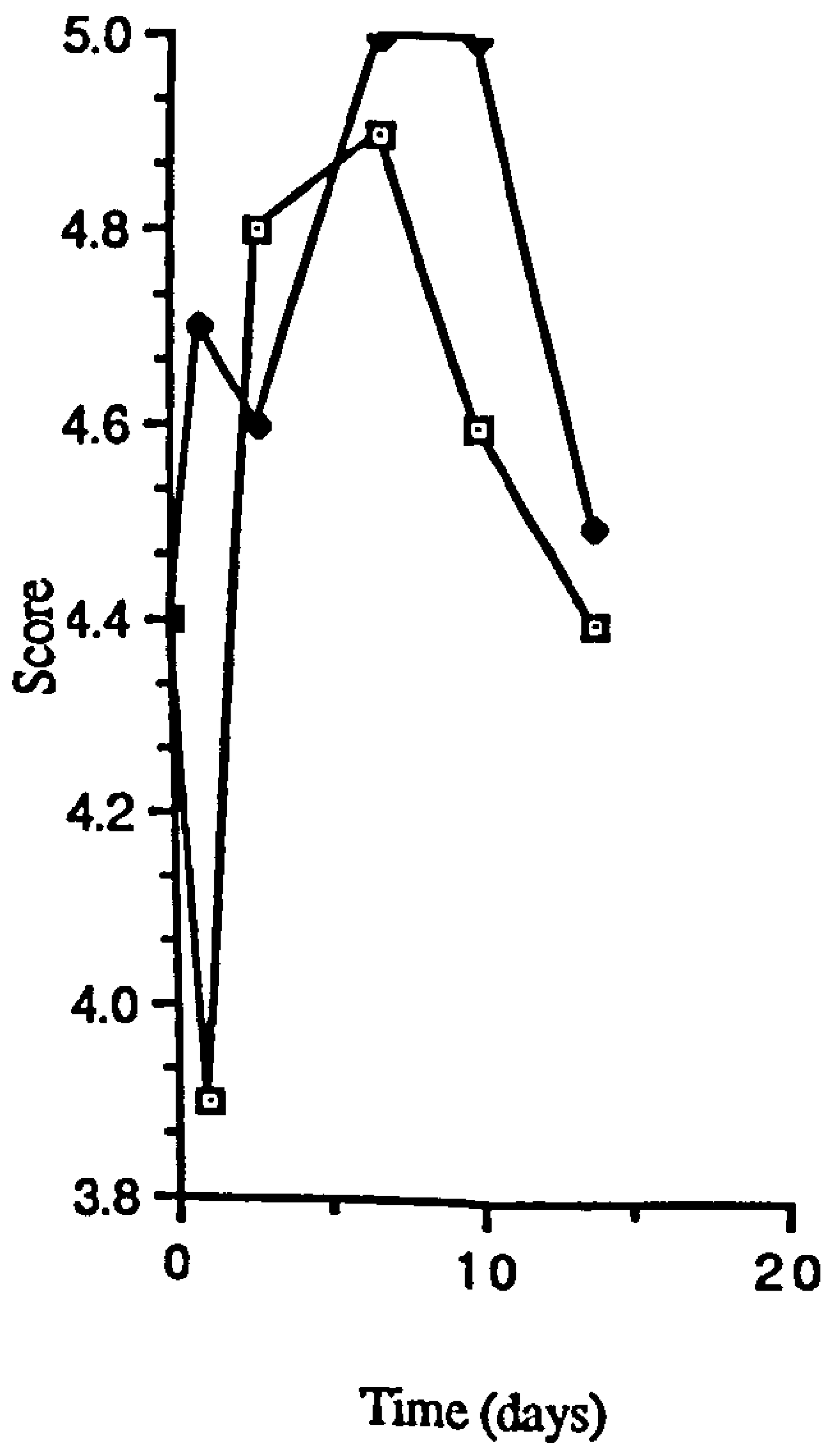


Fig. 8.8 Judges Mean Scores for Colour of Conditioned (⊡) and Lactic Acid Treated (♦) Bovine Muscles (mean of 8 judges, for *psoas major* (a), mean of 10 judges for *gluteus medius* (b), and mean of 9 judges for *serratus ventralis* (c) and 3 replications).

ventralis would be higher than that of either of the other 2 muscles studied. Overall acceptability of *psoas major* indicated a decrease due to conditioning and lactic treatment relative to the unconditioned sample (Fig. 8.9a). The 1 day lactic acid treated sample was perceived to be the lowest of all *psoas major* samples studied. After 14 days of treatment the conditioned sample was considered higher than the lactic acid treated sample. Overall acceptability of *gluteus medius* (Fig. 8.9b) was perceived to increase due to lactic acid treatment from 1 to 10 days. The 10 day lactic acid treated *gluteus medius* sample appeared to be highest (Fig. 8.9b) and was perceived to be considerably higher than the conditioned sample at this time. However, at 14 days of treatment, the conditioned sample was favoured over the lactic acid treated sample. Lactic acid treatment of *serratus ventralis* appeared to result in a less acceptable product at all times of treatment (Fig. 8.9c). *Serratus ventralis*, conditioned for 1 day was perceived to be highest of all samples studied.

8.3.10 Estimates of the Effects of Lactic Acid Treatment and Conditioning on Selected Sensory Attributes

Analysis of the data obtained from all sensory attributes for all 3 muscles, outlined above, seemed to indicate, in many of the cases studied, no beneficial effect from lactic acid treatment, prior to conditioning. Consideration of the results obtained from *serratus ventralis* for resistance to first bite (Fig. 8.5c), tenderness (Fig. 8.7c) and overall acceptability (Fig. 8.9c) suggested that the conditioned muscles were perceived to be higher than lactic acid treated samples and were, therefore, chosen as examples for further statistical analysis, to establish whether these organoleptic differences were of significance.

The estimates of the treatment effects and their convariance matrix were derived from an analysis of variance using the GLIM (Generalized Linear Model) package. Fig. 8.10 shows the differences between treatment effects (lactic acid and conditioning treatments) on resistance to first bite of *serratus ventralis* for the same number of days, together with a 95 % confidence interval for differences between treatments at a given number of days. Where the interval contains zero, it can be assumed that the effects are not signficantly different from each other at the 95 % level and vice versa. The results indicated that the lactic acid treated samples were significantly ($p < 0.05$) more resistant to first bite at 3 and 14 days of treatment than the conditioned samples, treated for equivalent times. No significant difference was observed, however, between lactic acid treated and conditioned samples for 1, 7 and 10 days of treatment (Fig. 8.10). Similar analyses were performed on the data obtained for tenderness (Fig. 8.11), overall acceptability (Fig. 8.12) and odour

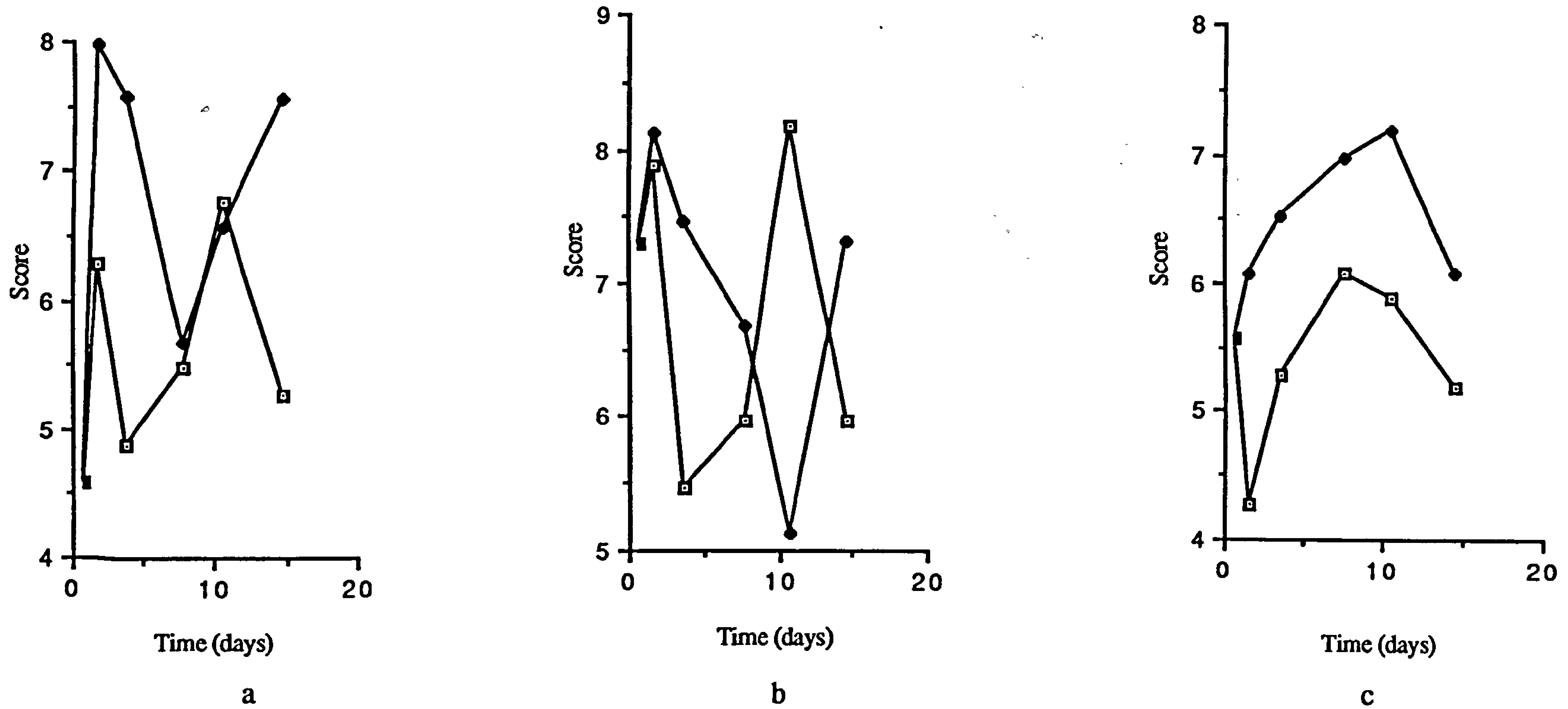


Fig. 8.9 Judges Mean Scores for Overall Acceptability of Conditioned (▣) and Lactic Acid Treated (♦) Bovine Muscles (mean of 8 judges, for *psoas major* (a), mean of 10 judges for *gluteus medius* (b), and mean of 9 judges for *serratus ventralis* (c) and 3 replications).

