AN ABSTRACT OF THE THESIS OF

Carlos Gonzalez-B for the degree of Doctor of Philosophy

in Food Science & Technology presented on July 6, 1982

Title: Effect of Pre-rigor Ultrapressurization on Bovine Intramuscular Connective Tissue

Abstract approved: ______________________
Allen F. Anglemier

Samples of pre-rigor sternomandibularis muscle from ten steers were pressurized at 15,000 psi for two min at 35°C to investigate the effects of pressurization and incubation time on the thermal stability of collagen. Immediately after pressurization, pH of the treated samples (pH 5.71) was significantly lower ($P < 0.05$) than the control samples (pH 6.85). The amount of labile collagen from the pressurized samples was 14.16% of the total collagen and was significantly ($P < 0.05$) higher than the control samples (10.24%).

Pressurization apparently accelerated the glycolytic enzymes causing a rapid drop in pH and reduced the strength of collagen fibers to allow for an increase in the thermolabile fraction. Pressurized samples showed a significantly greater ($P < 0.05$) release of enzymes (β-galactosidase, catheptic collagenase, cathepsin

C and elastase) from lysosomes than the control samples. The pH of the pressurized homogenates was significantly lower ($P < 0.05$) than the control samples.

The combined effect of the lysosomal enzymes, β-galactosidase and β-glucuronidase, on the ability of collagenase to solubilize collagen of pressurized muscle and intramuscular connective tissue isolated from pressurized samples was determined. It was found that the incubation of pressurized samples with lysosomal enzymes significantly decreased ($P < 0.05$) the thermal stability of collagen. The results provide strong evidence that lysosomal enzymes reduce the strength of muscle collagen and that pressurization does alter the configurational structure of connective tissue making it more susceptible to enzymatic attack.

Effect of Pre-rigor Ultrapressurization on Bovine Intramuscular Connective Tissue

by

Carlos Gonzalez-B

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

July 6, 1982

Commencement June 1983

APPROVED:

Professor of Food Science & Technology in charge of major

Head of Food Science & Technology

Dean of Graduate School

Date thesis is presented July 6, 1982

Typed by Donna Lee Norvell-Race for Carlos Gonzalez-B

TABLE OF CONTENTS

INTRODUCTION 1

REVIEW OF LITERATURE 4

Collagen Types. 5
Methods for Isolation and Characterization of Collagen Types. 7
Biosynthesis of Collagen. 10
Chemistry of Collagen Crosslinks. 12
Age Variation in Crosslinkages and Meat Tenderness. 14
Postmortem Changes in Connective Tissue Collagen. 15
Role of Lysosomal Enzymes in Connective Tissue Degradation 16
Effect of Pressurization on the Physico-chemical Properties of Muscle. 18

EXPERIMENTAL PROCEDURE 21

Intramuscular Connective Tissue Extraction. 21
Determination of Lysosomal Enzyme Activities. 22
β-Galactosidase Activity 23
Cathepsin C Activity 23
Catheptic Collagenase Activity 23
Elastase Activity. 23
Incubation of Muscle with Specific Enzymes. 24
Incubation of IMCT with Specific Enzymes. 24
Amino Acid Analysis of IMCT 25
Statistical Analysis. 25

RESULTS AND DISCUSSION 26

Effect of Pressurization on Muscle pH 26
Effect of Pressurization on Bovine Intramuscular Collagen . 28
Effect of pH on Yield of Intramuscular Connective Tissue. . 38
Effect of Pressurization on the Molecular Components of IMCT 48
Effect of Pressurization on the Release of Lysosomal Enzymes 51
Incubation of Pressurized Muscle and IMCT with Specific Enzymes 71
Amino Acid Composition of Pressurized IMCT. 74

CONCLUSION 76

BIBLIOGRAPHY 78

APPENDICES

A Isolation and Purification of Intramuscular Tissue 90

B Collagen Solubility (Heat Labile). 92

C Hydroxyproline Determination 94

D Assay for β-Galactosidase Activity 98

E Assay for Cathepsin C Activity 100

F Assay for Collagenase Activity 102

G Assay for Elastase Activity. 103

H Amino Acid Analysis of IMCT Sample 104

I Biuret Procedure 105

J Separation of IMCT Components by Lithium Dodecyl Sulfate/7.5-15% Polyacrylamide Gradient Gel Stabilized with 5-17% Sucrose Gradient. 107

LIST OF FIGURES

Figure		Page
1	Effect of pressurization and incubation time on the percentage of heat labile collagen of bovine sternomandibularis muscle stored at 2°C	31
2	Relationship between pH and yield of intramuscular connective tissue from bovine sternomandibularis muscle	42
3	Lithium dodecyl sulfate polyacrylamide gel gradient (7.5-15%) electrophoresis of pepsin-solubilized bovine intramuscular collagen	49
4	Specific activities of β-galactosidase in pressurized bovine sternomandibularis muscle	57
5	Specific activities of cathepsin C in pressurized bovine sternomandibularis muscle	58
6	Specific activities of catheptic collagenase in pressurized bovine sternomandibularis muscle	59
7	Activities of β-galactosidase in pressurized bovine sternomandibularis muscle	62
8	Activities of cathepsin C in pressurized bovine sternomandibularis muscle	63
9	Activities of cathepic collagenase in pressurized sternomandibularis muscle	64
10	Release of β-galactosidase activity in pressurized bovine sternomandibularis muscle	66
11	Release of cathepsin C activity in pressurized bovine sternomandibularis muscle	67
12	Release of catheptic collagenase in pressurized bovine sternomandibularis muscle	68

LIST OF TABLES

Table		Page
1	Effect of pressurization on the pH of bovine sternomandibularis muscle	27
2	Total collagen content of meat samples from bovine sternomandibularis muscle	29
3	Total collagen content and the percentage of heat labile collagen of bovine sternomandibularis muscle stored at 2°C for 72 hours	30
4	Total activity and percent release of β-galactosidase in pressurized bovine sternomandibularis muscle	34
5	Total activity and percent release of cathepsin C in pressurized bovine sternomandibularis muscle	35
6	Total activity and percent release of catheptic collagenase in pressurized bovine sternomandibularis muscle	36
7	Yield of intramuscular connective tissue (IMCT) of meat samples from bovine sternomandibularis muscle	39
8	Effect of pressurization on the pH of bovine sternomandibularis muscle and on the yield of intramuscular connective tissue (IMCT)	40
9	Effect of β-galactosidase, β-glucuronidase and collagenase on collagen solubility	46
10	Effect of β-galactosidase, β-glucuronidase and collagenase on isolated IMCT solubility	47
11	Effect of pressurization on pH values of bovine sternomandibularis muscle homogenates	52
12	Specific activities of β-galactosidase in pressurized bovine sternomandibularis muscle	54
13	Specific activities of cathepsin C in pressurized bovine sternomandibularis muscle	55

Table		Page
14	Specific activities of catheptic collagenase in pressurized bovine sternomandibularis muscle	56
15	Specific activities of elastase in pressurized bovine sternomandibularis muscle	61
16	Amino acid composition of collagen isolated from pressurized sternomandibularis muscle	75

Effect of Pre-rigor Ultrapressurization on
Bovine Intramuscular Connective Tissue

INTRODUCTION

Connective tissue is one of the components of meat associated with tenderness of the cooked product. Connective tissue in muscle is located in the endomysium, perimysium and epimysium, which collectively form a supporting network and respectively surround each individual muscle fiber, the muscle fiber bundles and the whole muscle. These connective tissue sheathes come together at the ends of the muscle to form thick tendons (Cassens, 1971).

The two principal connective tissue proteins are collagen, the major component, and elastin, a minor component. The amount of elastin is not closely associated with tenderness of meat (Cross _et al._, 1973). However, the total amount of collagen in muscle tissue (Bailey, 1972) and the percent of heat extractable collagen (Cross _et al._, 1973) are closely related to the toughness of meat.

A great deal of research has been conducted to determine the role of contractile proteins in meat tenderness and to improve meat tenderness through restriction of muscle shortening or through proteolytic alteration of the muscle proteins (Locker, 1960; Sharp, 1963; Hostetler _et al._, 1970, 1973; Smith _et al._, 1971; Parrish _et al._, 1969; Dutson _et al._, 1975). These studies were directed largely on the myofibrillar component of toughness, while relatively little work has been done on intramuscular collagen as a

component of toughness. This may be due to the slow rate of collagen degradation during postmortem storage, the lack of adequate research techniques, and the lack of knowledge regarding the mechanism of intramuscular collagen degradation. However, if the processes involved in collagen alterations during postmortem aging can be determined, we might be able to accelerate these postmortem alterations. The introduction of new techniques that could increase the eating quality of meat, especially tenderness, have received extensive attention in recent years. One of these techniques uses high pressure.

It has been demonstrated that pressurization of pre-rigor muscle significantly improves tenderness (MacFarlane, 1973; Kennick _et al_, 1980). The usefulness of this technique may be related to its possible effect upon the eating quality of meat as measured by changes in stroma proteins (collagen solubility). Several investigators have reported the effect of pressure on myofibrillar structure (Bouton _et al_., 1977; MacFarlane, 1974; MacFarlane and McKenzie, 1976). Others have studied the effect of pressure on the solubility of myofibrillar proteins (Ikkai and Ooi, 1966).

Objective measurements as well as subjective assessments have been used to determine the effectiveness of a pressure heat treatment in improving the tenderness of post-rigor muscles (Ratcliff _et al_., 1977). However, the reports of effect of the application of pressure on connective tissue or native collagen are very few or none, and the effect on these tissues cannot be discounted.

Knowledge of the effects induced by pressure on connective tissue is not only important but necessary for assessing the usefulness of the treatment as a technological process for tenderization, or as a research tool. A study of collagen changes in pressurized muscle may provide valuable information not only for assessing the value of pressure treatment as a technological process, but also for better understanding of the biochemistry and structural changes on muscle connective tissues and its relationship to muscle toughness.

The objectives of this study were:

1. to accelerate postmortem changes in muscle by applying high pressure and to determine the effect of high pressure and storage on the properties of intramuscular collagen.
2. to investigate the effect of high pressure treatment on the release of lysosomal enzymes in bovine skeletal muscle.
3. to determine the effect of high-pressure treatment on the susceptibility of muscle connective tissue to enzymatic digestion.
4. to determine the role of lysosomal enzymes in disruption of pressure treated muscle connective tissue.

REVIEW OF LITERATURE

Collagen accounts for one-third of human protein and is found primarily in bone, cartilage, skin and basement membrane. As a result almost every organ in the body contains at least a trace of this ubiquitous protein.

The collagen fibers in connective tissues are composed of tropocollagen molecules, which are rod-like macromolecules with a diameter of about 1.5 nm and a length of about 300 nm (Traub and Piez, 1971; Ramachadran, 1967; Prockop *et al.*, 1979). These molecules are built of three polypeptide chains known as α chains, each containing approximately 1000 amino acid residues and having a molecular weight of about 100,000. Each α chain is coiled in a left-handed helix, and the three α chains in each tropocollagen molecule are coiled in a right-handed superhelix (Ramachandran, 1967; Traub and Piez, 1971). The coiled-coil conformation provides great stability to chemical and enzymatical disruption. When collagen is denatured, the helical structure of collagen is lost and converted to a random coiled α (monomer), β (dimer) and γ (trimer) components, depending upon the number of crosslinks existing within the molecule.

A significant feature and characteristic of the amino acid composition of collagen is that glycine makes up about 33% of the total number of residues and the amino acids, proline and hydroxyproline, make up 25%. Another unique characteristic of collagen

is that it contains hydroxylysine (Ramachandran, 1967), an amino acid that does not occur in most other proteins (Schubert and Hamerman, 1968). The high contents of glycine, proline and hydroxyproline allow the formation of the triple-stranded super helix arrangement (Traub and Piez, 1971).

Each α chain of collagen contains a central helical region and a short nonhelical region at each end. The sequences of gly-pro-y and gly-x-hyp occur in equal proportions in the helical region, whereas the nonhelical amino terminal and carboxyl terminal peptides (about 10-25 residues each) do not contain the repetitive gly-pro-y or gly-x-hyp structure (Fessler and Fessler, 1978).

The principal carbohydrates present in vertebrate collagen are D-glucose and D-galactose, which are present as galactosyl residues and glucosyl-galactosyl residues linked glycosidically to the hydroxyl group of hydroxylysine (Spiro, 1969). The enzymes involved in the assembly of the hydroxylysine linked glucosylgalactose dissacharide units have been isolated recently (Spiro and Spiro, 1971a,b).

Collagen Types

Five distinct types of collagen have been separated from different tissue sources by fractional salt precipitation, after limited digestion with pepsin to cleave off the non-helical ends where crosslinks originate, and then purified by CM-cellulose chromatography. These five unique collagens are composed of seven

different α chains; α I(I), α I(II), α I(III), α I(IV), α_2, α_A and α_B have been determined to be different genetically. However, they probably undergo the same post-translational modification (Viidik and Vuust, 1980).

The collagen from bone, mature dermis and tendon contains two distinct α chains, α_1(I) and α_2, which are present in ratio of 2:1 (Piez, 1966). Collagen of this type is referred to as type I collagen, which is the most abundant type of collagen found in the body.

Type I collagen has been reported to contain about 6-8 hydroxylysine residues and a low carbohydrate content (Spiro, 1969). A second type of α_1 chain, α_1(II), is found in cartilage-specific collagen. It was first detected in chicken cartilage (Miller and Matukas, 1969), and is composed of three identical α chains which are genetically distinct from α_1(I) and α_2 (Miller and Matukas, 1974). This collagen has been designated type II with the chain composition of $[\alpha_1(II)]_3$. A third type of α_1 chain, α_1(III), occurs in collagen of infant dermis, vascular tissue and muscle tissue (Chung and Miller, 1974; Trelstad, 1974; Epstein, 1974; Bailey and Sims, 1977; Duance _et al_., 1977). The molecules are composed of three α_1(III) chains, and these chains are crosslinked in part by disulfide bonds. Basement membranes are believed to contain the fourth type of α_1 chain which has a high content of hydroxylysine, carbohydrate and both 3- and 4-hydroxyproline. The molecular composition of type IV collagen remains uncertain, and may vary among tissue sources. Type IV collagen

has been reported to contain three identical α chains in the basement membrane isolated from glomerulus and lens capsule (Kefalides, 1971), and two different α chains in the basement membrane collagen isolated from fetal membrane and muscle (Burgeson et al., 1976; Duance et al., 1977).

Type V collagen which may arise from basement membranes has been also isolated from skin, placenta and few other tissues (Burgerson et al., 1976; Chung et al., 1976). Type V collagen has been reported to contain two polypeptide chains: α_A and α_B (Bentz et al., 1978). All of the collagen types, except for type II, have been reported to exist in skeletal muscle (Bailey and Sims, 1977; Duance et al., 1977).

Methods for Isolation and Characterization of Collagen Types

Collagen molecules in tissue must be in a soluble form before they can be separated from each other or further purified. Type I collagen is readily obtained in soluble form by extracting skins or other tissues of young animals with neutral salt solutions or dilute acids (Piez, 1967). Collagen types II, III and IV are almost completely insoluble with these extractions. Several methods have been used to solubilize collagens from tissues including inhibition of crosslinkage formation by feeding the animal with β-aminopropionitrile (Miller and Matukas, 1969; Trelstad et al., 1970), or removing the nonhelical regions (which carry the crosslinks)

with partial pepsin digestion (Kefalides, 1971; Miller, 1972; Epstein, 1974; Chung and Miller, 1974; Trelstad, 1974; Burgeson *et al*., 1976).

Collagen species can be best separated in the native form from tissue extracts by differential salt precipitation at neutral pH. Type III precipitates at about 1.5-1.7 M NaCl (Epstein, 1974; Chung and Miller, 1974), type I at about 2.2-2.5 M NaCl. Type II and IV still remain soluble in 2.5 M NaCl solution but they can be precipitated at 4-4.4 M NaCl (Trelstad *et al*., 1972; Burgeson *et al*., 1976). Besides NaCl, some other precipitants such as amonium sulfate and ethanol can also be used for collagen fractionation (Trelstad *et al*., 1976).