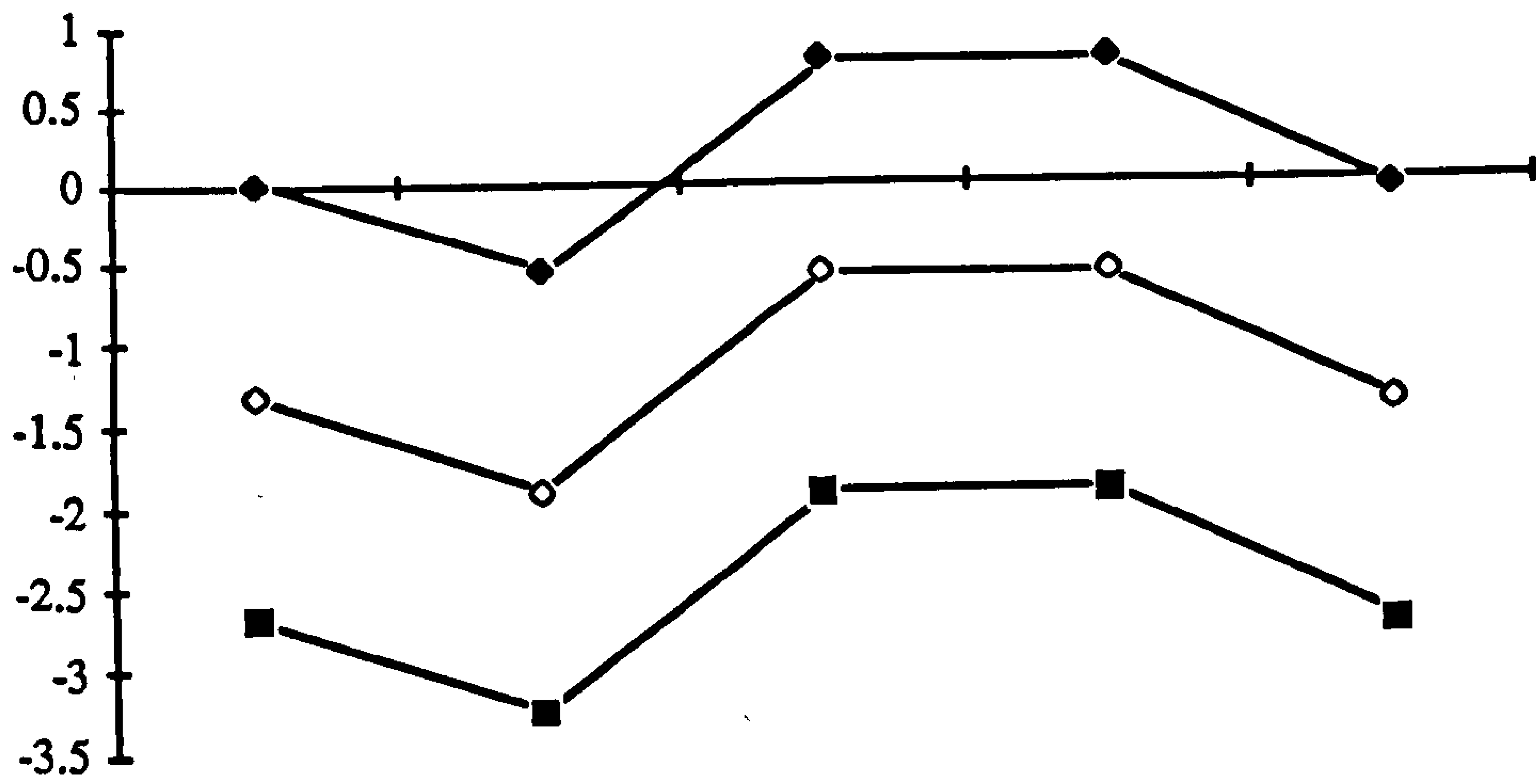


Fig. 8.10 Resistance - *Serratus Ventralis*

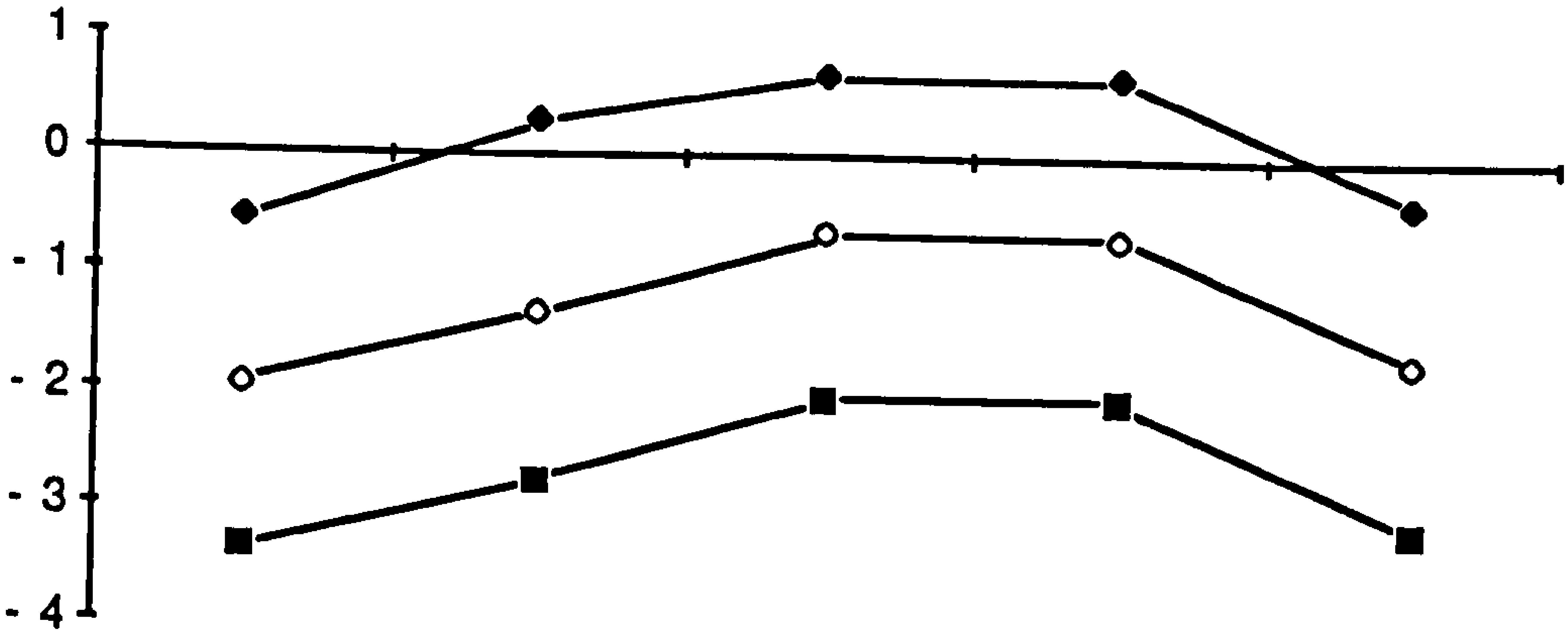


Fig. 8.11 Tenderness - *Serratus Ventralis*

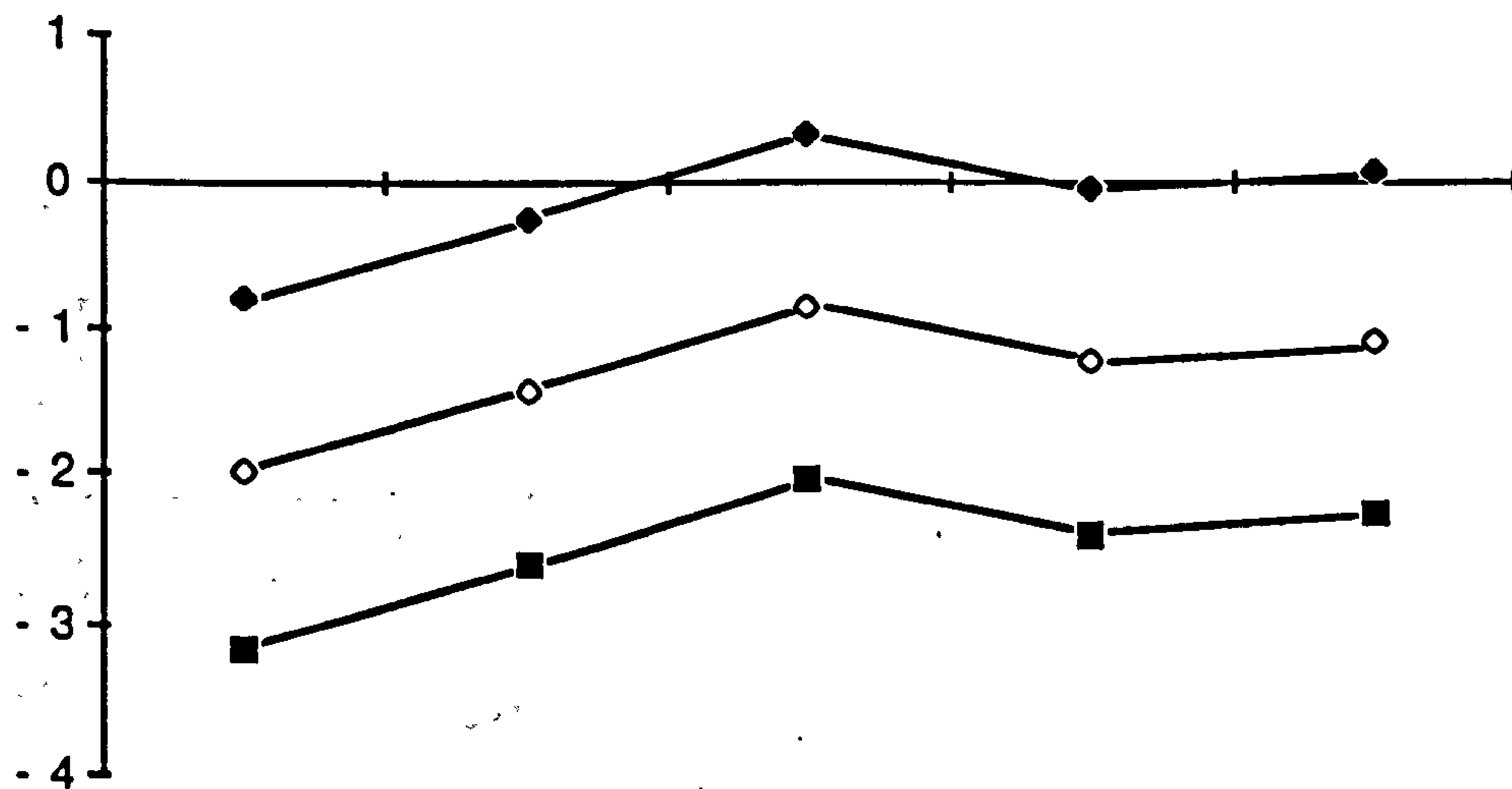


Fig. 8.12 Overall Acceptability - *Serratus Ventralis*

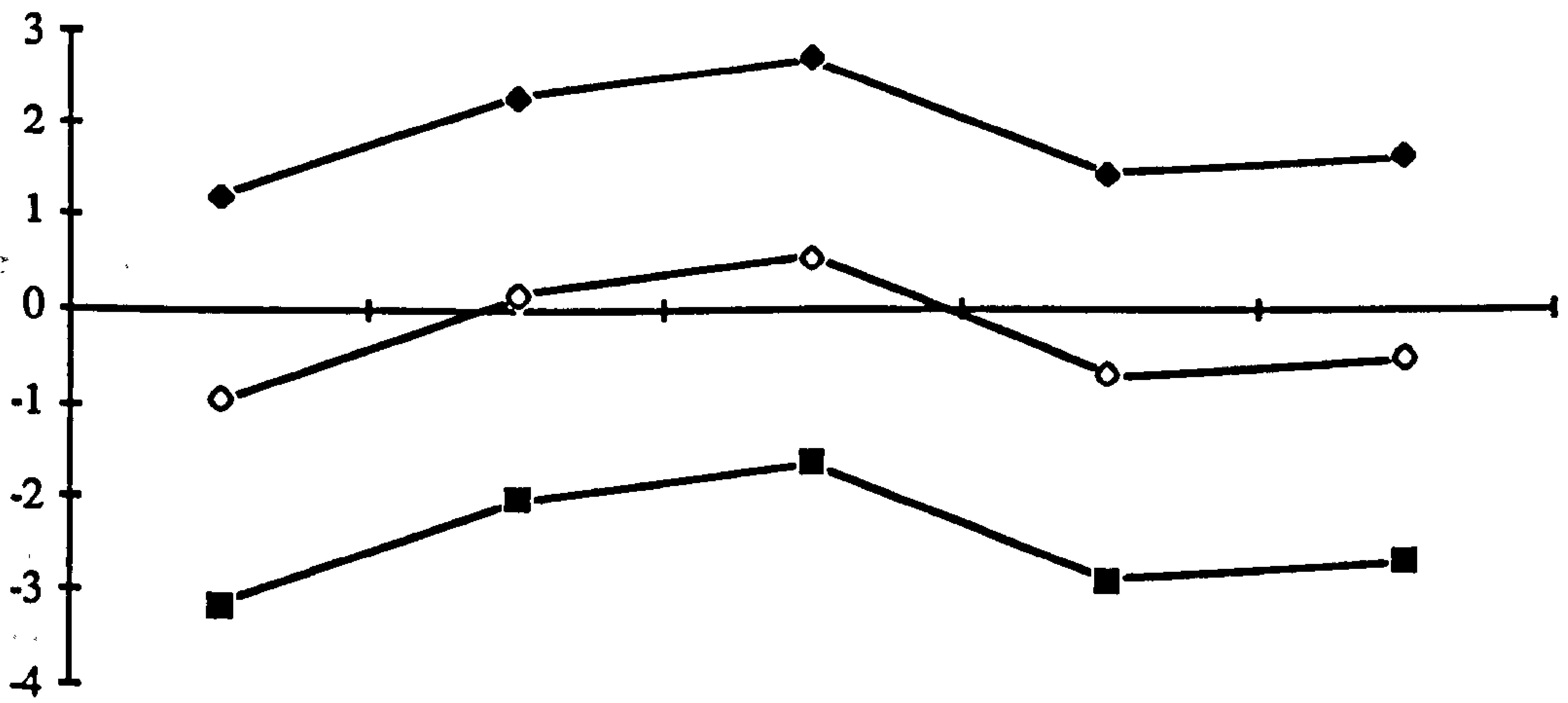


Fig. 8.13 Odour - *Serratus Ventralis*

(Fig. 8.13) of *serratus ventralis* to establish differences between treatment effects on these attributes for the same number of days. The results obtained for tenderness indicated that *serratus ventralis* meat samples, conditioned for 1 and 14 days were significantly higher ($p < 0.05$) than lactic acid treated samples, conditioned for equivalent times (Fig. 8.11). No significant differences between treatment effects on tenderness of this muscle were observed on samples, treated for 3, 7 and 10 days. The results obtained for overall acceptability of *serratus ventralis* indicated that conditioning for 1, 3 and 10 days produced a significantly ($p < 0.05$) more acceptable product than lactic acid treatment for these times (Fig. 8.12) while no signficant effect was observed on overall acceptability of this muscle, between conditioning and lactic acid treatments for 7 and 14 days. The differences in odour, of *serratus ventralis*, resulting from conditioning and lactic acid treatments were not signficant over the 14 days studied (Fig. 8.13).

Finally, to establish whether there were overall differences between lactic acid treated and conditioned samples for the 8 sensory attributes, the average effect due to lactic acid treatment was compared with the average effect due to conditioning, over all days (Fig. 8.14). As indicated above for Figs. 8.10 to 8.13, where the interval contains zero, it can be assumed that the effects are not significantly different from each other at the 95 % level and vice versa. The results indicated that resistance to first bite, tenderness, ease of chewing and overall acceptability of conditioned *serratus ventralis* samples were significantly higher ($p < 0.05$) than lactic acid treated samples, while the differences between all other attributes studied were not significant. This result was to be expected from the graphs of mean scores for these attributes from *serratus ventralis*, while those obtained from the other muscles studied would not be expected to indicate significant differenes between lactic acid and conditioning treatments. Furthermore, the inter-relationship between resistance to first bite, tenderness and ease of chewing and the strong influence of these 3 attributes on overall acceptability, together with the high correlation coefficients obtained between these 4 descriptive terms suggest that they might be perceived similarly by the judges.

8.4 DISCUSSION

Variability among the judges scores for the sensory attributes studied was highest for *serratus ventralis* in 75 % of the cases examined, when compared to variability of scores for *psoas major* and *gluteus medius*. Of the eight attributes studied, scores for colour and odour showed the highest variability for all three muscles examined. Acceptability is strongly related to personal preferences and individuals' preferences for 'rare', 'medium' or 'well done' steaks may have influenced their perception of 'acceptable' appearance.

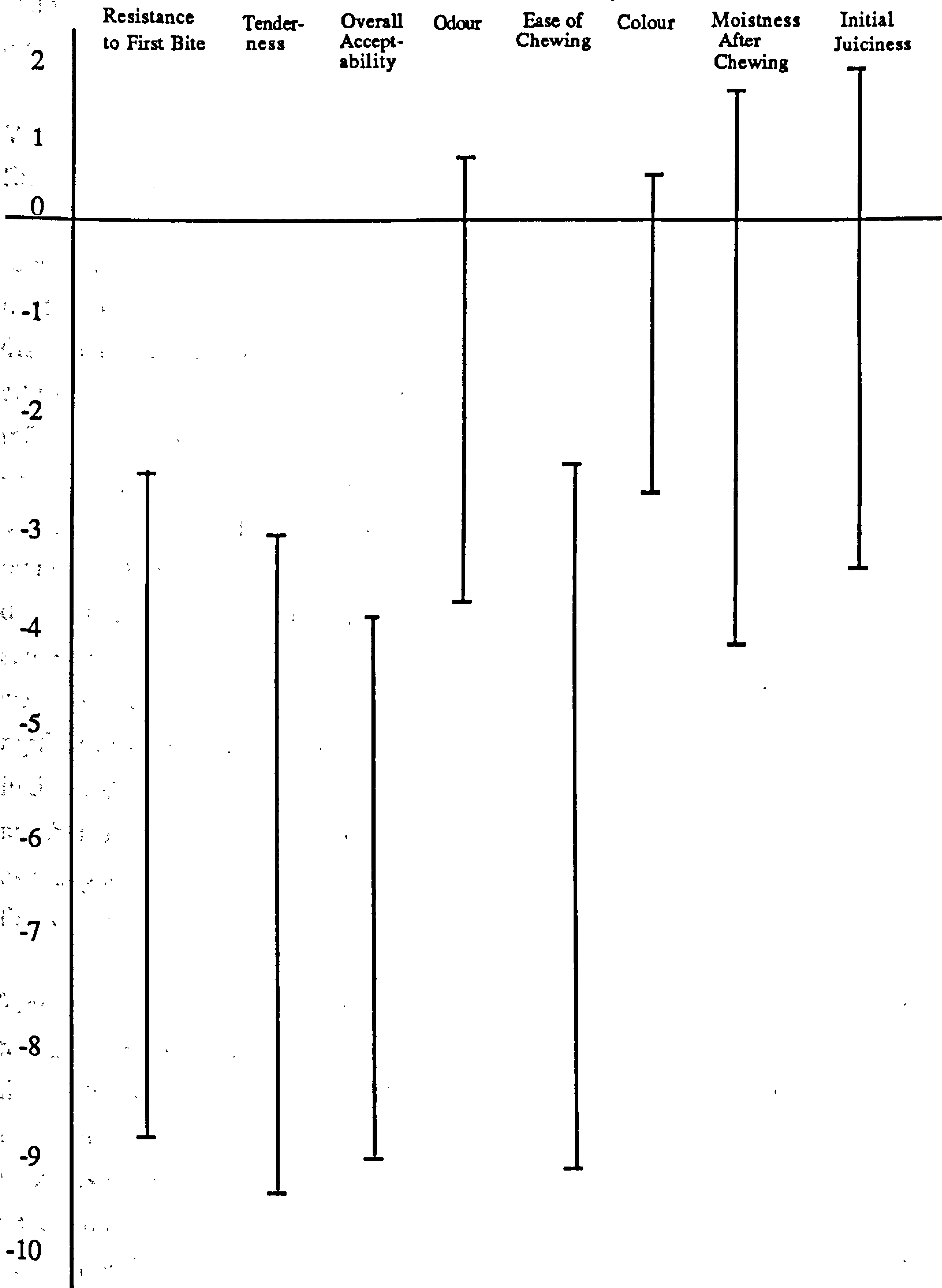


Fig. 8.14 Overall Effects of Treatments on Serratus Ventralis

Personal preferences do not tend to alter within a short time period, whereas an individuals perception of the degree of a particular attribute such as odour and juiciness may change, particularly in the absence of standards with which to anchor their judgements. In addition, odour is a complex attribute and may not have been adequately defined, while the olfactory response may also have become fatigued.

Variability among the scores of those factors relating to texture of the meat (resistance to first bite, ease of chewing, tenderness and hence, overall acceptability) was higher for *serratus ventralis* than for the other muscles studied. The high variability of the judges score for these attributes in *serratus ventralis* may have resulted from the higher content of randomly located connective tissue within this muscle. However, it was decided not to discard any judges' scores, as no judge was consistently poor in scoring all attributes from all muscles, and therefore discarding any judges' data would result in an overall loss of information. No significant interaction was found between the treatments of the meat (lactic acid treatment and conditioning) and the judges, which indicated that the effect of treatment of the meats was independent of any particular judge, and this interaction was therefore eliminated. The presence of an interaction between these variables would lead to difficulty in interpretation of results. Attempts to eliminate objective error due to the sequence of presentation of samples was achieved by random distribution of samples on each day, such that the sequence of presentation differed on each day of the study. Three replications were used in this study to evaluate the response consistency of the panel and individual judges, but higher numbers of replications are desirable. The higher the number of replications the more accurate is the result. Stone et al. (1974) suggested that as many as sixteen replications were necessary for quantitative descriptive analysis, but this value was reduced to four in a later publication (Stone et al. 1980).