The collagen chains obtained by salt fractionation can be further separated and then purified by ion-exchange chromatography and molecular sieve chromatography (Piez, 1967). The most commonly used ion-exchanger is carboxymethyl cellulose (CM-cellulose), which contains negatively charged groups at neutral pH and is a cation exchanger. Collagen is absorbed to the CM-cellulose and the retained collagen chains are eluted with a linear salt gradient to decrease the binding of anionic proteins. With a linear gradient from 0-0.1 M NaCl, both α_1(II) and α_1(IV) chains elute slightly earlier than the α_1(I) chains (Trelstad *et al*., 1970; Kefalides, 1971; Trelstad *et al*., 1972; Miller, 1972). On the other hand, type III collagen is present normally in extracts as the disulfide crosslinked trimer, $[\alpha_1(III)]_3$, and is eluted in a region between

α_1(I) and α_2 (Epstein, 1974; Chung and Miller, 1974; Trelstad, 1974).

Amino acid analysis is important in identifying and comparing the isolated collagen chains. Sodium doedcyl sulfate (SDS)-gel electrophoresis is also an effective method for analytical determination of collagen species (Kefalides, 1971; Trelstad, 1974; Eyre and Muir, 1975b; Burgeson _et al._, 1976). Since the migration rate of a protein on polyacrylamide gels containing SDS is determined primarily by the molecular weight of the protein (Weber and Osborn, 1969), both α_1(II) and α_1(III) chains have the same electrophoretic mobility as α_1(I) chains (Eyre and Muir, 1975b; Burgeson _et al._, 1976). The α_1(IV) chains isolated from anterior lens capsule collagen have a molecular weight (M.W.) about 140,000 and migrate slower than α_1(I) chains on SDS-gels (Kefalides, 1971). Despite the same electrophoretic mobility of α_1(III) and α_1(I), type III collagen is usually present as a trimer in samples without prior reduction of disulfide linkages (Chung and Miller, 1974). Thus, comparing gel electrophoresis band patterns of collagen extracts before and after reduction of disulfide linkages may provide another means for detecting the presence of type III collagen. Recently, Bailey and Sims (1977) were able to separate α_1(III) from α_1(I) chains on SDS-polyacrylamide gels by changing the buffer from phosphate pH 7.2 to Tris-borate pH 8.5.

The cyanongen bromide (CNBr) peptides, arising from cleavage of methionine residues by CNBr are useful in amino acid sequencing and also provide important information for characterizing and

comparing α chains (McClain, 1973). The CNBr peptides are usually separated and isolated by CM-cellulose or phosphocellulose ion-exchange chromatography and molecular sieve chromatography. Recently, Eyre and Muir (1975a), Scott and Veis (1976), and Scott *et al*. (1976) identified and quantitatively determined the CNBr peptides of soluble and insoluble collagens in SDS-polyacrylamide gels. They also demonstrated the possibility of selecting well-resolved individual peptides as indicators for identification and quantitation of specific types of collagen.

In the presence of ATP at acid pH, collagen molecules precipitate from solution to form segment long spacing crystallites (SLS), in which all the collagen molecules are aligned in parallel with their ends in registration (Bruns and Gross, 1973). Positive staining of SLS with phosphotungstic acid and uranyl acetate gives a characteristic band pattern under the electronmicroscope. The band pattern of SLS corresponds closely with the distribution of charged and uncharged amino acid residues along the tropocollagen molecule (Chapman, 1974). It is likely that electron micrographs of SLS may provide another way for identifying and comparing different types of collagen, since collagen of different genetic origins differ also in their SLS band pattern (Trelstad *et al*., 1979; Bruns and Gross, 1973).

Biosynthesis of Collagen

The biosynthesis of collagen has been recently reviewed by

Fessler and Fessler (1978), Bornstein (1974), Miller and Matukas (1974), Martin _et al_. (1975), Dutson (1976), and Prockop _et al_. (1979).

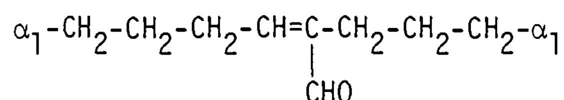
In general, collagen molecules are synthesized on a ribosomal complex containing mRNA, within the fibroblasts, osteoblasts and chondroblasts. The collagen protein is first synthesized as a precursor form, called procollagen, which contains additional peptide extensions on both ends of the chain (Tanzer _et al_., 1974; Davidson and Bornstein, 1975; Fessler and Fessler, 1978; Viidik and Vuust, 1980; Bornstein and Taub, 1979). Prior to secretion from the cell, these chains are further subjected to a series of post-translational alterations, including hydroxylation of certain proline and lysine residues by specific prolyl and lysyl hydroxylases and attachment of carbohydrate moieties to some of the hydroxylysine residues by galactosyl and glucosyl transferases (Bornstein and Taub, 1979). Both hydroxylation and glycosylation are initiated while the polypeptide chains are growing on the ribosomes and they are continued after the completed polypeptide chains are released from the ribosomes (Guzman _et al_., 1978; Fressler and Fressler, 1978). The three pro-α chains of the procollagen molecule are linked by interchain disulfide bonds, occurring at the carboxyl-terminal extension peptides (Byers _et al_., 1975; Fessler _et al_., 1975; Davison _et al_., 1975; Fessler and Fessler, 1978).

The complete triple helical procollagen molecule is secreted into the extracellular matrix, after which the excess terminal

peptides are removed by the action of procollagen amino and carboxy peptidase (Martin _et al_., 1975). Once terminal extension peptides are removed by cleaving, the molecule acquires the ability to aggregate into fibrils. The collagen fibrils are finally stabilized by the formation of covalent intra- and inter-molecular crosslinks.

Chemistry of Collagen Crosslinks

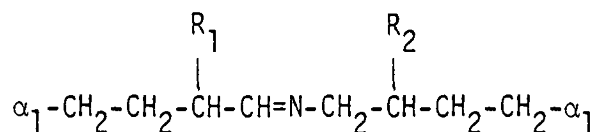
The intra-molecular crosslinks are produced in the nonhelical region of the collagen molecule (Traub and Piez, 1971) by joining two α chains to produce dimers designated β_{11} and β_{12} and three chains to produce a trimer designated γ_{112}. The initial stage in the formation of the intra-molecular crosslink involves the oxidative deamination of the ε-amino group of specific lysine residues by a copper requiring enzyme lysyloxidase to form reactive allysine, the δ-semialdehyde of α-aminoadipic acid (Gallop _et al_., 1972). Two allysine residues on adjacent chains can interact by aldol condensation to form the following unsaturated aldehyde:



$$\alpha_1\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH\text{=}\underset{\displaystyle CHO}{\underset{|}{C}}\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\alpha_1$$

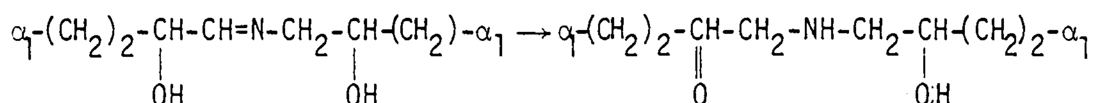
Aldehydes produced by the oxidative deamination of lysine or hydroxylysine can also interact with other lysine or hydroxylysine residues present in adjacent molecules within the fibrillar array to form the following Schiff base type of intermolecular crosslink

(Tanzer, 1973):



The R_1 and R_2 can be represented either H or OH. Four possible combinations of Schiff bases can be produced: lysinonorleucine (LNL), a reduced Schiff base product of lysine and allysine; dihydroxylysinonorleucine (DHLNL), a reduced Schiff base product of hydroxylysine and hydroxyallysine; hydroxylysinonorleucine (HLNL), a reduced Schiff base product, which can arise either from the condensation of lysine with hydroxyallysine or condensation of hydroxylysine with allysine.

The unreduced crosslink of dehydro-HLNL from hydroxylysine and allysine is a labile crosslink that is destroyed by heat, dilute acids and by certain aminothiols (Bailey _et al_., 1970), while the unreduced crosslinks of dehydro-HLNL, derived from hydroxylallysine and lysine, and dehydro-DHLNL are stable to heat and dilute acid. It has been suggested that the unreduced crosslinks of dehydro-DHLNL are probably stabilized by undergoing the Amadori rearrangement to form a more stable ketonic structure (Bailey _et al_., 1974):



Two allysine condensed aldol condensation products can produce

a more complex inter-molecular linkage by combining with the ε-amino group of hydroxylysine, generating hydroxymerodesmosine (Tanzer *et al*., 1973; McClain, 1976), two allysine condensed aldol condensation products also combine with the side chain of a histidine residue producing aldol histidine or with both histidine and hydroxylysine to give histidinohydroxymerodesmosine (HHMD) (Tanzer, 1973; McClain, 1976). HHMD was also called "fraction C" by Bailey (Bailey *et al*., 1970) who detected it in bovine achilles tendon and found it is one of the other labile crosslinks of collagen.

Age Variations in Crosslinkages and Meat Tenderness

It is well known that native collagen fibers show a progressive increase in tensile strength and insolubility during the process of collagen maturation. It has been shown that the percent of total collagen that is soluble is related with tenderness of meat from animals of different ages (Hill, 1966; Carmichael and Lawrie, 1967; Bailey, 1972), and the solubility of collagen is dependent upon the extent of crosslinking in the collagen (McClain, 1976). As the number of crosslinks increase with increasing animal age, the collagen is less soluble and the meat less tender (Goll *et al*., 1964; Carmichael and Lawrie, 1967; Herring *et al*., 1967).

Bailey (1972) reported that thermally denatured collagen from the young animal exudes from the meat during thermal contraction and sets into a gel on cooling, while in older animals all the denatured

collagen is crosslinked and is retained within the muscle. Shimokomaki et al. (1972) attributed the change in the tenderness of meat in older animals to an increase in the proportion of thermally stable bonds between collagen molecules. These authors have shown that the proportions of the reducible crosslinks in bovine muscle increased greatly during the first few months of growth, followed by a gradual decrease throughout the life span. Similar changes have been reported for human tendon collagen (Fujii and Tanzer, 1974). These authors have shown a progressive decrease in the content of the borohydride reducible crosslinks with age, and suggested this change is probably due to a conversion of reducible crosslinks to a stable non-reducible form.

Postmortem Changes in Connective Tissue Collagen

Due to the triple helical nature of collagen, it is generally assumed that proteolytic enzymes have little effect on native collagen. Sharp (1963) reported no differences in hydroxyproline extractability in samples of bovine muscle stored up to 172 hours at 37°C.

Herring et al. (1967) reported that collagen solubility did not increase during 10 days of postmortem aging. Despite the fact that release of hydroxyproline from connective tissue collagen does not increase, Kruggel and Field (1971) and Pfeiffer et al. (1972) noticed that structural changes occurred at the molecular level of collagen during aging. They reported that with increased postmortem time there was an increase in the number of detectable low

molecular weight collagen components. However, the nature of these low molecular weight components and the cause of the release was not made clear by these investigations.

Role of Lysosomal Enzymes in Connective Tissue Degradation

Lysosomes are a nearly universal cell organelle that contain hydrolytic enzymes which carry out the digestion of cellular parts and macromolecules which have yet to be digested or which resist digestion (Tappel, 1969; Barret, 1969).

The existence of lysosomes has been demonstrated in many muscle tissues (Parrish and Bailey, 1967; Canonico and Bird, 1970; Stagni and DeBernard, 1968; Ono, 1970), and it has been suggested that lysosomal enzymes are partly responsible for the proteolytic degradation of muscle components during postmortem aging (Dutson and Lawrie, 1974; Ono, 1970; Moeller *et al*., 1976, 1977).

The presence of collagenase and elastase activity in muscle lysosomes which are known to be responsible for the metabolism of collagen and elastin, respectively, has been reported by Goll (1965). Collagenase is the only enzyme known to have the ability to attack the helical region of the native collagen molecule under physiological conditions of temperature and pH (Gross and Nagai, 1965). It attacks the collagen molecule at a particular point about three-quarters from the N-terminal end of the molecule to form the characteristic TC_A (3/4 of the original length) and TC_B

(1/4 of the original length) fragments. These fragments can be further digested into smaller fragments by the collagenase itself.

More recently, some other enzymes have been purified and reported to have the ability to degrade connective tissue. Cathepsin D, the most active protease in skeletal muscle (Parrish and Bailey, 1967), has been reported to be the enzyme mainly responsible for the breakdown of cartilage proteoglycan at acid pH. Cathepsin D may also act synergistically with the collagenase to accelerate collagen degradation (Dingle _et al_., 1971).

Cathepsin B_1, the thio-dependent lysosomal protease, is able to degrade native collagen and cartilage proteoglycan at acid pH (Burleigh _et al_., 1974; Morrison _et al_., 1973). Another enzyme isolated from spleen neutrophils and referred to as cathepsin G has been shown to degrade collagen and cartilage proteoglycan at neutral pH (Burleigh _et al_., 1974). Other lysosomal enzymes such as hyaluronidase, β-glucuronidase, β-galactosidase (Aronson and Davidson, 1968; Dutson and Lawrie, 1974; Ono, 1970; Canonico and Bird, 1970; Kopp and Valin, 1980) may also play an important role in degrading the collagen mucopolysaccharide matrix.

Since postmortem conditions cause a rupture of the lysosomal membrane (Dutson and Lawrie, 1974; Moeller _et al_., 1976, 1977; Ono, 1970), it seems likely that some proteolytic breakdown of muscle connective tissue may occur during postmortem aging. However, since some of the connective tissue disruption by lysosomal enzymes was demonstrated at either low or high pH conditions (both

are unlikely to be present in postmortem muscle tissues), and cartilage, skin or tendon were used as substrates, the involvement of these enzymes in postmortem aging of muscle connective tissue is still questionable.

Effect of Pressurization on the Physicochemical Properties of Muscle

It is generally accepted that the degree of tenderness is greatly influenced by the state of the connective tissue and the contraction state of the fibrillar proteins (Bouton and Harris, 1972a,b; Marsh and Leet, 1966). Hydrostatic pressure has been reported to influence the development of tension in muscle (Johnson and Eyring, 1970). The effect of various hydrostatic pressures at different temperatures on pre-rigor samples of sheep and ox muscle has been reported (MacFarlane, 1973). His work indicates that pressures of 103-138 MN/m^2 at 30-35°C resulted in a rapid drop in pH, indicating that pressurization accelerated glycolysis, and that pressurization had a substantial tenderizing effect. It was suggested that the cause of the tenderizing effect of the pressure treatment was that myofibrillar structure was ruptured by thick filaments of the severely contracted muscle being forced into the Z discs.

Late in 1976, McFarlane *et al*. reported that pressurized pre-rigor beef muscle samples shortened by a mean of 40-42% and had fluid loss of 2-3% compared with samples that underwent thaw

rigor and shortened by a mean of 51% and had a fluid loss of approximately 16%. It was suggested that as well as contraction, an additional factor, possibly a weakening of filaments by pressure induced F-G actin transformation, may have occurred during the pressurization treatment.

Kennick _et al_. (1980) demonstrated that pressurization of pre-rigor muscles increased the rate of glycolytic reactions and improved tenderness significantly. The application of high pressure to homogenates of sheep meat in saline solutions increased water-holding capacity and promoted the solubilization of proteins (MacFarlane, 1974). The rate of pressure-induced solubilization of meat proteins depends on such factors as temperature, pH and the nature and concentration of salt (MacFarlane and McKenzie, 1976). These authors indicated that the amount of myofibrillar proteins solubilized by pressurization generally was greater at 0°C than 30°C, that KI salt was more effective than KCl and Na CH_3-COOH salts, and that a pH of 6 and higher showed a marked increase on the solubility of pressurized samples.

Bouton _et al_. (1977) have shown that pressure in the order of 100 MN/m^2 applied for 2.5 min or longer to post-rigor muscle heated to 40-60°C improved the tenderness of meat. They indicated that the tenderizing effect was similar to that obtained from pressurization of pre-rigor muscle. These authors reported that pressure-heat treatment was effective to overcome toughness associated with cold shortened muscle. They proposed that pressure causes

dissociation of the myofibrillar proteins and that further heating denatures the proteins so that they are unable to reassociate upon release of pressure.

The effectiveness of pressure-heat treatment in improving the tenderness of post-rigor muscle has been determined by using a combination of objective measurements (Warner-Bratzler peak shear, adhesion and Instron compression) and subjective assessment (Ratcliff et al., 1977).