Training of the judges and conducting the experiment at the same time each day attempted to eliminate the subjective error due to differences between what the judges perceived to be the qualities of the product and what they actually were. Schiffman et al. (1981) recommended the use of an undifferentiated line scale rather than a scale structured with numbers or descriptive terms, because judges lose sight of the task at hand while they debate the meanings of words like 'somewhat' or 'fairly'. In addition, many subjects feel uncomfortable with segmenting the line with words. The questionnaire was designed to assess the affect on textural properties relating to tenderness of lactic acid treatment of meat prior to conditioning. In addition, it was necessary to identify the effect of lactic acid treatment on attributes other than texture, which play an important role in overall acceptability of meat, e.g. colour and odour.

The pattern which emerged from analysis of odour scores from *psoas major, gluteus medius* and *serratus ventralis* due to conditioning and lactic acid treatment from 1 to 14 days revealed that this attribute was not significantly different due to the treatments The odour of the 14 day conditioned *gluteus medius* and *serratus ventralis* samples was favoured over the conditioned samples while for *psoas major*, the lactic acid treated sample was favoured over the 14 day conditioned sample.

The scores obtained for initial juiciness of the three muscles suggested that this attribute was not significantly affected by lactic acid treatment. The most favoured of the *gluteus medius* sample for this attribute was the 10 day lactic acid treated sample. Initial juiciness of the 14 day conditioned *psoas major* and *gluteus medius* samples was perceived to be higher than lactic acid treated samples while for *serratus ventralis* the 14 day lactic acid treated sample was favoured over the conditioned sample. High correlation coefficients were found between initial juiciness and moistness after chewing, indicating a strong relationship between these 2 attributes. The most favoured was the 10 day lactic acid treated sample from *gluteus medius*.

The overall effect of treatment of *serratus ventralis* for moistness after chewing from 1 to 14 days by conditioning and lactic acid was not significant. The most favoured samples for moistness after chewing were the unconditioned *psoas major*, the 10 day lactic acid treated *gluteus medius* and the 1 day conditioned *serratus ventralis*. At 14 days of treatment, the conditioned sample was favoured over the lactic acid treated sample for all 3 muscles. However, the differences perceived for this attribute from *serratus ventralis* were negligible.

During consideration of the structure of the experiment, it was decided to use untreated meat as a control, rather than meat injected with water or saline solutions as it was difficult to predict the effects of any solution on the texture of the meat. However, it is interesting to note in the light of this, that the meat injected with lactic acid to a final weight 10 % greater than original meat weight was not perceived to be initially more juicy or more moist after chewing than uninjected meat. This may indicate that the excess liquid was lost on cooking while inconsistencies of the judges' scores may also have led to this result.

Results from scores of resistance to first bite, ease of chewing and tenderness were found to be highly correlated. These 3 descriptive terms relate to the texture of the meat which would be expected to be strongly influenced by the mechanical status of the connective tissue. Resistance to first bite was significantly affected by lactic acid and conditioning treatments. This attribute was favoured in the 10 day lactic acid treated *psoas major* and

gluteus medius muscles over muscles conditioned for 10 days. Conditioned *serratus ventralis* was favoured over lactic acid treated samples at all times of treatment studied. In all 3 muscles studied resistance to first bite was favoured at 14 days of treatment in conditioned samples over to the lactic acid treated samples.

The graphs obtained from scores of ease of chewing of the 3 muscles were similar to those obtained for resistance to first bite for each muscle. Ease of chewing was significantly affected by conditioning and lactic acid treatment of *serratus ventralis*. The conditioned sample was found to be significantly ($p < 0.05$) higher than the lactic acid treated sample with respect to ease of chewing. At 14 days of treatment, conditioned samples were favoured over the lactic acid treated samples for all 3 muscles studied. The difference between the 2 treatments at 14 days with regard to ease of chewing was greatest for *serratus ventralis*.

The descriptive term, tenderness, encompasses the 2 preceding terms, resistance to first bite and ease of chewing, and the graphs obtained from scores of tenderness of the 3 muscles corresponded to those obtained for the 2 preceding descriptive terms for each muscle. Conditioned *serratus ventralis* muscles were perceived to be significantly ($p < 0.05$) more tender than lactic acid treated samples. The unconditioned *psoas major* was perceived to be the most tender of all treatments to *psoas major*. This result is surprising in the light of previous findings that conditioning has a positive effect on tenderness as discussed in Chapter 1 (section 1.5.4). Inter-muscle variability may have accounted for this discrepency. However, for *gluteus medius* and *serratus ventralis*, tenderness of lactic acid treated and conditioned muscles increased from the unconditioned to the 14 day treated samples. In all 3 muscles examined, tenderness of the conditioned samples at 14 days was favoured over the lactic acid treated samples. The difference was found to be greatest between the 2 treatments at 14 days for *serratus ventralis*.

It was considered important to monitor meat colour over the 14 days of treatment, to identify any adverse changes which may have occurred due to lactic acid treatment of the muscles. It was found that meat colour was not significantly affected by conditioning and lactic acid treatment. However, in all 3 muscles studied, meat colour was found to be favoured in the 14 day conditioned sample over the lactic acid treated sample for this time.

The descriptive term, overall acceptability used in this study was designed to take into account all attributes of the meat. Overall acceptability was significantly affected by lactic acid treatment and conditioning of *serratus ventralis*. Considering the high correlations obtained between this parameter and others affected by treatment of all muscles (ease of

chewing and tenderness) this result was to be expected. Unconditioned *psoas major* was found to be the most acceptable of all treated *psoas major* muscles. Muscles frozen immediately after slaughter of the animals were termed unconditioned muscles as described in Chapter 2 (section 2.3). As discussed in Chapter 1 (section 1.5.2.2), an exceeding fast rate of ATP breakdown and of rigor mortis onset ensues on thawing, leading to highly undesirable meat. Therefore, this result is contrary to previous opinion on the effects of conditioning on meat and may be explained by variability of the judges scores and by inter-muscle variability. Conditioning of *gluteus medius* and *serratus ventralis* for 14 days resulted in a more acceptable product than unconditioned muscles. The 10 day lactic acid treated *gluteus medius* muscle was considered the most acceptable while 10 day of conditioning produced the least acceptable product for *gluteus medius* muscle. However, in all 3 cases examined, the 14 day conditioned sample was found to be more acceptable than the 14 day lactic acid treated muscles.

Consideration of the results obtained in this study suggest that panel score and inter-muscle variability may have had an important effect on the actual results. Amerine et al. (1965) suggested the use of small trained sensory panels for production of less variation in replicated judgements than large untrained panels, for measuring differences between samples. The voluntary nature of the taste panel employed in this study may have had an important bearing on the outcome. A preliminary training session was undertaken with the taste panel volunteers where all aspects of the study to be undertaken were discussed. However, a more rigorously trained professional meat taste panel may have produced more consistent results. In addition, a higher number of replications of the taste panel study may also have produced more accurate results. Inter-muscle variability resulting from extrinsic and intrinsic factors, including sex, age, plane of nutrition, inter-animal variability and pre-slaughter manipulation, as discussed in Chapter 1 (section 1.2.1), may have added to variation within the results for each muscle type studied. It would be more desirable to use one muscle of each type for all treatment times. However, the quantities of meat required for this study necessitated the use of a number of muscles.

The results of the sensory study suggest that no benefit was gained from pre-rigor lactic acid injection of muscles. The results presented in Chapter 7, however, suggest that pre-rigor lactic acid injection had a significant role to play in solubilization of perimysial collagen which might have been expected to result in a more tender product. It may be concluded that the effect of treatment of pre-rigor muscles with 0.1 M lactic acid on collagen degradion may be sufficiently small not be be perceived by the taste panel. However, inconsistencies of the judges' scores may have led to an inaccurate result in this study.

Gault (1984) found that marinading, upon which the concept of lactic acid injection is based, resulted in increased meat tenderness from 0.01 N to 1.0 N acetic acid. Maximum swelling and tenderizing benefits of marinading were achieved when meat pH had reached 3.44. In this study, a meat pH value of 3.46 was reached at 3 h after injection of 0.8 M lactic acid (Chapter 7) at which time meat colour was deemed unacceptable. The objective of this study was aimed at producing meat tenderizing effects without adverse colour changes which would result in an unacceptable appearance. It was found that 0.2 M lactic acid treatment caused unacceptable meat colour changes at 1 h after injection. Future work on the effects of treatment of 0.1 M to 0.2 M lactic acid are required, to establish whether signficiant tenderizing benefits are attained from pre-rigor muscle lactic acid injection, without the adverse colour, flavour and odour changes associated with the process of marinading.

CHAPTER 9

FINAL DISCUSSION AND CONCLUSIONS

In this study, initial investigation of the gross connective tissue fraction of bovine muscle indicated that this fraction was significantly affected by conditioning. Further studies, using various techniques of solvent extraction, allowed the identification of suitable extraction methods for 'dissection' of intramuscular connective tissue into its subfractions- the perimysium and endomysium. It was found by examination of CNBr peptides, resolved by SDS-polyacrylamide gel electrophoresis, that SDS and urea washing yielded clean connective tissue preparations. In addition, these reagents were efficient extractants of solubilized perimysial collagen. These methods therefore were used to investigate the effects of conditioning on both perimysial and endomysial fractions from a range of beef muscles.

Yields of solubilized perimysial material were significantly lower ($p = 0.096$) for unconditioned muscles than for conditioned muscles. The collagen component of the perimysial fraction was also significantly affected by conditioning. The amount of solubilized perimysial collagen from unconditioned muscles was significantly lower ($p = 0.015$) than from conditioned muscles, the latter being approximately three times greater than the former. This is the first categorical deomonstration of the proteolysis of collagen in meat during conditioning. However, only non-dialyzable collagen was quantified in these studies and the lost dialyzable fraction may have indicated that significantly more collagen was solubilized due to conditioning than that observed in this work.

Psoas major, the highest quality muscle investigated (Dransfield, 1977) gave the highest yield of solubilized collagen due to conditioning and other good quality muscles indicated a similar trend. However, some of the poor quality muscles studied revealed similar increases on conditioning. It was therefore concluded from the results of this study that there was no correlation between the yields of proteolyzed soluble perimysial collagen due to conditioning and known muscle quality. However, this does not take account of the relative amounts of dialyzable collagen fragments which may have been higher in more tender muscles. In terms of the integrity of the residual insoluble collagen remaining after conditioning, SDS-polyacrylamide gel electrophoresis showed no major changes in the major peptide bands due to conditioning, suggesting that any damage incurred to the insoluble perimysial fractions remaining after conditioning must be minor.