The effect of hydrostatic pressurization on the protein quality has been reported by Elgasim and Kennick (1980). These authors demonstrated that pressurization does not affect the apparent biological value or net protein utilization of meat, but significantly improves protein digestibility. Bouton et al. (1978) have shown that pressure-heat treatment of connective tissue, had an opposite effect to heat-induced changes and concluded that the pressure-heat treatment produces tenderization equivalent to that obtained from heat treatment alone.

EXPERIMENTAL PROCEDURE

Muscle samples were obtained from ten steers slaughtered at the Oregon State University Meat Science Laboratory, using normal commercial procedures.

Pre-rigor sternomandibularis (STM) muscles were excised immediately after the dressed carcasses were split longitudinally and washed (within 1 hr of exsanguination). The treatment sample was removed from one side of the split carcass, vacuum-sealed in a Cry-O-Vac bag and placed in the pressure chamber (10.2 cm diameter and 30.5 cm long). The chamber was filled with warm water (35°C), closed and a pressure of 15,000 psi (103.5 MNm^{-2}) was applied for 2 min according to the procedure of Kennick and Elgasim (1980). The matching muscle (untreated) from the other side of the carcass served as control.

Intramuscular Connective Tissue Extraction

After being pressure-treated and trimmed of all adhering fat and connective tissue, muscles were ground through a 4.5 mm plate and the pH determination performed by using a probe electrode.

A modification of the procedure of Field (1970) described in Appendix A was used to extract crude intramuscular connective tissue (IMCT) from 50 g of each ground muscle sample. The isolated tissue was weighed and the yield expressed as a percent of the wet weight of muscle tissue. The moisture content was determined

by standard procedure (AOAC,1970).

The solubility (heat lability) of collagen was determined using a modification of the method of Hill (1966) outlined in Appendix B. The hydroxyproline content of the sample was determined colorimetrically according to the procedure of Neuman and Logan (1950) as described in Appendix C.

The isolated intramuscular connective tissue was solubilized by pepsin according to the procedure of Chung and Miller (1974), Appendix J. This solubilized material was separated by lithium dodecyl sulfate (LDS)-polyacrylamide gel electrophoresis (PAGE) as described by Broglie *et al*. (1980), Appendix J. Silver staining of the separated collagen components was performed by the procedures of Wray *et al*. (1981), Appendix J.

Determination of Lysosomal Enzyme Activities

Ten grams of each ground muscle sample was placed in 50 ml of 0.25 M sucrose solution containing 0.02 M KCl and centrifuged at 12,100 X G for 20 min at 3°C. Supernatants were collected and stored in Erlenmeyer flasks (capped) at 2°C. Samples to be used in the enzyme assays were taken from these stock solutions. These supernatants are referred to hereafter as the supernatant preparations.

After each centrifugation and removal of supernatants pellets were resuspended in 50 ml of the homogenizing solution containing 0.01% Trition X-100 (v/v) and homogenized for 20 sec at full speed

in a Virtis homogenizer. Homogenates were centrifuged at 12,100 X G for 30 min. Supernatants were stored in capped Erlenmeyer flasks at -5°C until assayed for enzymatic activity. These supernatants are referred to hereafter as the sediment preparation.

β-Galactosidase Activity

Supernatant fractions were assayed for β-galactosidase by a colorimetric procedure using lactose as substrate (Sigma Chemical Company, procedure for product No. G-6008) (Appendix D).

Cathepsin C Activity

Supernatants fractions were assayed for cathepsin C activity by the method of Goldberg and Rutenberg (1956) using Gly-Phe-2-naphthylamide as the substrate (Appendix E).

Catheptic Collagenase Activity

The method used to detect the presence of catheptic collagenase in the supernatant fraction was based on the procedure by Anderson (1969) with minor modifications (Appendix F).

Elastase Activity

Supernatants were assayed colormetrically for elastase activity using orecin-impregnated elastin as the substrate according to the procedure described by Sachar (1955) (Appendix G).

Activities of the above enzymes were also determined on the sediment fractions in the exact manner as for the supernatants fractions, except the reaction mixture contained 0.01% Triton

X-100 for all samples.

Protein concentration of the supernatants and sediments were determined by the biuret reaction of Gornall et al. (1949) (Appendix I). Determination of pH of supernatant and sedimentable fractions was performed immediately after they were obtained.

Incubation of Muscle with Specific Enzymes

Samples (4 g) from both freshly excised and pressure-treated STM muscles were suspended in 12 ml of one-fourth strength Ringer's solution homogenized, and divided into two groups. One group contained no added enzymes in the incubation solution, while the other group contained either collagenase (120 Sigma units) or collagenase (120 Sigma units) plus β-glucuronidase (250 Sigma units) and β-galactosidase (250 Sigma units). To determine the synergistic effect of β-glucuronidase and β-galactosidase on collagenase activity, samples were incubated in a water bath at 40°C for 1 hr. After incubation, samples were transferred to 77°C water bath and incubated for another 70 min to extract heat labile collagen.

The effect of each of these enzymes on muscle connective tissue was then determined by its ability to solubilize collagen (Hill, 1966) (Appendix B).

Incubation of IMCT with Specific Enzymes

IMCT samples (50 mg) were suspended in 12 ml of a one-fourth strength Ringer's solution and homogenized. One group of samples

contained no added enzymes in the incubation solution, while the other group contained either collagenase (120 Sigma units) or β-glucuronidase (250 Sigma units) plus β-galactosidase (250 Sigma units) and collagenase (120 Sigma units) to determine the synergistic effect of β-glucuronidase and β-galactosidase on collagenase digestability. The effect of these enzymes on IMCT was then determined by their ability to solubilize collagen (Hill, 1966) (Appendix B).

Amino Acid Analysis of IMCT

Samples for amino analysis were obtained according to the procedures described in Appendix A. Samples were hydrolyzed in 6 N HCL and prepared according to the procedure described in Appendix H. Amino acid analysis was carried out on a Beckman 120-B amino acid analyzer equipped with an automatic integrator at the Department of Biochemistry and Biophysics at Oregon State University.

Statistical Analysis

Data were analyzed using a paired t-test and the mean separation was prepared using Dunett Multiple Range test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Effect of Pressurization on Muscle pH

At the time of the initial sampling conducted immediately after completion of the pressure treatment (about 35 min postmortem), pH of the pressurized samples ranged from 5.62 to 5.82. The mean pH value was 5.71 for these 10 samples (Table 1). The mean pH value for the 10 control samples was 6.85. These values ranged from 6.45 to 7.10. Thus, the treated samples showed an average pressure-induced decline of 1.14 pH units which was significantly ($P < 0.05$) lower than that for the controls.

Pressure treatments have been shown to induce pH changes (Johnson *et al*., 1954). The drop in muscle pH following pressurization was found to be dependent on the amount of pressure, temperature used during pressurization and the time of treatment (MacFarlane, 1973). He also reported that a very rapid drop in pH was an indication that postmortem glycolysis was greatly accelerated by pressurization. Kennick *et al*. (1980) reported on pH changes in pressurized sheep longissimus dorsi and semimembranosus muscles and beef semitendinosus muscle. These authors indicated that the 1 hr pH of treated muscles was 5.80 which was very close to their 24 hr pH of 5.65 and the 1 hr pH of the controls was 6.50 which fell at a normal rate to pH 5.65 at 24 hr. They suggested that the 1 hr pH of the treated samples indicated that glycolysis was essentially completed by the end of the pressure treatment.

TABLE 1. Effect of pressurization on the pH of bovine sternomandibularis muscle[a]

Animal #	pH Pressurized	pH Control
1	5.82	6.85
2	5.65	7.00
3	5.55	6.75
4	5.80	7.00
5	5.66	7.10
6	5.69	6.86
7	5.76	7.00
8	5.62	6.65
9	5.75	6.83
10	5.75	6.45
Mean[b]	5.71 ± 0.09[c]	6.85 ± 0.20[d]

[a]Values determined on ground muscle.

[b,c,d]Means in the same row bearing different superscripts are significantly different ($P < 0.05$).

The pH changes in the present study with beef sternomandibularis muscle (Table 1) followed patterns similar to those reported by MacFarlane (1973) and Kennick et al. (1980).

Effect of Pressurization on Bovine Intramuscular Collagen

The collagen content of the 10 meat samples used to determine total collagen ranged from 7.98 to 12.23 mg/g (Table 2). The average collagen content was 9.39 mg/g. This value compares reasonably well with those reported by Hill (1966) who found approximately 1.02-1.64% collagen in bovine sternomandibularis muscle.

The effects of pressurization and incubation times on the percent of soluble collagen are shown in Table 3. Ten different samples from those above were used for this study. A slight increase in the mean value of percent soluble collagen was found with increasing incubation time, however, the differences were not significant ($P > 0.05$). Mean values for total collagen content and percentage of heat-extractable collagen from samples stored at different postmortem times from both the pressurized and control samples are presented in Table 3. The total amount of collagen was not significantly different among the treatment groups ($P > 0.05$).

The effect of pressure treatment and aging time on collagen solubility is shown in Figure 1. Pressurized samples showed a significantly higher percentage ($P < 0.05$) of heat labile collagen than control samples (Table 3). The collagen solubility data

TABLE 2. Total collagen content of meat samples from bovine sternomandibularis muscle[a]

Sample Number	Total collagen
1	9.60
2	8.47
3	12.23
4	8.74
5	8.83
6	8.88
7	7.98
8	8.52
9	11.33
10	9.33
Mean	9.39 ± 1.35

[a]Expressed as mg collagen/g sample.

[b]Average of two replications.

TABLE 3. Total collagen content and the percentage of heat labile collagen of bovine sternomandibularis muscle stored at 2°C for 72 hours

Sample No.	Total collagen[a]		Soluble collagen[b]	
0 Hrs	Pressurized	Control	Pressurized	Control
1	8.065	8.196	12.75	8.00
2	7.978	8.110	13.62	9.60
3	8.156	7.766	14.44	12.90
4	8.681	8.274	14.39	10.18
5	8.808	8.409	15.63	10.56
Mean	8.329 ±0.38[c]	8.151 ±0.24[c]	14.16 ± 1.07[d]	10.24 ± 1.77[e]
72 Hrs				
1	9.325	9.932	15.45	13.32
2	8.500	8.391	17.90	15.70
3	11.373	10.240	12.35	11.50
4	10.385	10.312	17.10	12.12
5	8.636	9.406	15.52	10.40
Mean	9.640 ± 1.22[c]	9.654 ± 0.79[c]	15.66 ± 2.12	12.60 ± 2.02

[a]Expressed as mg collagen/g sample.

[b]Yield of heat labile collagen as percentage of total collagen.

[c]Means for the total collagen content bearing a common superscript are not significantly different ($P > 0.05$).

[d,e]Means for the soluble collagen in the same row bearing a different superscript are significantly different ($P < 0.05$).

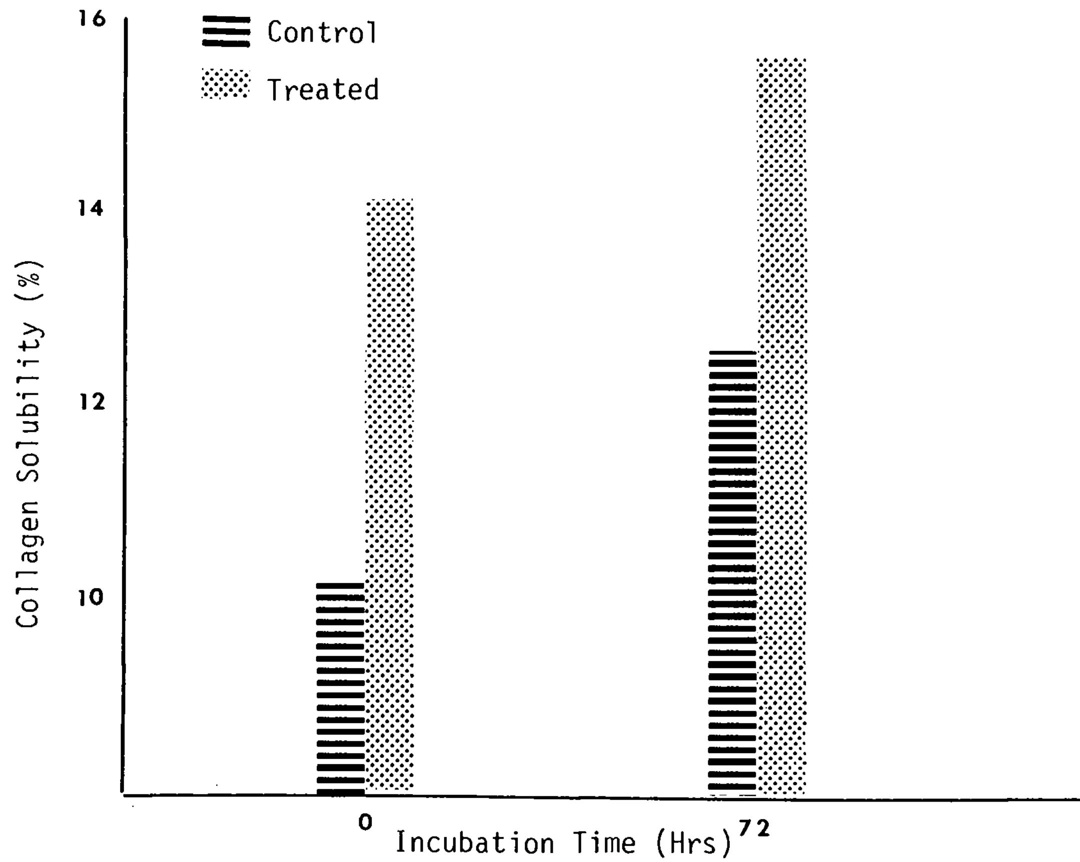


Figure 1. Effect of pressurization and incubation time on the percentage of heat labile collagen of bovine sternomandibularis muscle stored at 2°C

showed that control samples stored for 72 hr exhibited a collagen solubility similar to treated samples at 0 hrs. In agreement with the results in this study, Hill (1966) reported values approximately of 8.47 ± 0.97% heat labile collagen in sternomandibularis muscle from steers 22 months old. Herring et al. (1967) observed no difference between collagen solubility from tissue aged 5 to 10 days. Neither collagen solubility nor collagen content was significantly affected by postmortem contraction state. Similarly, Pfeiffer et al. (1972) reported that the percent of heat labile intramuscular and epimysial collagen obtained from normal and stretched muscle, aged 1 and 21 days, was not significantly different. In contrast to these reports, Kruggel and Field (1971) showed that intramuscular collagen from normal and stretched muscle aged 21 days had significantly less collagen solubilized by 5M guanidine hydrochloride solution than did intramuscular connective tissue (IMCT) from muscles aged 1 day.

Most of the above-mentioned studies indicated that intramuscular collagen from muscles subjected to aging and/or stretching had suffered structural changes at the molecular level. These changes were attributed to cleavage of crosslinks between the polypeptide chain of the collagen molecule. Bouton et al. (1978) observed a significant reduction in shear force simply due to modification of rigor bonds formed between the myosin heads and actin. However, from the results of present studies, it is likely that changes in connective tissue collagen may also have been a factor

in reduction of shear force of heat-pressure treated samples. High-pressure treatment has been shown in this study to accelerate release of lysosomal enzymes (Tables 4, 5, and 6) which may cause disruption of crosslinks involved between collagen molecules or between collagen and surrounding mucopolysaccharides.

It is well-established that mechanical modification of collagen fibers alters some of the physical characteristics of this muscle component. Rigby (1964) has shown that extension of rat tail tendon beyond 3% lowered its thermal shrinkage temperature, thus supporting the proposal that stretching of excised collagen is capable of producing structural changes in collagen. He postulated that this decrease was due to mechanical disruption of the polar, amorphous region of the molecule responsible for covalent bonding. His theory was that since the polar regions of the molecule were more disordered than the remainder, they would be weaker mechanically and the first to disrupt. Kruggel and Field (1971) also showed that stress could affect collagen crosslinkage and muscle tenderness.

Hydrostatic pressure has been reported to influence the development of tension in muscle (Johnson _et al_., 1954), the tension being increased or decreased according to the temperature at which the muscle was pressurized. Also, the tension-temperature relationship appears to be influenced by the animal species from which the muscle was obtained. The studies of Marsh and Leet (1966) indicated that muscle shortened by about 40% of its rest length should show maximum toughness. In contrast to their report, MacFarlane

TABLE 4. Total activity[a] and percent release[b] of β-galactosidase in pressurized bovine sternomandibularis muscle

Sample No.	Total activity		% Release	
	Pressurized	Control	Pressurized	Control
1	3.910	3.339	72.07	28.12
2	3.648	3.292	77.85	33.17
3	3.561	3.277	64.00	26.00
4	3.340	3.079	67.30	30.00
5	3.910	3.603	72.63	27.28
6	3.757	3.517	66.27	37.87
7	3.758	3.648	66.90	34.12
8	3.998	3.561	72.68	38.64
Mean[c]	3.735 ± 0.21 ★★	3.414 ± 0.19	69.96 ± 4.79 ★★★	31.90 ± 4.80

[a]Total activity represents the sum of activities in the supernatant and sedimentable fraction expressed as nanomoles of product released per min per ml of sample.

[b]Percent of total activity which is present in supernatant fraction.

[c]Level of significance between pressurized and control samples:

★ $P < 0.05$

★★ $P < 0.01$

★★★ $P < 0.005$

TABLE 5. Total activity[a] and percent release[b] of cathepsin C in pressurized bovine sternomandibularis muscle

Sample No.	Total activity		% Release	
	Pressurized	Control	Pressurized	Control
1	11.76	9.88	69.00	40.20
2	11.45	10.20	80.00	40.78
3	10.41	8.89	78.00	49.70
4	10.30	8.22	70.00	51.90
5	10.80	10.10	72.20	39.10
6	11.05	10.22	66.80	31.30
7	10.72	11.60	70.80	51.12
8	8.90	11.24	73.00	50.00
9	8.95	10.00	69.80	50.00
10	9.30	10.40	64.00	40.00
Mean[c]	10.36 ± 1.01	10.03 ± 1.03	71.36 ± 4.79 ★★★	44.40 ± 7.00

[a]Total activity representsthe sum of activities in the supernatant and sedimentable fraction expressed as nanomoles of product released per min per ml of sample.

[b]Percent of total activity which is present in supernatant fraction.

[c]Level of significant differences between pressurized and control samples: ★ $P < 0.05$; ★★ $P < 0.01$; ★★★ $P < 0.005$.

TABLE 6. Total activity[a] and percent release[b] of catheptic collagenase in pressurized bovine sternomandibularis muscle

Sample No.	Total activity		% Release	
	Pressurized	Control	Pressurized	Control
1	2.541	2.596	70.00	39.10
2	1.626	2.383	62.40	34.10
3	2.338	2.134	73.90	40.40
4	2.846	2.333	67.80	34.84
5	1.976	2.285	60.40	33.34
6	2.668	2.033	66.60	30.00
7	1.727	1.985	73.50	38.53
8	2.135	1.524	71.42	43.30
Mean[c]	2.232 ± 0.442	2.159 ± 0.324	68.25 ± 4.94 ★★★	36.70 ± 4.35

[a]Total activity represents the sum of activities in the supernatant and sedimentable fraction expressed as nanomoles of product released per min per ml of sample.

[b]Percent of total activity which is present in supernatant fraction.

[c]Level of significant differences between pressurized and control samples: ★ $P < 0.05$; ★★ $P < 0.01$; ★★★ $P < 0.001$.

(1973) reported that pressurized muscle shortened by 40% of its rest length had a shear value less than one-third that of muscle contracted by about 25%. He concluded that cold-shortened toughness could be overcome by pressurization. More recently, similar results were reported by Kennick _et al_. (1980) who observed muscle contraction of 30-50% of the on-carcass length upon pressurization. A highly significant reduction in shear force values for all pressurized muscles was also noted. They suggested rather strongly that pressurization improves tenderness significantly.

The existence of an interaction between contractile filaments and connective tissue has been suggested by Dranifield and Rhodes (1976). Rowe (1974) reported that two changes were taking place in the collagen network with muscle shortening. First, it appears that the collagen fibers were pulled out and lengthened at the expense of the crimp and, secondly, the angles of collagen fibers relative to the long axis of the muscle fibers changed with contraction.

Contraction of muscle fibers beyond the ability of the changing angles (Kennick _et al_., 1980) of inextensible collagen fiber of the endomysium to compensate for the increased diameter of the muscle fiber may cause physical breakdown of the collagen fibers. Kennick _et al_. (1980) showed that contraction caused by the pressure treatment decreased toughness associated with the endomysium and that pressurization may modify the structure of muscle fibers as well as the connective tissue.

From the results of this study and the above evidence, it appears that contraction induced by high pressure may play a role in increasing collagen solubility and reduce toughness associated with connective tissue.

Effect of pH on Yield of Intramuscular Connective Tissue

The initial yield of intramuscular connective tissue (IMCT) was 9.32% of the wet weight muscle tissue (Table 7). On a dry basis, this is equivalent to approximately 3.6% collagen (Table 8). This value compares reasonably well with those reported by Carmichael and Lawrie (1967) who found approximately 1.2-2.2% collagen in bovine longissimus dorsi muscle. It appears that the isolation method employed was effective in recovering most, if not all, of the IMCT from the muscles.

Data of Table 7 also reveal that the yield of bovine IMCT after pressurization was 6.64% which was approximately 50% lower than that of 9.32% for the control. This difference was significant at the 5% level of probability. This value agrees with the data reported by McClain _et al_. (1970) who found the yield of bovine IMCT at 24 hr had declined to a value approximately 50% lower than that at 0 hr. Similar data for porcine longissimus dorsi muscles at 0 and 72 hr postmortem were reported by Kauffman _et al_. (1964) and McClain _et al_. (1970). Data in Table 8 show that the pressure-induced drop in pH was accompanied by

TABLE 7. Yield of intramuscular connective tissue (IMCT) of meat samples from bovine sternomandibularis muscle

Animal No.	IMCT yield[a]	
	Pressurized	Control
1	9.00	12.00
2	8.00	10.50
3	7.60	9.30
4	4.70	9.00
5	5.20	8.00
6	6.30	8.70
7	5.30	9.20
8	4.60	7.40
9	7.50	7.80
10	8.20	11.30
Mean	6.64 ± 1.61[b]	9.32 ± 1.51[c]

[a]Expressed as percent of the wet weight of muscle tissue.

[b,c]Means in the same row bearing a different superscript are significantly different ($P < 0.05$).

TABLE 8. Effect of pressurization on the pH of bovine sternomandibularis muscle and on the yield of intramuscular connective tissue (IMCT)

Animal No.	IMCT yield[a]		pH	
	Pressurized	Control	Pressurized	Control
1	3.87	4.80	4.85	6.85
2	3.12	4.41	5.70	6.90
3	2.66	4.32	5.60	6.90
4	2.21	3.33	5.80	7.10
5	2.20	3.52	5.71	7.20
6	2.34	3.10	5.68	6.90
7	2.10	3.70	5.73	6.80
8	2.00	2.59	5.65	6.60
9	2.85	3.10	5.80	6.85
10	3.40	4.00	5.75	6.57
Mean	2.67 ± 0.62[b]	3.68 ± 0.69[c]	5.72 ± 0.80[d]	6.68 ± 0.27[e]

[a]Expressed as g IMCT dry weight basis.

[b,c]Means for the IMCT yield in the same row bearing a different superscript are significantly different ($P < 0.05$).

[d,e]Means for the pH in the same row bearing a different superscript are significantly different ($P < 0.05$).

a decrease in IMCT recovery. This finding is illustrated in Figure 2 which shows plots of yield versus pH. It appears that the effects of pressurization on the isolation of intramuscular connective tissue are similar to those observed when samples were aged post-mortem.

According to Le Chatelier's principle (Johnson *et al*., 1954) the application of pressure favors reactions that result in decreased volume of a system. One of the mechanisms they reported to be involved in a change of volume through which high pressure would be expected to influence the stability of a protein is by affecting the amount of zwitterion formation. Ionization is usually accompanied by a volume decrease; because of electrostriction, the water molecules are attracted closer to the charged ions and groups so as to make the total volume less than before ionization.

High pressure treatment induced significant changes in muscle pH as shown in Table 1. Changes in pH affect both the amount of ionization and the strength of hydrogen bonds. This results in a possible change of volume as well as conformational and configurational changes in muscle proteins.

The extracellular proteins, particularly collagen whose isoelectric point lies near pH 7.2 and provides the mechanical strength to muscle, undergo a change opposite to that of contractile proteins. It will be positively charged at the post-pressurization pH of muscle. The development of similar charges may contribute to unfolding of the triple helical structure of

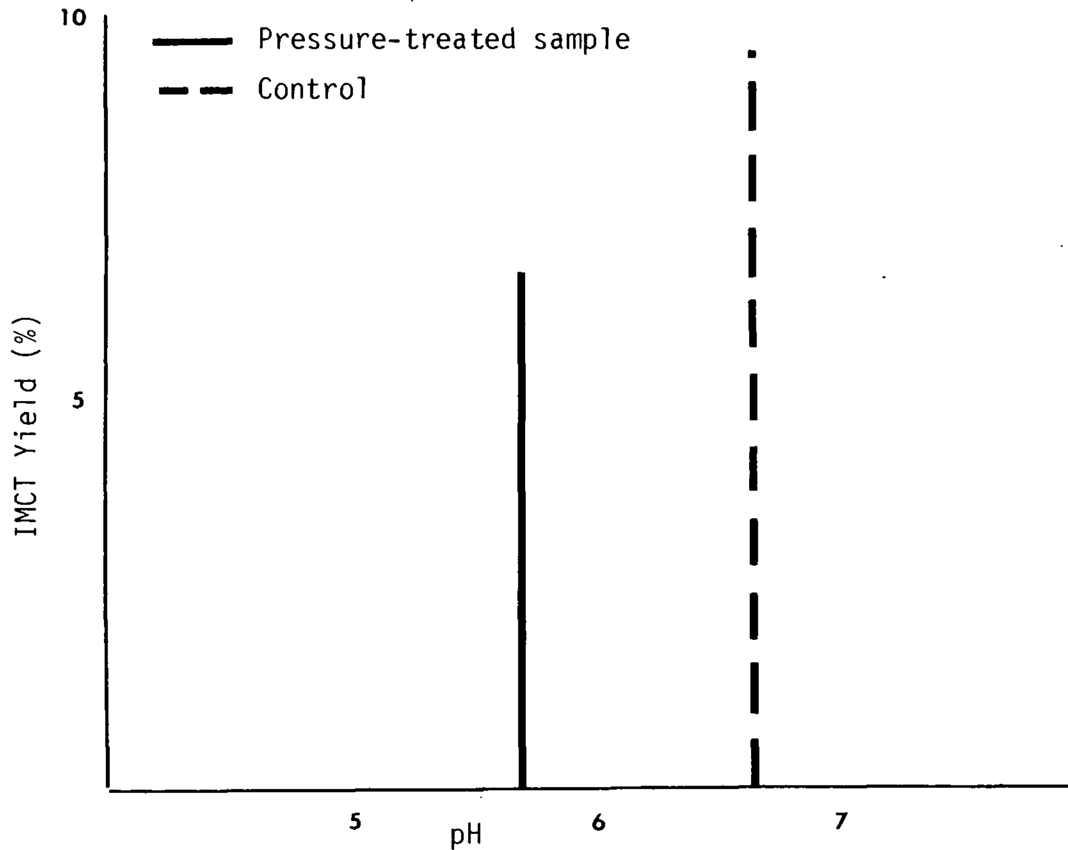


Figure 2. Relationship between pH and yield of intermuscular connective tissue from bovine sternomandibularis muscle*

*Note: Yield expressed as a percent of the wet weight of muscle tissue.

collagen by coulomb repulsion. Also, the accumulation of lactic acid may break the acid-labile electrovalent or ionic crosslinkages. This would make the collagen fibrils more pliable and soft. Consequently, these changes may expose those functional groups that were not previously available for binding water dipoles due to steric hindrance. In this process, hydration of collagen fibrils may occur if pH is less than 6.0, by absorbing free water dipoles displaced from intracellular myofibrils during pressure treatment. The absorption of Cl^- ions (present in the extracellular space) by cationic collagen fibrils may further facilitate the swelling and hence increase tenderness. Because the overall charge on the cell membrane is positive, anions like PO_4^{3-} released by enzyme catabolism of nucleotides and creatine phosphate would be expected to penetrate readily into the extracellular space and may contribute to the swelling of cationic collagen fibrils at a pH lower than 5.6. In addition to the volume changes, the structural changes in the polypeptide chains of collagen may allow H_2O to enter the crosslink region causing disruption of aldol linkages. This is possible because the aldols were originally subject to dehydration, resulting in formation of an α, β-unsaturated system (Fieser and Fieser, 1956). Roberts et al. (1971) have suggested that the α, β-unsaturated aldols can be reversed easily to free aldols by the addition of H_2O. Furthermore, it may be that conformational changes resulting from pressurization could allow enzymatic activity to occur. The normal helical structures of native

collagen are resistant to proteolytic degradation because of the tight packing of molecules within the fiber. A loosening of the network caused by swelling or stretching would allow enzymes to penetrate. Another possibility for the increase in collagen solubility in pressurized samples may be the result of structural changes combined with the low post-treatment pH may cause sufficient denaturation to allow some non-specific proteolytic activity. Houck et al. (1968) found proteases in sterile organ cultures of rat skin that solubilized 30% of the insoluble collagen when incubated at 56°C for 16 hr at a pH of 3.6. Whitaker (1964) stated that there are proteolytic enzymes with an optimal pH near 5.0 which are capable of hydrolyzing muscle proteins. Laakkonen et al. (1970) observed collagenase activity to be present in meat and concurred that some collagenolytic activity occurs in meat during aging.

The lysosomal enzymes cathepsin B_1 and collagenolytic cathepsin degrade native collagen by removing the telopeptides that carry the inter-chain crosslink (Etherington, 1974). This author found that cleavage of soluble, monomeric collagen occurs in the pH range of 3-6. The degradation of collagen in solution by cathepsin B_1 has been reported to occur at pH close to 5.4 (Burleigh et al., 1974). Etherington (1976) reported that cathepsin B_1 and collagenolytic cathepsin from bovine spleen act synergistically, and there might be differences between their specific action on the collagen substrate. Dingle et al. (1971)

indicated that cathepsin D is the enzyme mainly responsible for the breakdown of proteoglycans during the autolysis of chicken and rabbit cartilage. Fruton and Mycek (1956) reported that the pH optimum for cathepsin is near 7, although the activity of the enzyme does not fall off sharply in the pH range of 4.5-7.5. Kopp and Valin (1981) have shown that the incubation of collagen fibers with muscle lysosomal enzymes at 37°C, pH 5.5, affects the thermal stability of muscle collagen significantly, increasing the thermolabile fraction. They suggested that the muscle lysosomal fraction contains an enzyme system which can reduce the strength of muscle collagen at pH 5.5. It appears from the above evidence and the results shown in Tables 3, 9 and 10 and Figure 1 that an increase in the amount of soluble collagen in this study may be due to a combination effect of molecular changes in IMCT brought about by mechanical pressure and a low pH (5.8) in muscle and enzymatic activity, specifically lysosomal enzymes.

This portion of the present investigation has served to emphasize that the intramuscular connective tissues are altered during pressurization. It is possible that the connective tissue proteins are changed physically by the pressure and by the drop in pH, becoming more susceptible to fragmentation and thus resulting in a diminishing yield of recovery. In addition to these effects, the degradative action of cathepsin B_1 and collagenolytic cathepsin have been shown to be responsible for the release of collagen monomers from native collagen (Burleigh *et al.*, 1974;

TABLE 9. Effect of β-galactosidase, β-glucuronidase and collagenase on collagen solubility

Enzyme added	Number of samples	Collagen solubility[a]	
		Pressurized	Control
Collagenase[b]	6	35.56 ± 3.14[f]	30.77 ± 3.30[g]
Collagenase[b] plus β-galactosidase[c]	6	37.49 ± 2.53[f]	31.72 ± 2.46[g]
Collagenase[b] plus β-galactosidase[c] plus β-glucuronidase[d]	6	38.80 ± 1.98[f]	33.94 ± 2.15[g]★[e]

[a]Values are means ± standard deviation, collagen solubility is expressed as a percentage of total collagen.

[b]Contained 120 Sigma units of collagenase/4 g of muscle sample.

[c]Contained 250 Sigma units of β-galactosidase/4 g of muscle sample.

[d]Contained 250 Sigma units of β-glucuronidase/4 g of muscle sample.