The application of the urea extraction method to endomysial preparation proved unsuccessful. However, application of the method of Light & Champion (1984) for endomysial extraction which employed SDS in the procedure, was successful for investigation of conditioning effects on this connective tissue fraction. This study revealed that higher yields of solubilized endomysial material resulted from conditioning. This method proved much more efficient for solubilization of endomysial material than the urea and sequential methods previously employed for endomysial extraction. The collagen component of solubilized endomysial fractions was also found to be increased in all muscles examined due to conditioning. High quality muscles examined in this study revealed high yields of solubilized endomysial collagen before and after conditioning relative to the poorer quality muscles studied. However, the percentage collagen solubilized by conditioning was lower for higher quality muscles. The fact that there were variations in the extent of perimysial and endomysial collagen solubilization suggests that the proteolytic process is random and uncontrolled. No correlation was therefore apparent between high incidence of proteolyzed soluble endomysial collagen due to conditioning and known muscle quality, an observation previously apparent for perimysial collagen.

Future work on the solubilization of intramuscular connective tissue collagen during conditioning is required to gain a complete understanding of the nature of the solubilized perimysial and endomysial collagen. This may be achieved by comparison of all solubilized collagen (dialyzable and non-dialyzable) from unconditioned with conditioned muscles. Direct acid hydrolysis of solubilized fractions, enrichment of acid and neutral amino acids by ion-exchange chromatography and quantification of hydroxyproline in the total soluble fraction would overcome the problems of loss of the dialyzable fraction encountered in this study. Collection and enrichment of the dialyzable fraction, obtained by the perimysial and endomysial extraction procedures outlined in this thesis and characterization of the collagen component of this would furnish further knowledge on the extent of proteolysis during conditioning. SDS-polyacrylamide gel electrophoresis and immunoblotting techniques with specific anticollagen antibodies could be applied to determine which collagen types are solubilized during conditioning.

Further studies on the extent of damage to endomysial collagen during conditioning with model conditioning systems *in vitro* using cathepsins are required for verification of damage to type III collagen during conditioning. This could be achieved by incubation of insoluble endomysium obtained from unconditioned muscle with cathepsin preparations and electrophoretic assessment of the damage to types I and III collagen after various extents of treatment with cathepsins. Study of conditioning effects on endomysial

collagen using model protease systems and comparison of the results with *in situ* effects due to normal conditioning should provide further information on changes to this fraction during conditioning. The work carried out in this study examined effects of conditioning on endomysial types I and III collagen. In addition, it would be of interest to investigate the effects of conditioning on type IV collagen from endomysium.

Type IV collagen can be released from purified endomysium by pepsin digestion (Light & Champion, 1984). It would be interesting to compare yields of endomysial type IV collagen obtained from unconditioned muscles with yields from conditioned muscles. If type IV collagen is damaged on conditioning, then pepsin treatment would cause excessive degradation and lower yields would be expected from conditioned samples. The damage incurred by endomysial type IV collagen may also result in altered peptide maps on electrophoresis and could lead the determination of the activity of cathepsins against this collagen type. Problems encountered in basement-membrane research arise from the fact that in normal tissues like placenta, kidney and muscle, basement membrane components are highly insoluble and are present in only very small quantities (Kefalides et al., 1979). Suitable model systems for *in vitro* studies of basement membrane collagen have included the mouse EHS (Engelbreth-Holm-Swarm) tumour (Orkin et al., 1977) which produces large amounts of basement membranes during subcutaneous propagation and rat yolk sac tumours (Wewer, 1982; Martinez-Hernandez et al., 1982). Incubation of these structures with cathepsins in a model conditioning system and elucidation of the damage and quantification of the damage incurred to type IV collagen would furnish information on the susceptibility of this protein to cathepsin action. 2-D NEPHGE investigation of the model conditioning system and comparison with the real *in situ* situation may also provide further evidence of catheptic action on type IV collagen during conditioning of meat.

In addition to type IV collagen, basement membranes may be composed of up to 50 individual proteins (Timpl & Dziadek, 1986). Evidence for the effects of conditioning on the non-collagenous components of basement membranes is lacking. A number of histochemical techniques have been utilized to enhance the visualization of basement membranes. Periodic acid Schiff (PAS) reagent or silver stains have been used because they reveal a heavily stained linear structure in the basement membrane region. These stains are not specific for the basement membrane, however, and what they identify at the light microscopic level include part of the adjacent connective tissue reticular fibres, microfibrils and ground substance (Martinez-Hernandez & Amenta, 1983; Vracko, 1982). A more sensitive technique is electron microscopy. The pattern and electron density of the basement membrane zone depends on the staining methods utilized. Cytochemical stains and cationic dyes including alcain blue, lysozyme and ruthenium red (Caulfield &

Farquhar, 1976; Caulfield, 1978; Kanwar & Farquhar, 1979) have been used successfully to label anionic sites within basement membranes, while tannic acid enhances the contrast of the basement membranes (Simionescu & Simionescu, 1976). Electron microscopic comparisons of basement membrane isolated from unconditioned muscle with conditioned muscles, may reveal alterations to muscle basement membrane components during conditioning of meat. This work could be extended to investigate the extent of damage at the perimysial/endomysial junction by using frozen sections of whole meat samples.

Conditioning resulted in alterations to the insoluble endomysial collagen fraction. New peptide material was observed on the one-dimensional CNBr peptide maps obtained from insoluble endomysium from *gastrocnemius* and *supraspinatus* . However, no alterations in the CNBr-peptide maps obtained from *psoas major* and *extensor capri radialus* were observed. The endomysium is that connective tissue fraction representing the first collagenous barrier and line of attack to liberated lysosomal cathepsins during the conditioning process and as such, may be expected to be significantly altered during conditioning. The fact that alterations to the insoluble endomysial fraction due to conditioning were observed by one-dimensional SDS-polyacrylamide gel electrophoresis of endomysial, but not perimysial collagen, obtained from some muscles, might suggest more extensive proteolysis of this connective tissue fraction due to conditioning. However, it was unclear whether the new peptide material evident on endomysial CNBr-peptide maps was collagenous. Ratios of types I : III collagen obtained from insoluble endomysial fractions were found to increase on conditioning. These results indicate that type III collagen is preferentially destroyed during the conditioning process. Bailey et al. (1979) found a correlation between a higher content of type III collagen and toughness, while Deethardt & Tuma (1971) suggested that type III collagen fibres were more resistant to cooking than other collagenous fibres. However, the results of Wu et al. (1981) suggested that type III collagen is more resistant to proteolytic attack than type I collagen, while the results of the present study seem to indicate that type III collagen is more susceptible to proteolysis. Type III collagen from human skin becomes more resistant to pepsin degradation with age (Lovell et al., 1987), while in young humans it was consistently solubilized by pepsin. In the present study, it was concluded that it may be the location of type III collagen which resulted in the high susceptibility of the molecule to proteolysis during conditioning. The type III collagen triple helix is absent from the surface of mature large diameter fibrils (Nowack et al., 1976; Lovell et al., 1987) but was observed on fibrils of small diameter, suggesting that, on maturation the type III collagen molecule becomes buried within the fibril. The collagen used in the present study was obtained from young animals, therefore its location to the exterior of the fibril may have resulted in increased susceptibility to proteolysis during conditioning.

Immunofluorescent localization of types I, III, IV and V collagen as previously described (Bailey et al., 1979) in perimysium and endomysium obtained from unconditioned and conditioned muscles and localization of types I and III and I and V cofibres may provide the necessary evidence of alterations to specific collagen types during conditioning.

Time course studies of the effects of pepsin and spleen cathepsin preparations on degradation of purified perimysium from unconditioned muscle indicated the intractable nature of the residual insoluble perimysial matrix remaining after conditioning. Solubilization studies on perimysium indicated that the effect of normal conditioning observed *in situ* can be mimicked *in vitro* by cathepsin treatment. However, subtle proteolytic damage may not be detected by one-dimensional SDS-polyacrylamide gel electrophoresis techniques at this time. Severe treatment of insoluble perimysium by cathepsin preparations resulted in changes to the major peptide bands, visible as broadening and 'fuzziness' of bands, decreased staining intensity, loss of high molecular weight material and a significant reduction in quantity of all peptides when compared to untreated perimysium. 2-D electrophoresis, employing NEPHGE-SDS indicated that cathepsin treatment of insoluble perimysium for 24 h resulted in loss of several peptide spots and appearance of at least one new spot when compared to the unconditioned material. The model system described here could be used for further studies of catheptic action on bovine connective tissue using the immunofluorescent techniques mentioned above to establish the susceptibility of types I, III, IV and V collagen to catheptic action *in vitro* and extension of this observation to the *in situ* events in meat.

Insoluble perimysial collagen obtained from conditioned muscle indicated an altered peptide pattern, when compared to the unconditioned material. Furthermore, the results indicated a link between *in situ* conditioning effects and cathepsin treatment *in vitro* of insoluble perimysial collagen, evidenced as loss of 2 common peptides from conditioned and cathepsin treated insoluble perimysium, providing direct evidence of cathepsin action on intra-muscular collagen during conditioning. The 2-D protease system using secondary chymotrypsin digestion showed reproducible evidence for the effect of conditioning on the high molecular weight peptides of the insoluble collagenous matrix, where enzymic 'clips' are known to occur in the telopeptide regions of the collagen molecule (Etherington & Evans, 1977; Etherington, 1980). This is further good evidence for the role of cathepsins in meat collagen degradation during conditioning. In addition, the high molecular weight aggregates formed from the C-terminal end of the collagen molecular are involved in stabilizing mature matrix collagen (Light & Bailey, 1980b, c), therefore, alterations to this region would be expected to have a significant effect on the mechanical status of connective tissue collagen. The polymeric peptide poly $\alpha 1CB6$,

contains the mature collagen cross-link (Light & Bailey, 1980b, c). Muscle cathepsins act on the telopeptide regions of the collagen molecule, therefore, the nature of polyα1CB6 isolated from unconditioned muscles would be expected to differ greatly from conditioned muscles.

Alterations to perimysial and endomysial collagen were observed due to conditioning, evident as the appearance of many additional peptides resulting from secondary chymotrypsin digestion of the high molecular weight material. Generation of new peptide material in the MW range 40 000 - 50 000 was observed for both connective tissue fractions. In addition, it was shown that conditioning resulted in loss of at least 2 peptides from perimysial collagen, due to conditioning. These results further highlight the role of conditioning in degradation or depolymerization of high molecular weight material and suggest the need for future work on this region. Peptide mapping and partial sequencing of fragments isolated from insoluble collagen from conditioned meat would be expected to reveal alterations due to catheptic action on these regions during conditioning. In addition, HPLC analysis of the solubilized peptide material from conditioned or from the model protease system meat may provide further information on the nature of catheptic action during conditioning. Identification of the specific collagen types altered during conditioning and sites of cleavage of the collagen molecules warrants further work. As mentioned earlier, the technique of 'Western blotting' which employs immunological detection of proteins, separated by SDS-polyacrylamide gel electrophoresis after transfer to nitrocellulose has been applied to assessment of protein degradation in post-mortem muscle (Bandman & Zdanis, 1988) and may be suitable for detection of the specific collagen types altered during conditioning.