[e]Means in the same column are the mean differences in collagen solubility as compared with collagenase only added samples: ★ $P < 0.05$.

[f,g]Means in the same row bearing a different superscript are significantly different ($P < 0.05$).

TABLE 10. Effect of β-galactosidase, β-glucuronidase and collagenase on isolated IMCT solubility

Enzyme added	Number of samples	Collagen solubility	
		Pressurized	Control
Collagenase[b]	6	67.62 ± 1.28	64.62 ± 1.78
Collagenase[b] plus β-galactosidase[c]	6	69.63 ± 1.38★	66.39 ± 1.88
Collagenase[b] plus β-galactosidase[c] plus β-glucuronidase[d]	6	70.96 ± 0.85★[f]	66.91 ± 2.03★[g]

[a]Values are means ± standard deviation, collagen solubility is expressed as a percentage of total collagen.

[b]Contained 120 Sigma units of collagenase/50 mg of IMCT sample.

[c]Contained 250 Sigma units of β-galactosidase/50 mg of IMCT sample.

[d]Contained 250 Sigma units of β-glucuronidase/50 mg of IMCT sample.

[e]Means in the same column are the mean differences in collagen solubility as compared with collagenase only added samples: ★ P 0.05.

[f,g]Means in the same row bearing a different superscript are significantly different ($P < 0.05$).

Etherington, 1976). These activities may have increased the susceptibility of collagen to depolymerization by other proteases at acidic pH. Since these collagen fragments are soluble in neutral salt solutions (Eisen et al., 1968), it is likely that the decrease in yield of IMCT may be caused by a loss of connective tissue during the isolation procedure.

Effect of Pressurization on the Molecular Components of IMCT

Intramuscular collagen components were isolated after limited pepsin digestion. The electrophoretic banding pattern of untreated samples revealed a characteristic mammalian collagen chain composition. The presence of α_1, α_2, β_{11}, β_{12} chains in addition to the γ and higher molecular weight components are evident in Figure 3.

Electrophoretic analysis (Figure 3) showed a high amount of γ-chains and higher molecular weight components which are the main components of type III collagen. Also observed was a high concentration of α_1- and α_2-chains indicative of the presence of type I collagen. These two types of collagen are present in muscle tissue (Bailey and Sims, 1977).

The above results suggest that the pepsin-solubilized collagen from IMCT is a mixture of different types of collagen. Despite some similarities in the electrophoretical properties observed between pressurized and untreated samples, the electrophoretic pattern of the pressurized sample showed a component with a high

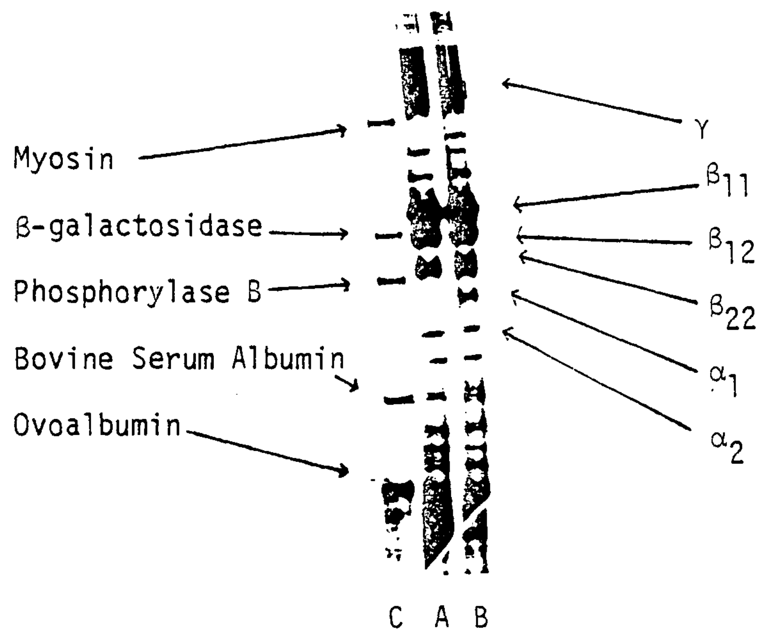


Figure 3. Lithium dodecyl sulfate polyacrylamide gel gradient (7.5-15%) electrophoresis of pepsin-solubilized bovine intramuscular collagen [(a) unpressurized; (b) pressurized; (c) standards]

molecular weight of approximately 160,000, and a low molecular weight component of approximately 80,000 (Figure 3). Both of these components were absent in the electrophoretic pattern of the untreated samples. These results indicate that the increase in number of low molecular weight components was accompanied by a decrease in high molecular weight chains. The possible reduction of some disulfide intrachain crosslinks and/or decrease in resistance to peptide digestion apparently occurs with pressure treatment.

Although the nature of the causes of collagen changes is not completely clear, Dutson and Lawrie (1974), Moeller _et al_. (1976) and Ono (1979) have shown that postmortem conditions cause a rupture of the lysosomal membrane. Disruption of these membranes is greatly enhanced by high temperature conditioning (Moeller _et al_., 1976, 1977). It has been reported that cathepsin D is able to hydrolyze proteoglycans (Dingle _et al_., 1971) and that cathepsin B_1 can degrade collagen by attacking the crosslinks in the non-helical region of collagen molecule to give a β- to α-conversion (Burleigh _et al_., 1974). Also a collagenolytic lysosomal enzyme has been shown to depolymerize and solubilize the polymeric collagen fibrils at an acid pH (Etherington, 1976). Other lysosomal enzymes such as hyaluronidase, β-glucuronidase and β-galactosidase also may have important roles in degrading the collagen mucopolysaccharide matrix (Aronson and Davidson, 1968; Dutson and Lawrie, 1974; Ono, 1970; Canonico and Bird, 1970). Thus, it is possible that lysosomal enzymes are responsible for the post-pressurization

degradation of muscle collagens. This could result in the release of more collagen components from collagen fibers during postmortem aging either by breaking collagen crosslinkages or by breaking the interaction between tropocollagen and the surrounding ground substance.

Effect of Pressurization on the Release of Lysosomal Enzymes

The objective of this phase of the study was to determine the influence of pressurization on the amount of free and bound lysosomal enzyme activities and to determine the effect that changes in lysosomal enzyme activities may have on connective tissue.

Mean pH values determined from aliquots of crude enzyme preparations are shown in Table 11. A rapid rate of pH decline ($P < 0.05$) was observed at 2 hr postmortem for pressurized samples (pH 5.6) as compared to the controls (pH 6.67). This is in agreement with the results of MacFarlane (1973) and Kennick et al. (1980) who found an increased rate of postmortem glycolysis associated with pressure treatment; thus, a faster rate of pH decline.

In the present study, no differences in pH values were observed between pressurized samples and the controls once the optimal pH was reached (after 24 hr of storage).

Activities of β-galactosidase, cathepsin C, catheptic collagenase and elastase were determined from samples collected after pressurization. The activity of the supernatant fraction

TABLE 11. Effect of pressurization on pH values of bovine sternomandibularis muscle homogenates[a,b]

Time	Pressurized		Control	
	Free enzyme supernatant	Sedimentable fraction	Free enzyme supernatant	Sedimentable fraction
2 Hr	5.60 ± 0.10[c]	5.50 ± 0.13[c]	6.67 ± 1.89[d]	6.57 ± 0.11[d]
24 Hr	5.43 ± 0.03	5.41 ± 0.04	5.50 ± 0.98	5.44 ± 0.08

[a]Determined on supernatant and sedimentable fractions.

[b]Means obtained from 10 samples.

[c,d]Means on the same row bearing a different superscript are significantly different ($P < 0.05$), compared with the treated samples.

represents the amount of enzymes released from the lysosomes (free activity) while the sedimentable fraction is indicative of the amount of enzyme retained within membranous structures (bound activity). The sum of the activities in the supernatant and sedimentable fractions (after membrane disruption by Triton X-100) was used to obtain total activity and represents the activity of 1 ml of sample supernatant. Percent free activity is the percent of total activity present in the supernatant fraction.

The specific activity of β-galactosidase, cathepsin C, catheptic collagenase are shown in Tables 12, 13 and 14, respectively. The paired t-test was used to determine statistical differences between the mean enzyme activity of the pressurized and control samples. The data also are plotted in Figures 4, 5 and 6, as the means of eight animals for β-galactosidase, catheptic collagenase, and ten animals for cathepsin C.

The free specific activities (supernatant fraction) of β-galactosidase and catheptic collagenase obtained from pressurized samples were significantly higher ($P < 0.01$) than those of the controls. The sedimentable specific activities (particulate fraction) were significantly lower in the pressurized samples (Tables 12 and 13, and Figures 4 and 6) than the controls. An increase in free enzyme-specific activities observed in the pressurized samples suggested that a larger portion of the enzyme was released during pressurization.

Similar to the differences between pressurized and control

TABLE 12. Specific activities of β-galactosidase[a] in pressurized bovine sternomandibularis muscle

Sample No.	Free activity		Sedimentable activity	
	Pressurized	Control	Pressurized	Control
1	447.00	303.00	245.00	308.00
2	437.00	266.00	207.00	272.00
3	364.00	286.00	288.00	375.00
4	357.00	250.00	230.00	369.00
5	436.00	309.00	267.00	327.00
6	301.00	213.00	278.00	276.00
7	333.00	237.00	246.00	338.00
8	337.00	225.00	273.00	312.00
Mean[b]	376.50 ± 55.8 ★★★	261.12 ± 35.8	254.25 ± 27.0 ★★★	322.12 ± 38.2

[a]Specific activity of β-galactosidase is expressed as picomoles of product released per min per mg protein.

[b]Level of significant difference between pressurized and control samples: ★ $P < 0.05$; ★★ $P < 0.01$; ★★★ $P < 0.005$.

TABLE 13. Specific activities[a] of cathepsin C in pressurized bovine sternomandibularis muscle

Sample No.	Free activity		Sedimentable activity	
	Pressurized	Control	Pressurized	Control
1	1.306	1.276	0.795	0.761
2	1.410	1.016	0.587	0.745
3	1.289	1.451	0.520	0.699
4	1.173	1.138	0.612	0.682
5	1.205	1.130	0.755	0.768
6	1.087	1.050	0.833	0.858
7	0.921	0.953	0.686	0.718
8	0.833	1.071	0.504	0.792
9	0.726	0.819	0.677	0.714
10	0.789	0.761	0.686	0.880
Mean[b]	1.073 ± 0.24	1.066 ± 0.20	0.665 ± 0.11 ★	0.760 ± 0.06

[a]Specific activity of cathepsin C is expressed as nanomoles of product released per min per mg protein.

[b]Level of significant difference between pressurized and control samples: ★ $P < 0.05$; ★★ $P < 0.01$.

TABLE 14. Specific activities[a] of catheptic collagenase in pressurized bovine sternomandibularis muscle

Sample No.	Free activity		Sedimentable activity	
	Pressurized	Control	Pressurized	Control
1	215	163.00	168	200.00
2	195	154.00	128	221.00
3	200	141.00	152	181.00
4	254	154.00	191	214.00
5	175	239.00	173	184.00
6	329	174.00	164	177.00
7	195	217.00	96	210.00
8	242	176.00	128	135.00
Mean[b]	225 ± 49.2 ★	177.05 ± 22.8	150 ± 30.7★	190.20 ± 27.6

[a]Specific activity of catheptic collagenase is expressed as picomoles of product released per min per mg protein.

[b]Level of significant difference between pressurized and control samples: ★ $P < 0.05$; ★★ $P < 0.01$.

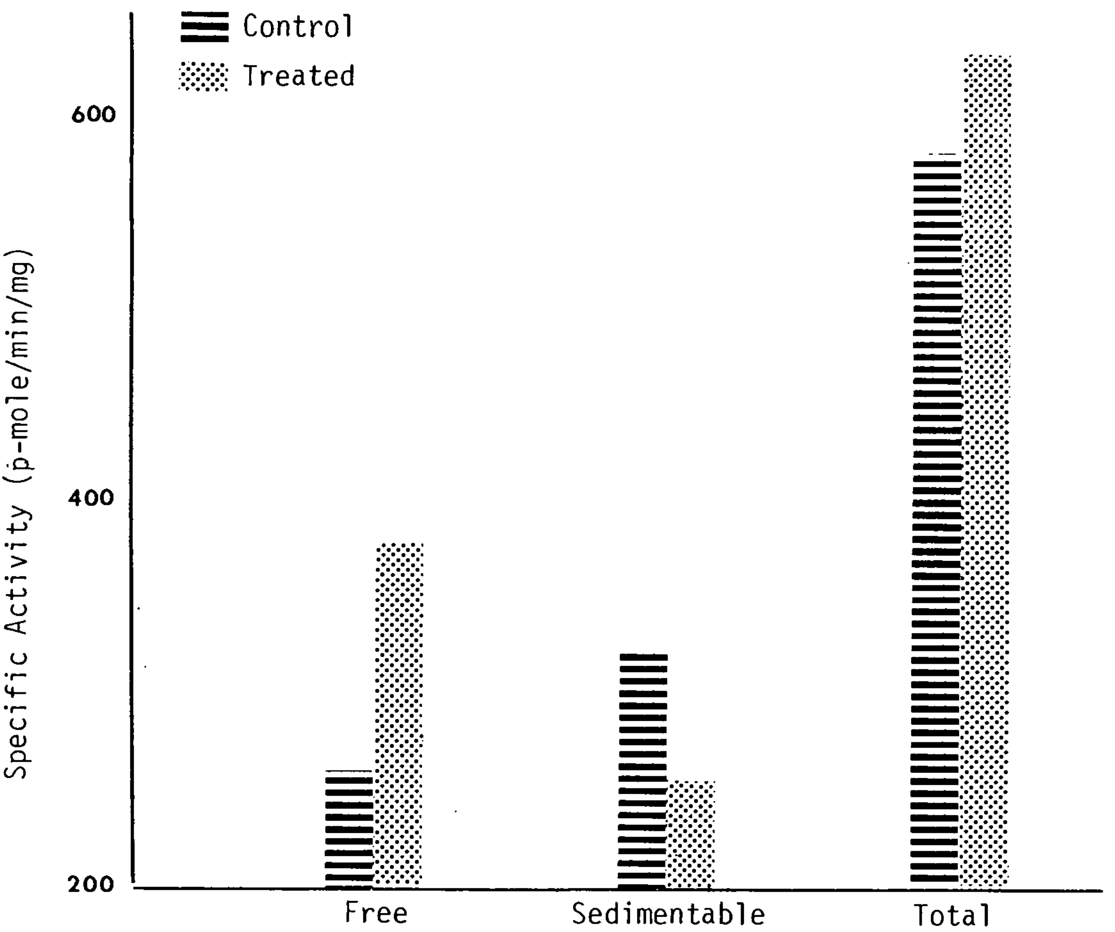


Figure 4. Specific activities of β-galactosidase in pressurized bovine sternomandibularis muscle

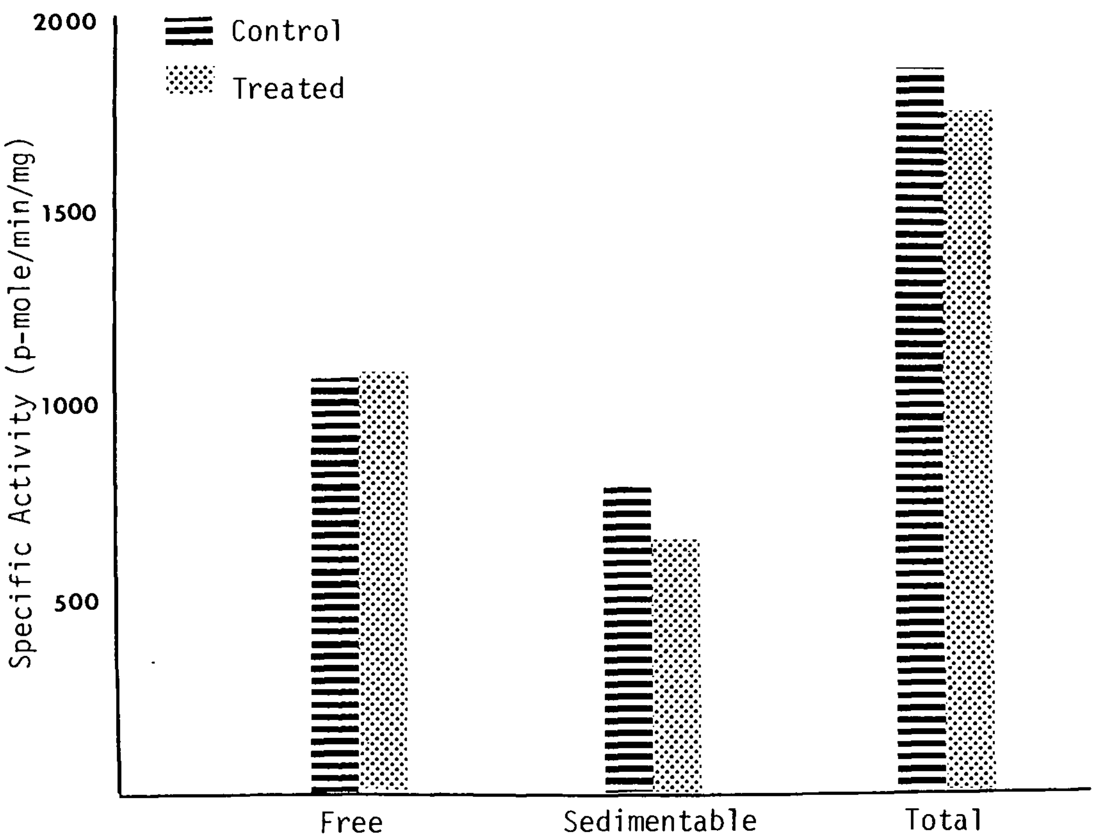


Figure 5. Specific activities of cathepsin C in pressurized bovine sternomandibularis muscle

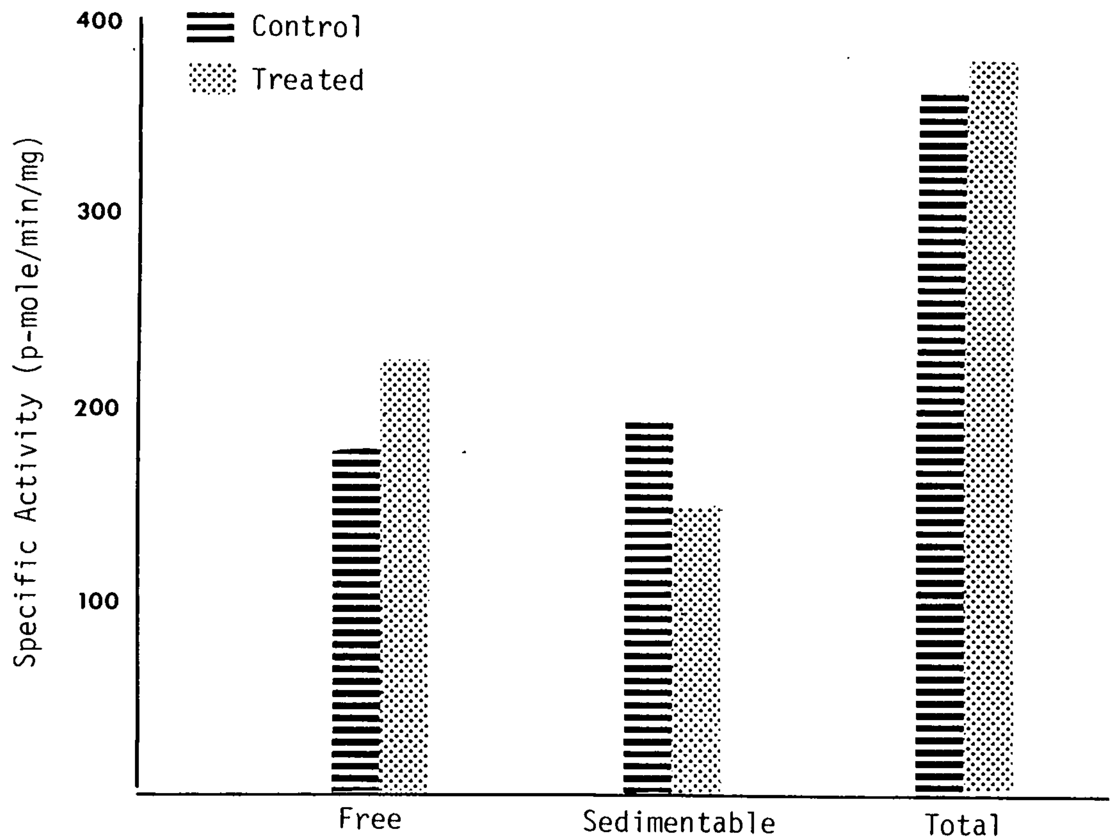


Figure 6. Specific activities of catheptic collagenase in pressurized bovine sternomandibularis muscle

samples in the specific activities of β-galactosidase and catheptic collagenase from sternomandibularis muscle, the specific activity of cathepsin C was less in the sedimentable fraction for pressurized and control samples (Table 3). In contrast, the free specific activity of cathepsin C in the supernatant fractions obtained from pressurized samples was essentially equal to that of the control samples. Differences in the specific activity of cathepsin C in the sedimentable fractions were significantly different ($P < 0.05$) between the pressurized and control samples.

Table 15 shows the differences between pressurized and control samples for the specific activity of elastase. Due to the low level or absence of specific activity in several samples, these data are inconclusive. One comment should be made about the method for the determination of elastase activity. Although some pressurized samples gave small absorbance readings in the elastase assay, the values were outside the range of reliability of the spectrophotometer. Table 15 shows the extent of hydrolysis for some samples but not for all.

It should be noted that the free and total activity of β-galactosidase, cathepsin C, and catheptic collagenase were greater in the pressurized samples than in the controls (Figures 7, 8 and 9). However, the sedimentable activity was higher in the control samples. This increase in free activity could be due to a faster release of enzymes bound to membranes or to an increase in enzyme activity caused by pressurization or a combination of both.

TABLE 15. Specific activities[a] of elastase in pressurized bovine sternomandibularis muscle

Sample No.	Free activity		Sedimentable activity	
	Pressurized	Control	Pressurized	Control
1	0.038	0.029	0.021	--
2	0.034	0.008	0.009	--
3	0.038	0.030	0.012	--
4	0.044	--	0.015	--
5	0.027	--	--	--

[a]Specific activity of elastase is expressed as mg of product released per 20 min per mg protein.

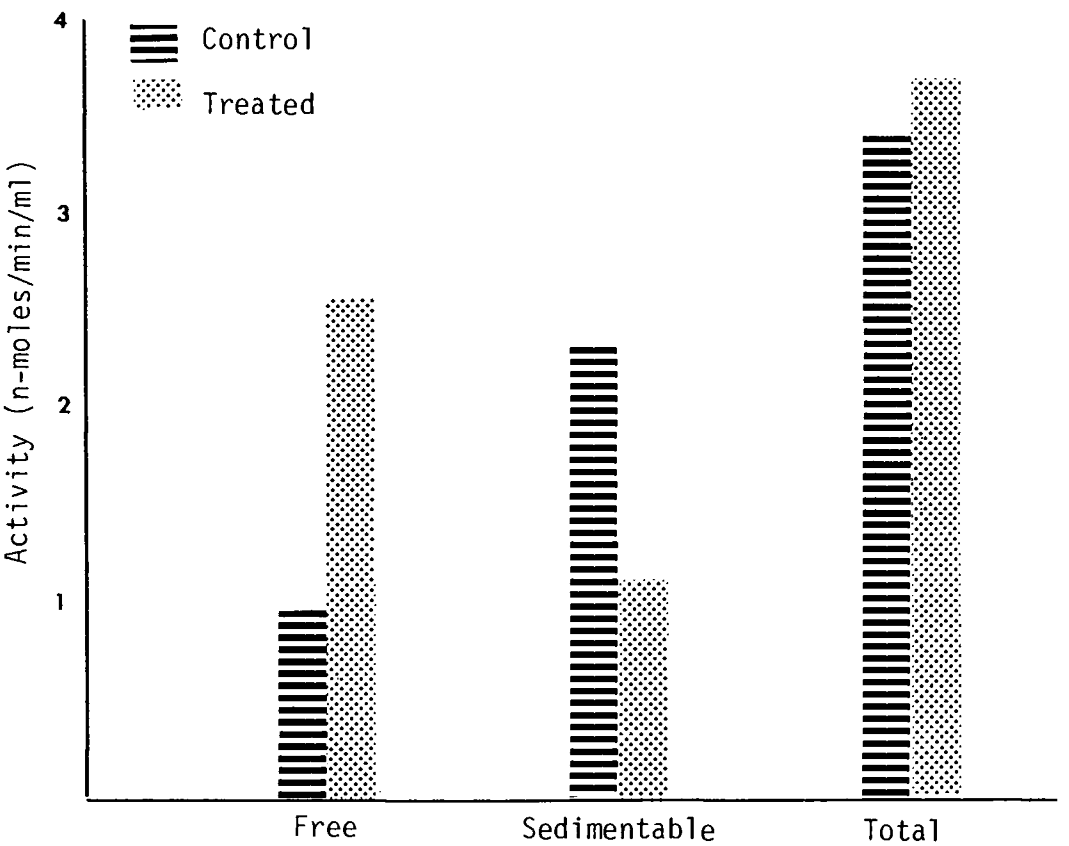


Figure 7. Activities of β-galactosidase in pressurized bovine sternomandibularis muscle

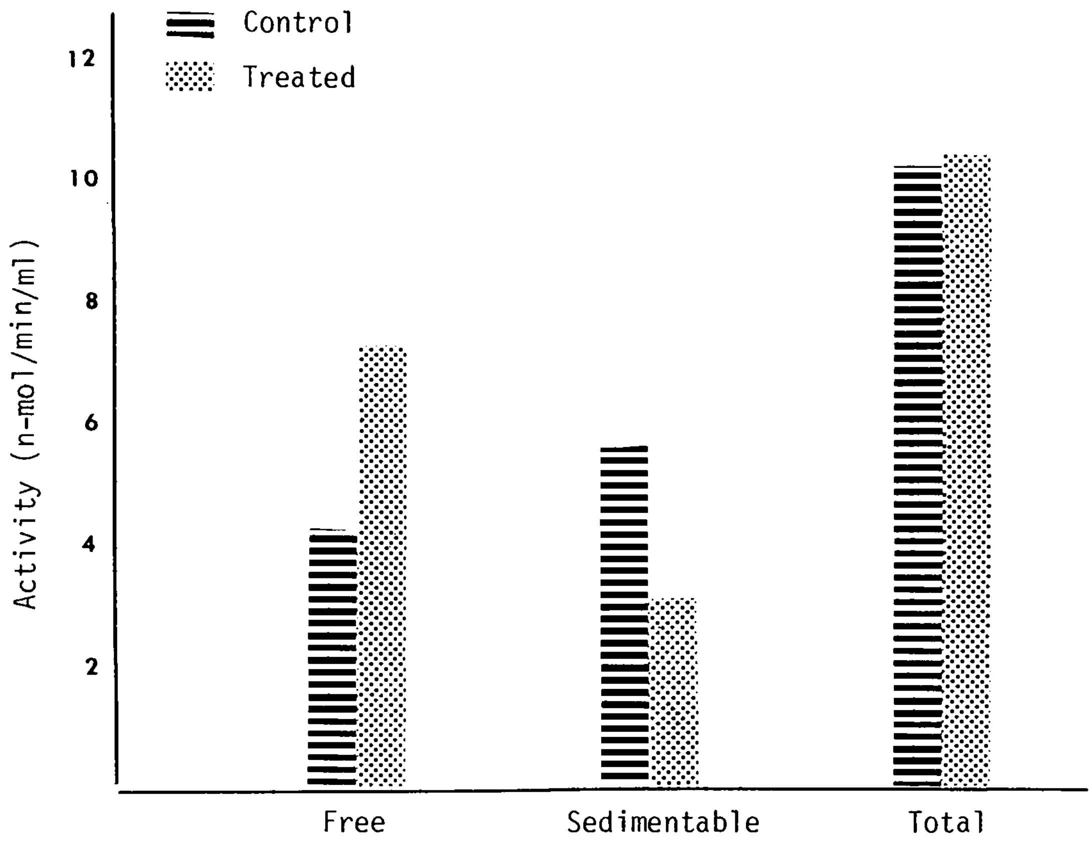


Figure 8. Activities of cathepsin C in pressurized bovine sternomandibularis muscle

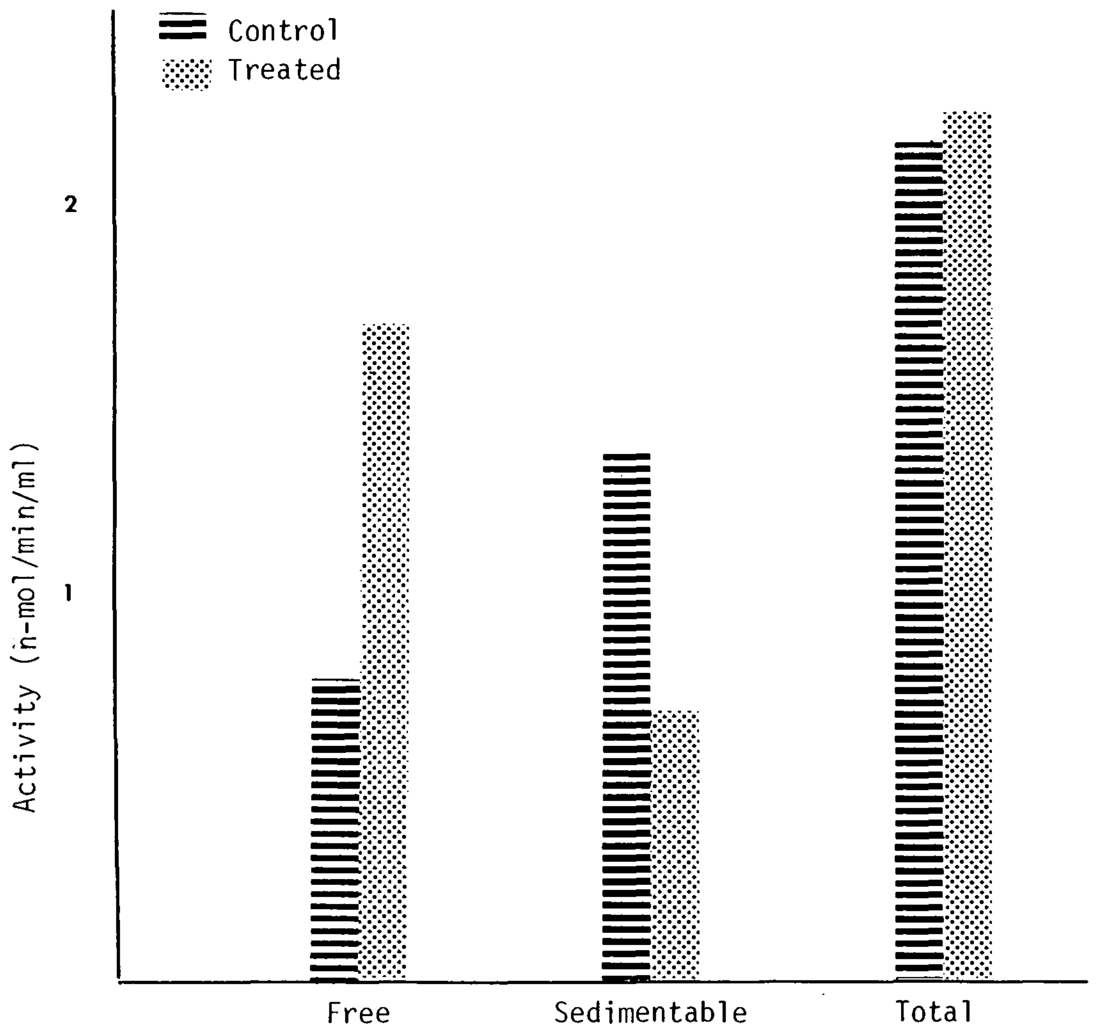


Figure 9. Activities of catheptic collagenase in pressurized bovine sternomandibularis muscle

Total activity of catheptic collagenase and cathepsin C were slightly higher (Tables 5 and 6, and Figures 8 and 9) in the pressurized than in the control samples. A significant ($P < 0.01$) increase in total activities of β-galactosidase (Table 4 and Figure 7) was observed in the pressurized samples.