Investigation of accelerated conditioning effects as a potential meat tenderizing method by 0.1 M lactic acid injection of pre-rigor muscle caused rapid pH decline to a minimum of pH 5.3 only 3 h after injection, after which it reached a plateau value of pH 5.5, 8.75 h after injection. Normal pH decline results in attainment of pHu approximately 24 h after slaughter. Pre-rigor lactic acid injection caused no effect on the onset of rigor mortis, monitored by ATP decline in post-mortem muscle. Percentage solubilized perimysial collagen was significantly higher ($p < 0.05$) from lactic acid treated bovine muscles than untreated muscles, suggesting a role for pre-rigor lactic acid injection on collagen solubilization during the subsequent conditioning period. In all cases, 0.1 M lactic acid injected muscles attained the level of solubilized perimysial collagen reached after 14 days of normal conditioning in reduced time, ranging from 3 to 4 days for *psoas major*, 12 to 13 days for *gluteus medius* and 6 to 7 days for *serratus ventralis*. These results may suggest that pre-rigor lactic acid injection of muscles caused rapid activation of muscle

cathepsins resulting in early attainment of conditioning effects on the collagen of these muscles. A survey of comparisons of the high molecular weight peptide region on CNBr/chymotrypsin maps of lactic acid treated and untreated muscles, conditioned from 1 to 14 days, revealed increased incidence of collagen degradation in this region due to lactic acid treatment, although this varied among the muscles studied. Similar effects to those observed in perimysium obtained from muscle conditioned for 14 days were apparent in lactic acid treated *serratus ventralis*, conditioned for only 3 days. Increased incidence of high molecular weight material, resulting from secondary chymotrypsin digestion was evident from perimysium obtained from muscles conditioned normally for 14 days and treated with lactic acid prior to conditioning for 3 days. The results of this study suggested that the use of pre-rigor lactic acid injection may lead to accelerated conditioning effects in bovine muscles. This would be of considerable benefit to meat producers both in terms of the economics of storing meat during conditioning and the quality of the final product.

From a sensory point of view, however, no beneficial effect on tenderization of beef was observed from pre-rigor lactic acid injection. Variability of the judges scores for the sensory attributes studied was high, and indicated that the results of this small study might not be reliable. The work presented in this thesis on lactic acid treatment of pre-rigor muscles must be considered preliminary, however. Injection of 0.1 M lactic acid results in a decrease of pH to 5.3 at 3 h after injection. Although injection of 0.2 M lactic acid resulted in a decrease in meat pH from 7.1 to 4.43, only 1 h after injection, meat colour was considered unacceptable. Gault (1984) found a six-fold increase in tenderness over the narrow concentration range 0.01 N to 0.1 N acetic acid and a further three-fold increase in tenderness with increasing marinade strength. Maximum swelling characteristics of the meat and tenderizing benefits were achieved when meat pH had fallen to pH 3.44. In the present study, pH 3.46 was reached only 3 h after injection of 0.8 M lactic acid. As the present work was aimed at development of a tenderizing method which did not adversely affect the appearance of the uncooked product, the lower lactic acid concentration ranges were studied only, because of the adverse meat colour changes observed with higher concentration of lactic acid. Further studies on lactic acid concentrations between 0.1 M and 0.2 M which bring pH values lower than that obtained by 0.1 M lactic acid injection, without causing adverse colour changes are required, where the effects on tenderization may be expected to be perceived by sensory assessment.

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APPENDIX 1

1. Scores of Odour *(psoas major)*

	Treatment										
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	3.6	4.0	3.8	3.6	5.6	7.1	4.3	5.0	4.5	6.25	7.9	5.1
2	6.6	3.3	7.0	7.4	7.6	7.6	7.7	7.0	4.2	5.3	3.8	6.1
3	6.9	6.4	5.6	6.6	7.2	5.7	5.5	5.8	7.1	6.6	7.7	6.5
4	8.4	6.7	6.8	7.5	9.0	6.6	8.4	6.8	7.7	7.0	7.7	7.5
5	7.6	7.1	6.3	6.8	6.5	7.8	7.2	7.2	6.6	6.0	7.9	7.0
6	8.3	9.4	5.6	8.9	6.5	9.8	7.8	7.7	6.8	5.2	9.0	7.7
7	8.6	7.7	6.9	6.2	7.0	7.1	7.3	7.4	6.3	6.9	8.0	7.2
8	2.9	3.4	4.1	3.6	2.6	4.2	3.5	2.9	2.5	2.3	1.9	3.1

2. Scores of Odour *(gluteus medius)*

	Treatment										
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	8.7	6.3	7.6	7.0	6.4	6.8	5.0	6.8	6.3	7.9	6.2	6.8
2	7.6	10.1	7.6	7.8	8.4	6.9	7.0	7.0	7.4	7.4	6.7	7.6
3	3.7	9.1	4.8	3.4	3.0	6.0	0.0	0.0	9.5	0.0	10.7	6.3
4	7.0	7.8	6.0	6.9	6.7	6.8	7.0	7.1	6.9	6.9	6.4	6.9
5	6.05	7.9	7.2	6.8	6.3	6.85	6.9	6.5	7.05	7.3	6.85	6.9
6	8.6	8.4	11.1	3.2	5.5	6.1	7.3	8.7	6.3	8.5	11.3	7.7
7	7.1	5.1	4.5	4.6	5.5	5.1	5.9	6.1	4.1	6.0	5.6	5.4
8	2.4	3.6	2.2	2.0	2.0	2.9	3.3	2.4	2.3	2.2	2.9	2.6
9	4.3	5.0	3.8	6.2	5.4	3.7	6.0	4.1	3.8	4.2	5.5	4.7
10	10.6	11.85	10.4	10.55	10.95	10.55	11.55	11.25	10.95	12.35	10.75	11.0

*Mean of 3 replicates

3. Scores of Odour *(serratus ventralis)*

	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	5.85	5.65	6.9	5.35	6.65	4.75	6.05	6.2	6.35	6.75	5.7	6.0
2	5.0	5.2	5.6	5.7	7.6	5.8	6.4	6.8	6.2	6.3	7.3	6.2
3	5.0	4.5	7.85	7.7	4.6	4.85	3.7	3.45	5.7	9.3	5.6	5.7
4	6.8	6.7	6.9	7.8	6.8	6.7	6.9	6.8	6.9	8.4	6.9	7.0
5	5.0	6.15	6.55	6.2	6.1	5.5	6.15	6.75	6.2	6.35	6.2	6.1
6	7.6	2.8	10.1	11.4	9.5	9.3	8.7	10.9	7.1	9.3	10.4	8.8
7	4.3	3.5	3.1	4.1	3.6	4.6	4.3	2.9	4.3	5.3	2.1	3.8
8	3.3	3.1	2.5	3.0	2.5	2.2	2.1	2.5	3.5	3.0	2.7	2.8
9	2.3	2.7	2.8	2.2	2.7	2.8	3.3	3.2	2.5	2.6	4.2	2.8

4. Scores of Initial Juiciness *(psoas major)*

	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	4.5	4.4	4.1	6.1	7.9	5.2	4.6	5.2	5.2	8.95	7.5	5.8
2	6.6	11.4	10.5	8.8	10.2	9.7	3.9	6.9	3.9	2.8	3.2	7.1
3	4.6	7.6	5.9	5.8	6.7	3.4	5.4	6.8	6.9	5.9	6.3	5.9
4	2.9	8.1	6.6	7.6	7.0	2.7	4.8	8.1	7.6	5.6	4.2	5.9
5	5.5	6.3	5.1	5.7	7.7	2.7	3.4	6.3	6.7	6.6	5.0	5.5
6	5.1	8.8	5.5	4.2	10.0	7.0	8.4	8.1	8.1	6.7	5.4	7.0
7	3.8	7.5	3.4	5.2	5.0	6.0	4.6	5.8	3.9	3.6	6.6	5.0
8	2.1	5.9	4.7	6.0	3.7	2.3	6.9	9.2	1.5	6.0	5.3	4.9

*Mean of 3 replicates

5. Scores of Initial Juiciness *(gluteus medius)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	6.0	5.1	5.9	6.2	5.9	5.5	5.5	7.9	7.2	4.9	4.9	5.9
2	4.8	10.2	10.5	10.1	5.5	5.4	5.1	6.9	8.3	3.1	7.2	7.0
3	5.0	5.9	4.8	4.6	4.6	7.0	0.0	0.0	6.5	0.0	0.0	5.5
4	5.3	6.0	5.6	5.1	7.3	3.6	3.6	6.4	5.8	3.7	3.1	5.0
5	4.2	5.0	3.05	3.2	4.05	3.2	3.95	3.4	4.85	4.35	5.4	4.1
6	5.3	6.7	4.7	2.6	5.6	4.1	1.9	6.8	8.2	3.0	3.3	4.7
7	1.8	5.5	5.0	2.2	6.3	3.4	4.3	5.9	8.1	2.3	7.9	4.8
8	3.9	3.7	4.5	3.5	4.8	3.6	6.0	9.2	5.2	5.1	3.0	4.8
9	3.9	6.9	4.0	4.8	3.5	3.4	3.8	5.3	6.8	5.1	5.7	4.8
10	5.15	10.0	6.9	5.75	7.55	9.55	5.5	8.2	8.05	4.7	6.3	7.1

6. Scores of Initial Juiciness *(serratus ventralis)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	6.3	5.9	8.4	5.95	6.65	6.75	3.95	5.05	7.8	7.05	6.25	6.4
2	8.2	9.6	8.0	10.0	9.5	7.3	8.2	9.6	9.8	8.4	7.0	8.7
3	6.95	5.3	7.1	7.75	4.45	5.45	6.25	6.0	8.3	6.8	5.45	6.3
4	5.8	6.5	5.6	8.0	7.3	7.1	6.3	7.1	6.7	6.4	6.7	6.7
5	3.25	2.75	7.5	3.35	6.55	4.1	2.65	6.85	4.25	4.5	3.4	4.5
6	4.6	5.1	9.4	11.8	2.6	9.3	6.2	9.7	9.2	5.5	5.8	7.2
7	3.9	3.0	4.1	5.2	6.3	2.9	3.4	5.4	6.6	5.8	2.5	4.5
8	3.0	3.3	2.8	2.6	3.6	3.3	3.5	3.2	4.4	3.6	4.0	3.4
9	3.4	3.1	3.7	3.6	3.5	3.0	5.0	5.2	4.5	3.1	4.9	3.9

*Mean of 3 replicates

7. Scores of Moistness after Chewing *(psoas major)*

	Uncond -itioned	Treatment Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	5.1	5.3	5.3	6.8	9.7	8.2	5.2	6.2	6.1	11.0	8.8	7.1
2	9.3	11.0	9.3	3.1	9.9	6.3	4.2	9.6	3.7	2.7	3.1	6.6
3	7.1	9.2	7.8	9.5	9.0	6.1	9.5	9.0	9.1	8.8	9.1	8.6
4	3.3	7.7	8.4	7.9	6.8	3.0	7.1	8.7	6.9	5.8	7.1	6.6
5	8.1	7.0	5.0	4.8	7.3	10.4	10.1	7.2	9.5	10.4	9.0	8.1
6	4.2	8.5	6.5	5.4	10.7	7.3	8.9	7.3	9.2	7.5	5.6	7.4
7	3.3	8.2	10.2	8.0	6.6	9.8	10.2	6.6	6.8	7.4	11.6	8.1
8	3.7	8.2	5.6	7.0	6.5	3.6	7.8	6.0	6.0	7.8	8.2	6.4

8. Scores of Moistness after Chewing *(gluteus medius)*

	Uncond -itioned	Treatment Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	7.4	7.1	7.8	7.7	8.2	6.4	6.8	10.3	7.6	6.4	6.4	7.5
2	5.8	10.1	8.1	10.6	6.2	4.5	10.1	9.7	8.8	3.7	7.6	7.7
3	7.5	9.4	7.5	7.0	9.6	8.8	0.0	0.0	11.0	0.0	0.0	8.7
4	4.0	5.3	5.9	5.3	8.4	3.8	4.5	7.7	6.1	3.7	4.9	5.4
5	4.1	4.15	3.75	4.9	6.1	3.4	4.1	4.05	5.6	4.95	8.35	4.8
6	6.3	7.7	5.9	3.4	7.2	4.3	6.1	7.4	7.3	3.9	5.4	5.9
7	7.3	8.2	7.1	5.7	8.2	4.9	6.8	7.1	10.0	3.1	10.0	7.1
8	5.5	9.5	6.1	6.5	7.0	7.8	9.8	10.2	6.3	7.0	8.5	7.7
9	5.5	8.1	4.6	5.5	5.2	4.0	6.3	6.6	8.2	6.0	6.2	6.0
10	8.25	10.7	7.6	9.45	9.9	8.9	9.7	8.3	8.45	5.95	6.5	8.5

*Mean of 3 replicates

9. Scores of Moistness after Chewing *(serratus ventralis)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	7.9	7.65	10.35	8.35	7.55	9.1	6.0	7.55	8.75	9.3	9.55	8.4
2	9.2	9.2	7.8	10.0	9.6	8.5	7.3	9.7	10.4	7.8	7.6	8.8
3	7.05	6.65	9.0	8.7	4.6	6.9	7.3	6.4	9.4	8.2	7.05	7.4
4	5.5	5.7	5.2	9.0	7.8	8.2	6.8	7.7	8.4	9.4	8.9	7.5
5	3.5	3.05	7.95	10.0	9.3	8.05	3.5	9.3	9.3	6.75	6.3	7.0
6	4.0	5.1	9.4	12.3	3.9	9.2	7.9	9.6	9.2	6.0	7.5	7.6
7	4.5	3.8	6.5	7.9	7.4	7.4	4.9	7.3	6.8	8.3	10.7	6.9
8	6.4	6.9	6.5	6.8	6.6	6.6	6.5	6.2	6.4	7.5	6.3	6.6
9	3.7	3.6	5.0	4.2	4.7	4.1	5.9	5.8	5.0	4.1	5.4	4.7

10.Scores of Resistance to First Bite *(psoas major)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	4.3	5.5	3.6	4.1	6.7	5.4	4.8	5.3	3.9	7.1	8.1	5.3
2	9.6	9.4	7.5	5.6	9.0	5.7	8.9	8.9	4.4	3.6	3.5	6.9
3	6.2	6.1	5.9	7.4	6.9	5.1	8.5	7.0	7.4	7.6	10.0	7.1
4	3.6	4.9	6.8	4.2	8.3	3.3	8.3	8.2	4.1	3.5	6.7	5.6
5	3.0	6.1	4.1	4.7	5.8	3.4	10.5	7.9	4.9	5.9	4.5	5.5
6	3.3	5.8	4.3	4.9	8.4	6.4	10.6	10.0	8.3	7.3	7.2	7.0
7	5.8	9.2	6.3	5.3	5.4	4.2	12.9	11.2	5.2	9.3	9.2	7.6
8	2.5	9.5	1.4	4.9	7.3	2.0	4.8	10.2	6.6	6.2	7.7	5.7

*Mean of 3 replicates

11. Scores of Resistance to First Bite *(gluteus medius)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	10.0	10.3	8.6	8.1	9.4	9.4	11.1	10.2	9.6	7.1	8.4	9.3
2	10.0	11.6	7.5	10.5	9.3	6.0	11.4	9.7	7.8	4.1	8.5	8.8
3	10.6	8.0	4.8	8.9	10.1	5.3	0.0	0.0	9.3	0.0	0.0	8.1
4	7.0	8.4	6.7	9.6	10.1	5.4	10.8	8.1	8.2	6.2	5.6	7.8
5	7.65	3.35	3.7	8.25	11.2	5.8	7.45	5.2	3.15	4.25	5.3	5.9
6	10.5	9.1	4.4	6.4	11.0	6.9	11.7	10.1	12.2	7.6	8.8	9.0
7	9.1	10.4	6.5	10.5	11.3	7.4	11.2	9.2	8.0	8.7	10.6	9.4
8	7.2	6.6	2.5	6.0	5.2	6.5	9.1	9.5	4.3	5.0	8.7	6.4
9	6.5	10.3	4.5	5.7	8.7	3.8	7.9	5.1	6.4	4.7	6.6	6.4
10	10.05	6.45	4.75	4.7	9.2	4.95	12.1	8.8	8.55	5.8	7.9	7.6

12. Scores of Resistance to First Bite *(serratus ventralis)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	3.8	4.4	4.95	4.55	3.7	4.35	4.05	4.3	7.75	5.7	4.65	4.7
2	8.2	8.1	7.2	9.6	8.3	7.3	7.4	10.3	9.6	8.0	6.4	8.2
3	7.8	6.55	6.9	7.0	4.8	3.1	6.95	3.85	7.55	7.5	6.5	6.2
4	6.7	4.0	4.4	5.9	7.0	5.7	6.9	6.9	5.6	7.6	6.9	6.1
5	5.5	2.15	3.3	2.4	3.9	3.6	2.7	5.85	3.25	4.6	3.65	3.7
6	4.0	1.3	1.7	7.1	7.2	2.5	1.2	7.9	7.0	3.2	4.8	4.35
7	10.2	6.2	6.5	9.6	8.0	4.6	10.0	9.2	9.0	10.3	7.1	8.2
8	1.3	1.7	2.2	2.0	1.7	1.9	2.8	2.1	3.5	3.3	4.0	2.4
9	4.7	4.0	4.1	4.0	4.0	4.0	6.1	5.8	4.9	4.8	5.5	4.7

*Mean of 3 replicates

13. Scores of Ease of Chewing *(psoas major)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	4.2	5.1	3.5	3.5	7.0	4.8	5.3	5.8	4.3	6.35	7.6	5.2
2	3.4	6.5	7.2	4.0	6.6	5.3	7.4	8.2	3.3	3.1	2.6	5.2
3	5.6	6.8	3.8	7.8	6.1	5.6	7.3	7.0	6.6	7.2	8.4	6.6
4	3.9	6.9	8.0	4.4	8.6	3.3	7.6	9.1	7.4	5.2	7.3	6.5
5	3.1	6.1	4.9	3.8	5.3	3.5	9.2	6.8	4.2	5.6	4.5	5.2
6	3.9	7.5	2.8	5.5	8.6	5.6	8.9	7.2	6.8	4.1	5.3	6.0
7	7.6	8.0	7.2	5.3	6.5	3.8	12.6	10.5	6.6	8.6	8.4	7.7
8	2.5	7.2	3.5	5.0	6.1	3.0	7.6	9.4	5.5	6.1	7.3	5.7

14. Scores of Ease of Chewing *(gluteus medius)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	9.3	9.0	8.8	7.3	8.7	8.3	10.5	9.9	8.9	6.7	7.6	8.7
2	7.8	11.4	8.3	10.6	9.6	6.2	11.4	9.5	7.9	4.4	7.2	8.6
3	9.0	6.6	6.2	8.4	10.1	5.4	0.0	0.0	7.8	0.0	0.0	7.6
4	7.4	9.1	6.3	9.0	9.3	5.8	10.5	7.1	8.7	5.5	5.7	7.7
5	6.8	3.7	4.1	6.5	10.05	6.1	7.65	6.45	3.75	4.1	5.1	5.8
6	8.8	8.3	1.9	5.5	9.1	6.8	8.5	8.1	6.9	5.6	4.6	6.7
7	7.6	10.4	5.6	8.9	10.7	5.6	11.3	8.9	8.7	5.0	10.1	8.4
8	8.4	9.7	4.5	6.3	6.6	8.8	9.0	10.3	6.1	5.1	8.2	7.5
9	6.3	9.8	4.0	5.1	8.9	3.7	8.7	5.4	6.1	4.5	8.1	6.4
10	10.2	9.35	5.65	6.1	9.35	7.3	10.5	8.95	8.25	6.9	7.5	8.2

*Mean of 3 replicates

15. Scores of Ease of Chewing *(serratus ventralis)*

	Treatment											
	Uncond-itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	$\bar{x}$
1	4.2	4.05	6.3	4.75	3.65	5.3	4.8	4.2	7.75	4.7	4.75	4.95
2	8.0	8.3	4.5	9.6	8.8	6.6	7.3	10.2	9.0	7.8	5.8	7.8
3	7.15	6.1	7.95	5.6	5.0	4.4	6.6	5.7	6.75	7.45	5.1	6.2
4	6.6	4.3	5.0	8.3	6.2	5.4	6.9	7.3	8.4	8.9	6.9	6.7
5	5.4	1.8	5.4	6.4	7.1	4.2	2.65	8.15	6.45	4.85	6.2	5.3
6	7.4	2.0	5.5	8.1	8.2	4.4	9.6	7.7	7.6	3.1	8.4	6.5
7	12.1	5.6	6.1	8.5	8.7	3.6	8.3	6.7	6.5	10.5	8.2	7.7
8	1.5	2.0	2.0	2.1	2.0	2.0	3.2	2.4	3.3	3.2	4.0	2.5
9	4.4	3.9	3.8	3.9	3.4	3.7	5.2	5.8	3.9	4.9	5.6	4.4