The release of β-galactosidase, cathepsin C and catheptic collagenase from the pressurized and control samples are presented in Tables 4, 5, and 6, respectively. The data also are plotted in Figures 10, 11 and 12. The percent of free enzyme activity increased rapidly to a value about two-thirds of the total activity after pressurization. The proportion of activity in the sedimentable fraction for the control samples was similar for both β-galactosidase and catheptic collagenase (Figures 7 and 9); however, the release of cathepsin C (Figure 11) from control samples exhibited a different trend than that observed for β-galactosidase and catheptic collagenase. These results are similar to those reported by Balasubramaniam and Deiss (1966) who observed a different rate in the release of acid phosphatase, arylsulfatase and ribonuclease from lysosomes. These investigators attributed the variation in the release of enzymes from the lysosome to differences in binding properties of these enzymes within the lysosome.

It was established earlier by Beaufay and deDuve (1959) that treatments which cause mechanical breakage of the membrane will lead to the "activation" of lysosomal enzymes. They also observed that the autolytic breakdown (thermal activation) which occurs

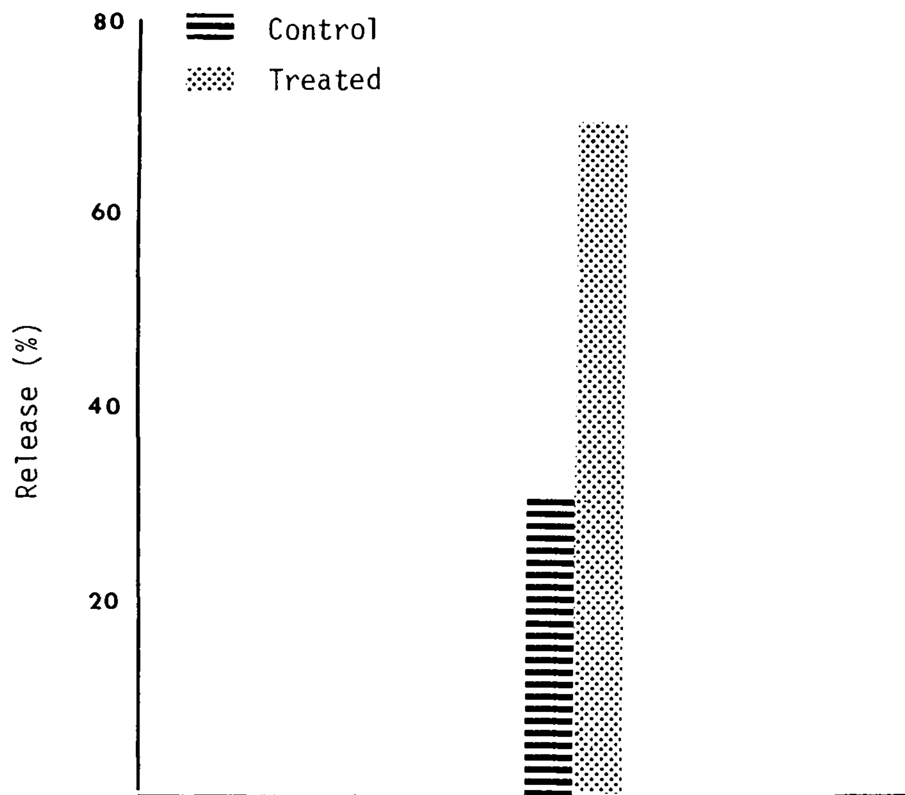


Figure 10. Release of β-galactosidase activity in pressurized bovine sternomandibularis muscle

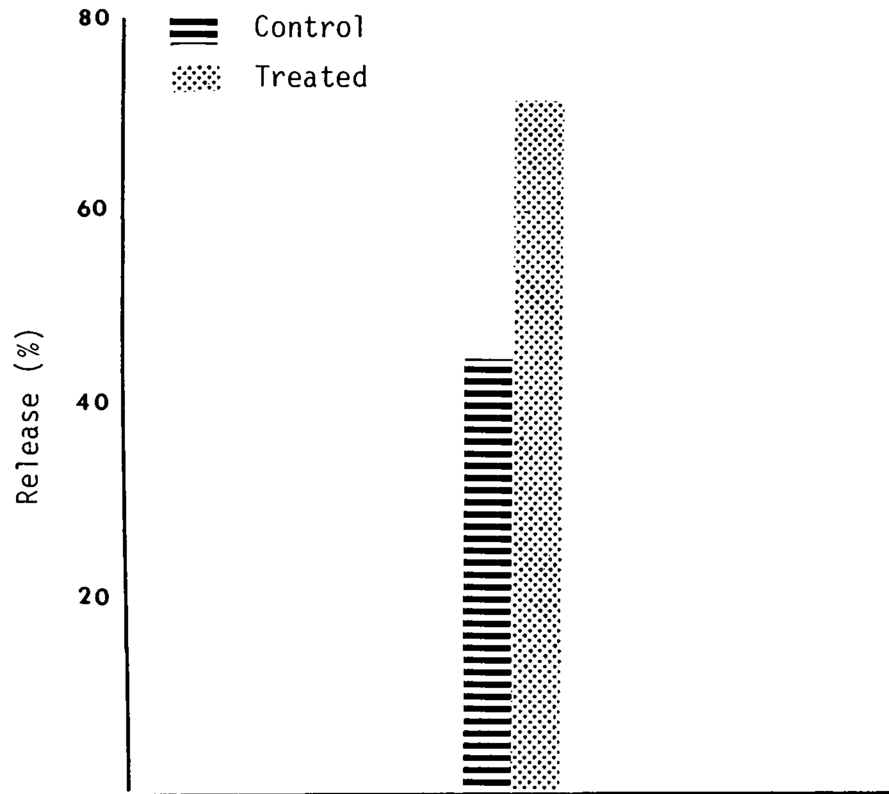


Figure 11. Release of cathepsin C activity in pressurized bovine sternomandibularis muscle

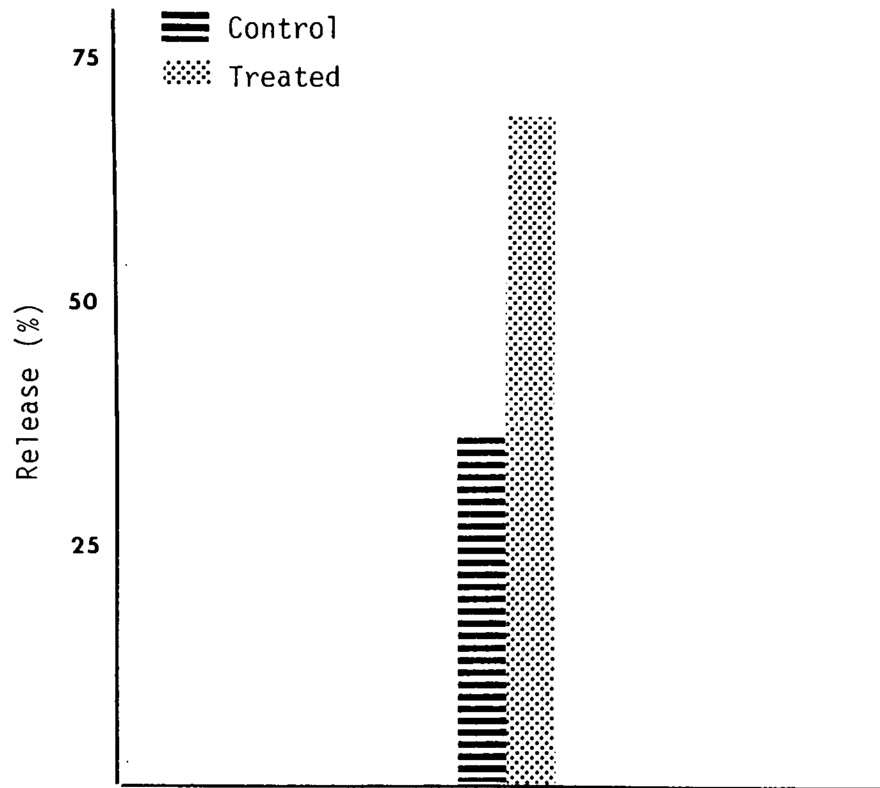


Figure 12. Release of catheptic collagenase in pressurized bovine sternomandibularis muscle

when particles are incubated at 37°C is favored by a low pH. The results from this study and previous studies by MacFarlane (1973) and Kennick et al. (1980) provide sufficient evidence that pressurization causes a very rapid drop in muscle pH.

The relation between pH and the availability of lysosomal enzymes has been discussed by Sawant et al. (1964). They reported that the lysosomal membrane has the characteristics of a charged membrane. Therefore, the increased availability of lysosomal enzymes (free enzymes) in the supernatant after pressurization may indicate that a positive charge on the protein part of the membrane at acid pH (pH 5.6) might be associated with this increased availability. Collagenase, β-galactosidase and cathepsin C were more available at acid pH, thus these enzymes might be bound to charged groups of the membrane. Similar results have been reported by Moeller et al. (1976, 1977). These authors observed a significant increase in the β-glucuronidase and cathepsin C unsedimentable activities when longissimus dorsi muscles samples were held at 37°C for 12 hr at pH 5.4.

Dutson and Lawrie (1974) observed that the specific activities of free β-glucuronidase decreased. From the above discussion it can be seen that a low pH in conjunction with high temperatures enhances the alteration of the lysosomal membrane and a faster release of acid hydrolases into the muscle tissue as was shown by a rise in the percentage of free enzyme activity (i.e., activity in the supernatant of the pressurized samples in this study).

One reason for the high activity in the supernatant of pressurized samples may be that disruption of the membrane occurs during pressurization leading to the activation of lysosomal enzymes. Another reason may be a greater increase of K, Na, Ca, Zn, and Fe ions in the sarcoplasmic fluid than in myofibrillar tissue; calcium ion is an activator of collagenase and the sodium ion is an activator of β-galactosidase. This increase is not surprising since with post-pressurization decrease in muscle pH, cations tend to dissociate from myofibrils because of weakening of the electrostatic force. Several workers (Arnold et al., 1956; Wierbicki et al., 1957) have found that Na, Ca, and Mg ions are released from the myofibrils during aging. Others also have confirmed the dynamic shift in these cations in aged meat (Berman and Swift, 1964). The results of Nakamura's (1973) work clearly showed that Ca is released from muscle tissue during postmortem aging as ATP is depleted. The Ca accumulating ability of the sarcoplasmic reticulum membranes has been shown to decrease gradually during postmortem aging as the result of pH decline (Greaser et al., 1969; Goll et al., 1971).

Studies by Kennick et al. (1980) have shown that high pressure treatments increased the percentage of exudate in muscle tissue. Van Den Berg et al. (1964) found increasing amounts of calcium in meat juices released during cooking of poultry meat. Laakkonen et al. (1970) reported that the exuded meat juices (drip) from bovine muscle showed a high collagenolytic activity.

From the above evidence and the results of the present study, the high pressure treatment results in a greater release of all four enzymes from the lysosomes.

Incubation of Pressurized Muscle and IMCT with Specific Enzymes

The purpose of this portion of the investigation was to study the influence of pressurization on collagen and its susceptibility to attack by lysosomal enzymes and to determine the synergistic role of glucosidases to degrade collagen in presence of collagenase.

The early study of Miller and Kastelic (1956) proposed that alteration in the ground substances may be involved in increasing tenderness. McIntosh (1967) suggested that the tenderizing effect of the postmortem aging is due, at least in part, to the chemical and biophysical changes in mucoproteins. These changes may be ascribed to lysosomal glycosidases (β-glucuronidase, β-galactosidase, α-manosidase, and β-N-acetyl glucosaminadase) which are thought to catabolize mucopolysaccharides (Tappel, 1966).

The effects of β-galactosidase and β-glucuronidase on collagen solubility were tested with fresh muscle samples and IMCT samples with the presence of collagenase in the incubation solution. A high enzyme concentration of β-galactosidase and β-glucuronidase with a short incubation time were used in order to reduce the action of other proteolytic enzymes present in muscle samples. These data are shown in Tables 9 and 10. A slight (but not

significant) increase in collagen solubility was observed in pressurized samples with added β-galactosidase. A much higher increase in collagen solubility (Table 9) was observed in pressurized samples with an added combination of β-galactosidase + β-glucuronidase (significant $P < 0.05$). Similarly, a slight increase in collagen solubility (Table 10) was observed when IMCT control samples were incubated with β-galactosidase in the presence of collagenase. The increase in collagen solubility by a combination of β-galactosidase and β-glucuronidase in the presence of collagenase was significant at the $P < 0.05$ level (Table 10). The increase in collagen solubility observed in the pressurized IMCT samples was higher than that observed for the control samples when incubated with β-galactosidase or a combination of β-galactosidases and β-glucuronidase (Table 10) in the presence of collagenase (both cases did show significant increase, $P < 0.05$).

Although β-galactosidase and β-glucuronidase can act on the ground substances of connective tissue to cause release of galactose and glucose residues from protoglycans without any direct effect on the collagen molecules, the observed increase in collagen solubility due to the combined action of collagenase with other enzymes indicates that β-galactosidase and/or β-glucuronidase may cause a release or exposure of collagen fibrils from surrounding mucopolysaccharides or proteoglycans to facilitate degradation by collagenase. The studies of Gelman and Blackwell (1974) indicated that the triple helical structure of collagen is stabilized by its

interaction with acid mucopolysaccharides. It has been suggested that proteoglycans are effective in protecting collagenase tissue from enzyme attack (Eyre and Muir, 1974; Osebold and Pedrini, 1976). The studies of Etherington (1977) showed that not only the number and location of the intermolecular crosslinks are important in determining the level of susceptibility of collagen to enzymatic digestion, but also the type and quality of associated proteoglycans are important in determining the degree of resistance of the collagen to enzymatic dissolution. More recently, Koop and Valin (1981) have reported that incubation of collagen fibers with lysosomal enzymes at 37°C, pH 5.5, affects significantly the thermal stability of muscle collagen. Wu et al. (1981) have suggested that lysosomal glycosidases may participate in the dissolution of collagen fibers during postmortem aging. Therefore, it is very likely that lysosomal glycosidases may have an important function in collagenolysis by degrading the associated proteoglycans components of the tissue matrix and ground substance to increase susceptibility of the collagen to degrading enzymes. Furthermore, the observed increase in collagen solubility due to the combined effects of high pressure and enzymatic attack indicates that pressurization may cause an exposure of collagen fibrils making them more susceptible to degradation by enzymes. This observation is supported by previous results in this study (Tables 4, 5, and 6, and Figures 10, 11, 12) in which pressurized samples showed an increased release of enzymes and/or

depolymerization of collagen components from collagen fibers.

Amino Acid Compoisition of Pressurized IMCT

The amino acid composition of untreated and pressurized collagens is given in Table 16. The data are typical for the composition of mammalian collagen, in which glycine makes up approximately 33% of the total amino acid residues while alanine and the imino acid, proline, each account for 11% (Dehm and Kefalides, 1978). Trace amounts of cystine may indicate the presence of type III and type IV collagens as reported by Dehm and Kefalides (1978). Although slight differences in amino acid content were observed between pressurized and unpressurized samples, pressurization does not seem to affect the amino acid content of collagen.

TABLE 16. Amino acid composition[a] of collagen isolated from pressurized sternomandibularis muscle

Amino acid residue	Pressurized	Control
Aspartic acid	2.45	3.02
Threonine	1.23	1.60
Serine	1.40	1.81
Glutamic acid	3.55	4.72
Proline	3.30	4.50
Glycine	9.32	9.90
Alanine	3.04	2.70
Cystine	0.05	0.20
Valine	1.21	1.61
Methionine	0.81	0.77
Isoleucine	0.67	1.53
Leucine	1.86	2.05
Tyrosine	0.73	0.65
Phenylalanine	0.52	0.95
Histidine	0.57	0.52
Lysine	1.20	1.71
NH_4	3.63	3.73
Arginine	1.09	1.85

[a]Expressed as mg/100 mg.

CONCLUSION

On the basis of these studies it was concluded that:

1. Pre-rigor pressurization of bovine sternomandibularis muscle samples at 15,000 psi for two min at 35°C resulted in significant decreases ($P < 0.05$) in muscle pH, and significant increases ($P < 0.05$) in heat labile collagen. Incubation of pressurized samples at 4°C for 72 hr resulted in a slight but not significant increase in heat labile collagen. These results indicate that some disruption of interchain crosslinks may have occurred as an initial result of pressurization. Collagen amino acid composition was not affected by pressurization.