16. Scores of Tenderness *(psoas major)*

	Treatment											
	Uncond-itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	$\bar{x}$
1	4.1	5.0	3.5	3.6	6.3	4.9	4.8	5.8	4.0	6.45	7.4	5.1
2	2.5	2.7	7.8	2.9	7.6	7.1	7.4	6.7	2.1	2.9	2.5	4.7
3	4.9	7.5	4.4	7.7	7.3	4.8	7.9	7.9	7.9	7.7	9.1	7.0
4	4.0	6.7	8.1	4.3	8.6	4.1	8.1	9.2	7.1	5.8	7.3	6.7
5	3.4	6.4	4.7	3.6	5.5	5.6	8.8	6.7	5.8	6.5	5.3	5.7
6	4.6	7.7	3.1	5.6	7.5	4.8	8.6	8.2	6.9	4.6	6.1	6.15
7	8.1	8.1	6.2	4.3	5.9	5.0	11.8	8.5	5.8	8.5	7.8	7.3
8	3.5	8.1	3.5	5.3	5.0	2.9	7.7	8.7	7.0	5.6	6.5	5.8

*Mean of 3 replicates

17. Scores of Tenderness *(gluteus medius)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	8.9	8.1	8.4	8.0	9.1	8.3	9.3	9.1	7.2	5.6	7.0	8.1
2	9.5	11.3	9.6	10.6	7.9	4.1	11.9	9.6	9.6	4.7	7.9	8.8
3	9.2	6.2	6.3	8.0	10.8	6.4	0.0	0.0	9.0	0.0	0.0	8.0
4	7.9	8.8	5.8	9.1	9.4	6.0	10.4	7.4	7.7	5.7	5.7	7.6
5	6.4	4.5	3.65	5.95	9.3	6.1	7.75	6.3	4.95	4.65	6.25	6.0
6	7.4	8.4	3.2	6.3	7.9	7.0	10.0	6.9	7.2	5.3	4.8	6.8
7	7.4	9.7	5.5	8.1	10.8	5.0	10.2	7.8	8.6	4.3	8.4	7.8
8	8.0	9.4	4.8	6.7	6.6	8.8	9.8	10.5	5.3	5.5	8.9	7.7
9	5.9	10.4	3.4	6.0	9.4	3.3	9.5	5.4	6.2	4.1	8.4	6.5
10	8.8	9.95	5.1	7.05	10.1	6.3	11.2	8.5	6.3	6.95	7.7	8.0

18. Scores of Tenderness *(serratus ventralis)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	4.0	4.45	5.65	4.3	3.55	4.85	4.6	4.65	7.85	4.85	6.0	5.0
2	7.9	8.9	5.0	8.5	8.0	6.1	7.65	11.0	8.8	7.7	6.5	7.8
3	7.65	5.7	8.35	5.6	4.55	3.8	6.7	5.65	7.35	6.8	5.1	6.1
4	6.8	5.2	4.9	7.3	6.1	5.3	7.6	7.9	7.6	8.5	5.8	6.6
5	5.35	1.75	5.15	5.65	6.35	4.5	2.65	7.8	6.1	5.75	5.85	5.2
6	7.2	2.6	6.7	8.2	7.3	4.6	8.5	6.4	8.6	4.4	7.7	6.6
7	12.1	4.3	6.5	7.6	8.4	3.2	7.5	6.0	6.5	9.7	7.6	7.2
8	2.0	2.0	2.3	2.2	2.3	2.5	3.1	2.6	3.5	3.3	3.4	2.65
9	4.0	3.2	2.7	3.5	2.8	3.4	5.2	5.6	4.0	4.9	5.7	4.1

*Mean of 3 replicates

19. Scores of Meat Colour *(psoas major)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	2.7	2.0	2.3	3.0	4.5	3.5	3.7	3.1	2.7	4.9	4.8	3.4
2	2.5	3.2	3.5	3.2	2.5	3.4	3.8	4.7	2.9	4.0	3.4	3.4
3	4.2	4.1	2.9	4.3	3.9	3.6	3.6	4.4	4.8	4.6	3.9	4.0
4	5.7	5.0	5.6	5.5	5.9	3.6	6.0	6.2	6.6	4.6	5.7	5.5
5	3.2	3.3	2.8	2.7	3.1	3.6	3.1	3.0	3.2	3.2	3.6	3.2
6	5.0	7.1	3.8	7.0	6.1	4.4	6.9	5.1	3.9	5.8	2.7	5.25
7	3.2	4.5	3.5	5.9	3.8	4.2	4.0	3.8	4.9	5.1	4.2	4.3
8	0.9	1.2	1.8	2.2	1.5	1.5	1.9	1.0	1.1	1.4	1.0	1.4

20. Scores of Colour *(gluteus medius)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	4.7	3.8	4.5	3.2	3.3	3.8	3.6	3.7	3.0	4.2	5.3	3.9
2	6.4	8.3	7.7	7.5	8.8	5.5	7.7	7.4	6.8	6.2	6.8	7.2
3	3.0	2.7	3.1	3.9	3.2	2.7	0.0	0.0	4.3	0.0	0.0	3.3
4	5.2	7.4	5.6	5.1	5.1	4.5	7.0	8.8	8.1	6.3	5.7	6.25
5	2.7	2.65	2.65	2.45	2.55	2.4	2.95	2.45	2.35	2.7	2.85	2.6
6	6.5	4.6	4.1	4.8	4.2	4.1	3.5	8.8	7.3	4.4	5.9	5.3
7	3.6	4.5	3.3	3.0	3.2	3.6	3.1	3.2	3.1	3.3	3.8	3.4
8	1.3	2.4	1.4	1.5	1.1	2.2	2.7	1.6	1.2	1.0	2.9	1.75
9	4.4	5.6	3.6	4.5	4.5	3.5	3.9	4.3	5.6	3.6	3.9	4.3
10	8.05	7.2	7.2	6.3	4.85	7.3	6.6	6.6	6.6	6.6	6.55	6.7

*Mean of 3 replicates

21. Scores of Colour *(serratus ventralis)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	3.05	3.2	4.45	3.6	4.4	3.55	3.95	3.6	3.75	3.9	4.35	3.8
2	7.0	7.7	6.5	6.9	7.1	7.3	7.75	7.9	7.5	6.6	7.5	7.25
3	4.75	4.05	6.65	4.6	4.75	4.3	4.6	3.35	5.4	7.0	4.25	4.9
4	6.5	5.5	5.7	7.1	6.3	5.8	6.8	7.9	9.1	7.9	5.3	6.7
5	3.5	2.15	4.8	5.2	3.45	3.35	3.95	4.15	5.1	3.4	3.8	3.9
6	5.5	4.0	5.4	8.1	4.2	7.0	6.3	4.7	4.8	6.3	6.0	5.7
7	4.3	3.7	3.3	4.2	5.4	3.0	3.3	3.7	3.3	4.4	3.1	3.8
8	2.1	2.2	2.8	2.0	2.1	2.5	2.4	2.3	2.9	2.3	2.6	2.4
9	2.7	2.8	3.9	2.8	3.3	2.8	3.6	3.4	3.1	3.1	3.4	3.2

22. Scores for Overall Acceptability *(psoas major)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	3.4	4.3	2.9	4.6	7.5	4.1	4.6	5.6	3.8	7.65	7.9	5.1
2	4.4	6.6	5.1	1.4	8.5	5.3	10.4	9.7	2.6	2.1	2.3	5.3
3	3.9	6.4	3.4	6.7	5.5	3.9	7.0	7.4	6.5	7.5	8.6	6.1
4	4.4	6.5	7.7	6.9	7.8	3.9	7.4	7.9	7.0	5.6	6.6	6.5
5	4.5	5.0	3.5	3.5	4.8	6.4	8.1	6.2	6.2	6.4	8.4	5.7
6	4.9	6.2	3.2	7.5	7.6	5.9	7.2	6.0	5.2	7.8	7.9	6.3
7	6.9	7.4	7.5	6.8	6.7	8.0	10.9	9.3	7.7	8.6	10.6	8.2
8	3.5	7.2	5.4	5.6	5.3	3.9	7.6	7.6	5.8	6.3	7.7	6.0

*Mean of 3 replicates

23. Scores of Overall Acceptability *(gluteus medius)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	8.5	6.9	5.8	6.3	7.0	7.2	7.9	8.8	5.6	5.0	6.2	6.8
2	9.0	10.9	9.3	7.7	9.4	5.7	11.7	9.9	7.0	2.8	6.9	8.2
3	6.1	7.1	4.1	5.7	9.5	4.0	0.0	0.0	10.5	0.0	0.0	6.7
4	7.1	8.4	6.5	6.9	7.8	5.0	9.3	7.5	7.1	5.9	6.3	7.1
5	6.35	3.7	3.25	6.15	8.65	5.9	6.45	5.3	4.45	5.05	6.45	5.6
6	7.3	6.2	6.3	3.9	7.3	7.1	4.7	7.7	6.7	8.4	10.7	6.9
7	7.3	8.7	4.6	5.0	8.7	5.1	8.6	4.8	6.6	2.8	6.8	6.3
8	8.2	9.5	5.0	6.2	6.4	8.9	9.8	10.4	6.4	5.8	9.1	7.8
9	5.0	7.3	3.4	4.5	8.6	3.2	7.2	4.4	5.4	3.6	6.4	5.4
10	7.35	9.1	6.2	6.4	7.7	7.0	6.8	8.0	6.3	6.1	6.45	7.0

24. Scores of Overall Acceptability *(serratus ventralis)*

	Treatment											
	Uncond -itioned	Conditioned (Days)					Lactic Acid Treatment (Days)					
Taster*	1	1	3	7	10	14	1	3	7	10	14	x̄
1	4.45	3.55	6.55	6.0	5.85	5.85	5.05	5.5	7.55	7.0	2.9	5.5
2	7.2	6.6	4.6	7.2	8.2	5.8	6.25	7.5	6.7	7.8	6.1	6.7
3	5.55	4.2	6.85	4.85	4.05	4.2	5.95	6.2	7.5	9.65	4.2	5.7
4	5.8	5.0	5.6	7.4	6.3	4.9	7.1	7.4	8.3	7.8	6.1	6.5
5	4.25	1.6	5.4	5.95	6.3	4.5	3.0	7.85	6.85	6.25	5.5	5.2
6	4.6	4.4	4.0	6.8	5.6	6.8	8.1	7.0	11.3	6.1	9.9	6.8
7	10.3	5.1	6.6	8.5	7.5	5.2	8.5	7.2	5.7	10.0	8.0	7.5
8	4.5	4.4	4.5	4.5	5.3	5.2	5.0	4.8	5.3	5.2	5.7	4.9
9	3.0	2.6	2.3	2.6	2.7	3.1	5.0	4.6	3.3	4.1	5.3	3.5

*Mean of 3 replicates