2. Pressurization results in a significant decrease ($P < 0.05$) in the yield of intramuscular connective tissue (IMCT) which was accompanied by a rapid drop in muscle pH. The loss of IMCT appears to be related to pH decline and/or the activation of lysosomal enzymes leading to an increase in neutral salt-soluble collagen.

3. The β-galactosidase activity of the pressurized samples was significantly higher ($P < 0.01$) than the controls. Total activities of catheptic collagenase, cathepsin C and elastase were slightly higher in the pressurized than the control samples. These data suggest that pressurization altered the degree of enzyme induction.

4. The distribution of enzyme activities between supernatant (free) and sedimentable (bound) lysosomal fractions varied per enzyme. These results indicate the extent to which these enzymes were bound to the membrane and to differences in the lability of enzymes to pressurization.

5. The addition of purified enzymes (β-galactosidase and β-glucuronidase) in the muscle incubation solutions resulted in a significant increase ($P < 0.05$) in the amount of thermal labile collagen. It is apparent that pressurization induced configurational changes in the collagen molecule making it more susceptible to enzymatic attack. It is also a strong indication that lysosomal enzymes play a role in the tenderization of meat.

6. The addition of purified lysosomal enzymes either β-galactosidase or β-galactosidase plus β-glucuronidase to IMCT incubation solutions resulted in a significant increase ($P < 0.05$) in the amount of thermal labile collagen. Hence, pressurization enhances the postmortem activity of lysosomal enzymes on connective tissue.

BIBLIOGRAPHY

Anderson, A. J. 1969 Effects of lysosomal collagenolytic enzymes, anti-inflamatory drugs and other substances on some properties of insoluble collagen. Biochem. J. 113:457.

Arnold, N., Wierbicki, E., and Deatherage, F. E. 1956. Postmortem changes in the interactions of cations and proteins of beef and their relation to sex and diethylstibestol treatment. Food Technol. 10:245.

Aronson, N. N. and Davidson, E. A. 1968. Digestive activity of lysosomes. II. The digestion of macromolecular carbohydrates by extracts of rat liver lysosomes. J. Biol. Chem. 243:4564.

Bailey, A. J. 1972. The basis of meat texture. J. Sci. Food Agri. 23:995.

Bailey, A. J. and Sims, T. J. 1977. Meat tenderness: Distribution of molecular species of collagen in bovine muscle. J. Sci. Food Agr. 28:565.

Bailey, A. J., Peach, C. M., and Fowler, L. J. 1970. Chemistry of the collagen crosslinks: Isolation and characterization of two intermediate intermolecular crosslinks in collagen. Biochem. J. 117:819.

Bailey, A. J., Robins, S. P., and Balian, G. 1974. Biological significance of the intermolecular crosslinks of collagen. Nature 251:105.

Balasubramaniam, K. and Deiss, W. P., Jr. 1965. Characteristics of thyroid lysosomal cathepsin. Biochim. Biophys. Acta 110:564.

Beaufay, H. and de Duve, C. 1959. Tissue fractionation studies. 9. Enzymatic release of bound hydrolases. Biochem. J. 73:603.

Bentz, H., Bächinger, H. P., Glanville, R. W., and Kühn, K. 1978. Physical evidence for assembly of α and β chains of human placental collagen in a single triple helix. Europ. J. Biochem. 92:563.

Berman, M. D. and Swift, C. E. 1964. Meat curing. The action of NaCl on meat electrolyte binding. J. Food Sci. 29:182.

Bornstein, P. 1974. The biosynthesis of collagen. Ann. Rev. Biochem. 43:567.

Barrett, A. J. 1969. Properties of lysosomal enzymes. In "Lysosomes in Biology and Pathology." J. T. Dingle and H. B. Fell (eds.), p. 245. North Holland Publ., Amsterdam.

Bornstein, P. and Traub, W. 1979. The chemistry and biology of collagen. In "The Proteins," Vol. 1. H. Neurath and R. L. Hill (eds.), p. 411. Academic Press, New York.

Bouton, P. E. and Harris, P. V. 1972a. The effect of cooking temperature and time on some mechanical properties of meat. J. Food. Sci. 37:140.

Bouton, P. E. and Harris, P. V. 1972b. A comparison of some objective methods used to assess meat tenderness. J. Food. Sci. 37:218.

Bouton, P. E., Harris, P. V., Macfarlane, J. J., and O'Shea, J. M. 1978. Pressure-heat treatment of meat: Effect on connective tissue. J. Food Sci. 43:301.

Bouton, P. E., Ford, A. L., Harris, P. V., Macfarlane, J. J., and O'Shea, J. M. 1977. Pressure-heat treatment of post-rigor muscle: Effects on tenderness. J. Food Sci. 42:132.

Broglie, R. M., Hunter, C. N., Delepelaire, P., Niederman, R. A., Chua, N., and Clayton, R. K. 1980. Isolation and characterization of the pigment-protein complexes of rhodopseudomonas sphaeroides by lithium dodecylsulfate/polyacrylamide gel electrophoresis. Proc. Natl. Acad. Sci. 77:87.

Bruns, R. R. and Gross, J. 1973. Band pattern of the segment-long spacing form of collagen. Its use in the analysis of primary structure. Biochem. 12:808.

Burgeson, R. E., El Adli, F. A., Kaitila, I. I., and Hollister, D. W. 1976. Fetal membrane collagens: Identification of two new collagen alpha chains. Proc. Natl. Acad. Sci. 73:2579.

Burleigh, M. C., Barrett, A. J., and Lazarus, G. S. 1974. Cathepsin B_1: A lysosomal enzyme that degrades native collagen. Biochem. J. 137:387.

Byers, P. H., Click, E. M., Harper, E., and Bornstein, P. 1975. Interchain disulphide bonds in procollagen are located in a large nontriple helical COOH-terminal domain. Proc. Natl. Acad. Sci. 72:3009.

Canonico, P. G. and Bird, J. W. C. 1970. Lysosomes in skeletal muscle tissue: Zonal centrifugation evidence for multiple cellular sources. J. Cell Biol. 45:321.

Carmichael, D. J. and Lawrie, R. A. 1967. Bovine collagen: I. Changes in collagen solubility with animal age. J. Food Tech. 2:299.

Cassens, R. G. 1971. Microscopic structure of animal tissues. In "The Science of Meat and Meat Products." J. E. Price and B. S. Schweigert (eds.). Freeman and Company, San Francisco.

Chapman, J. A. 1974. The staining pattern of collagen fibrils. I. An analysis of electron micrographs. Connective Tissue Res. 2: 137.

Chung, E., Rhodes, R. K., and Miller, E. J. 1976. Isolation of three collagenous components of probable basement membrane origin from several tissues. Biochem. Biophys. Res. Commun. 71: 1167.

Chung, E. and Miller, E. J. 1974. Collagen polymorphism: Characterization of molecules with the chain composition $[\alpha_1(III)]_3$ in human tissues. Sci. 183:1200.

Cross, H. R., Carpenter, Z. L., and Smith, G. C. 1973. Effects of intramuscular collagen and elastin on bovine muscle tenderness. J. Food Sci. 38:998.

Davidson, J. M. and Bornstein, P. 1975. Evidence for multiple steps in the limited proteolytic conversion of procollagen to collagen. Fed. Proc. 34:562.

Dehm, P. and Kefalides, N. A. 1978. The collagenous component of lens basement membrane. J. Biol. Chem. 253:6680.

Dingle, J. T., Barrett, A. J., and Weston, P. D. 1971. Cathepsin D: Characterization of immunoinhibition and the confirmation of a role in cartilage breakdown. Biochem. J. 123:1.

Dransfield, E. and Rhodes, D. N. 1976. Effect of post-rigor muscle length on the texture of meat. J. Sci. Food Agr. 27: 483.

Duance, V. C., Restall, D. J., Beard, H., Bourne, F. J., and Bailey, A. J. 1977. The location of three collagen types in skeletal muscle. FEBS Letters 79:248.

Dutson, T. R. 1976. Biosyntehsis and structure of collagen. Proc. 29th Recip. Meat Conf., p. 336.

Dutson, T. R. and Lawrie, R. A. 1974. Release of lysosomal enzymes during postmortem conditioning and their relationship to tenderness. J. Food Technol. 9:43.

Dutson, T. R., Smith, G. C., Hostetler, R. L., and Carpenter, Z. L. 1975. Postmortem carcass temperature and beef tenderness. J. Anim. Sci. 41:289 (Abstr.).

Eisen, A. Z., Jeffrey, J. J., and Gross, J. 1968. Human skin collagenase isolation and mechanism of attack on the collagen molecule. Biochim. Biophys. Acta. 151:637.

Elgasim, E. A. and Kennick, W. H. 1980. Effect of pressurization of pre-rigor beef muscles on protein quality. J. Food Sci. 45:1122.

Epstein, E. H., Jr. 1974. $[\alpha_1(III)]_3$ Human skin collagen: Release by pepsin digestion and preponderance in fetal life. J. Biol. Chem. 249:3225.

Etherington, D. J. 1974. The purification of bovine cathepsin B_1 and its mode of action on bovine collagen. Biochem. J. 137:547.

Etherington, D. J. 1976. Bovine spleen cathepsin B_1 and collagenolytic cathepsin: A comparative study of the properties of the two enzymes in the degradation of native collagen. Biochem. J. 153:199.

Etherington, D. J. 1977. The dissolution of insoluble bovine collagens by cathepsin B_1, collagenolytic cathepsin and pepsin: The influence of collagen type, age and chemical purity on susceptibility. Connective Tissue Res. 5:135.

Eyre, D. R. and Muir, H. 1974. Collagen polymorphism: Two molecular species in pig intervertebral disc. FEBS Letters 42:192.

Eyre, D. R. and Muir, H. 1975a. Characterization of the major CNBr-derived peptides of procine type II collagen. Connective Tissue Res. 3:165.

Eyre, D. R. and Muir, H. 1975b. Type III collagen: A major constituent of rheumatoid and normal human synovial membrane. Connective Tissue Res. 4:11.

Fessler, J. H. and Fessler, L. I. 1978. Biosynthesis of procollagen. Ann. Rev. Biochem. 47:129.

Fessler, L. I., Morris, N. P., and Fessler, J. H. 1975. Procollagen processing to collagen via disulfide-linked triple-stranded intermediate in chick calvaria. Fed. Proc. 34:562.

Field, R. A. 1970. A rapid connective tissue determination. Proc. Wes. Sec., Am. Soc. Anim. Sci. 21:242.

Fieser, L. F. and Fieser, M. 1956. "Organic Chemistry," 3rd ed., p. 208. Reinhold Publ. Corp., New York.

Fruton, J. S. and Mycek, M. J. 1956. Studies on beef spleen cathepsin C. Arch. Biochem. Biophys. 65:11.

Fujii, K. and Tanzer, M. L. 1974. Age-related changes in the reducible crosslinks of human tendon collagen. FEBS Letters 43:300.

Gallop, P. M., Blumenfeld, O. O., and Seifter, S. 1972. Structure and metabolism of connective tissue proteins. Ann. Rev. Biochem. 41:617.

Geldman, R. A. and Blackwell, J. 1974. Collagen-mucopolysaccharide intereaction at acid pH. Biochim. Biophys. Acta 342: 254.

Goldbarg, J. A. and Rutenberg, A.M. 1958. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. Cancer 11:283.

Goll, D. E. 1965. Postmortem changes in connective tissue. Proc. 18th Recip. Meat Conf., p. 161.

Goll, D. E., Hoekstra, W. G., and Bray, R. W. 1964. Age-associated changes in bovine muscle connective tissues: I. Rate of hydrolysis by collagenase. J. Food Sci. 29: 608.

Goll, D. E., Stromer, M. H., Robson, R. M., Temple, J., Eason, B. A., and Busch, W. A. 1971. Tryptic digestion of muscle components simulates many changes caused by postmortem storage. J. Anim. Sci. 33:963.

Gornall, A. G., Bardawill, C. S., and David, D. M. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751.

Greaser, M. L., Cassens, R. G., Briskey, E. J., and Hoekstra, W. G. 1969. Postmortem changes in subcellular fractions from normal and pale soft exudative procine muscle. 1. Calcium accumulation and adenosine triphosphatase activities. J. Food. Sci. 34:120.

Gross, J. and Nagai, Y. 1965. Specific degradation of the collagen molecule by tadpole collagenolytic enzyme. Proc. Nat. Acad. Sci. 54:1197.

Guzman, N. A., Graves, P. N., and Prockop, D. J. 1978. Addition of mannose to both the amino- and carboxyl-terminal propeptides of type II procollagen occurs without formation of a triple helix. Biochem. Biophys. Res. Commun. 84:691.

Hartley, B. S. and Shotton, D. M. 1971. Pancreatic elastase. In "The Enzymes," 3rd ed. P. D. Boyer (ed.), p. 323. Academic Press, New York.

Herring, H. K., Cassens, R. G., and Briskey, E. S. 1967. Factors affecting collagen solubility in bovine muscles. J. Food. Sci. 32:534.

Hill, F. 1966. The solubility of intramuscular collagen in meat animals of various ages. J. Food Sci. 31:161.

Hostetler, R. L., Landmann, W. A., Link, B. A., and Fitzhugh, H. A., Jr. 1970. Influence of carcass position during rigor mortis on tenderness of beef muscles: Comparison of two treatments. J. Anim. Sci. 31:47.

Hostetler, R. L., Landmann, W. A., Link, B. A., and Fitzhugh, H. A., Jr. 1973. Effect of carcass suspension method on sensory panel scores for some major bovine muscles. J. Food Sci. 38:264.

Houck, J. C., Johnston, J., and Jacob, R. A. 1968. Chemical biology of in vivo and in vitro wounds. Biochem. Pharmacol. 17:19.

Ikkai, T. and Ooi, T. 1966. The effect of pressure on F-G transformation of actin. Biochem. 5:1551.

Johnson, F. H. and Eyring, H. 1970. The kinetic basis of pressure effects in biology and chemistry. In "High Pressure Effects on Cellular Processes." A. M. Zimmerman (ed.), p. 2. Academic Press, New York.

Johnson, F. H., Eyring, H. and Polissar, M. J. 1954. "The Kinetic Basis of Muscular Biology," p. 286. John Wiley & Sons, New York.

Kauffman, R. G., Carpenter, Z. L., Bray, R. W., and Hoekstra, W. G. 1964. Interrelations of tenderness, chronological age, and connective-tissue fractions of porcine musculature. J. Agr. Food Chem. 12:504.

Kefalides, N. A. 1971. Isolation of a collagen from basement membranes containing three identical α-chains. Biochem. Biophys. Res. Comm. 45:226.

Kennick, W. H., Elgasim, E. A., Holmes, Z. A., and Meyer, P. F. 1980. The effects of ultrahydrostatic pressurization of pre-rigor muscle on post-rigor meat characteristics. Meat Sci. 4:33.

Kopp, J. and Valin, C. 1981. Research Note. Can muscle lysosomal enzymes affect muscle collagen postmortem? Meat Sci. 5:319.

Kruggel, W. G. and Field, R. A. 1971. Soluble intramuscular collagen characteristics from stretched and aged muscle. J. Food Sci. 36:1114.

Laakkonen, E., Sherbon, J. W., and Wellington, G. H. 1970. Low-temperature, long-time heating of bovine muscle. 3. Collagenolytic activity. J. Food Sci. 35:181.

Locker, R. H. 1960. Proteolysis in the storage of beef. J. Sci. Food Agric. 11:520.

Marsh, B. B., and Leet, N. G. 1966. Studies on meat tenderness. 3. The effects of cold shortening on tenderness. J. Food Sci. 31:450.

Martin, G. R., Byers, P. H., and Piez, K. A. 1975. Procollagen. Adv. in Enzymol. 42:167.

Macfarlane, J. J. and McKenzie, I. J. 1976. Pressure-induced solubilization of myofibrillar proteins. J. Food Sci. 41:1442.

Macfarlane, J. J. 1974. Pressure-induced solubilization of meat proteins in saline solution. J. Food Sci. 39:542.

Macfarlane, J. J. 1973. Pre-rigor pressurization of muscle: Effects on pH, shear value and taste panel assessment. J. Food Sci. 38:294.

McClain, P. E. 1973. Isolation and characterization of the cyanogen bromide peptides from the α_1 and α_2 chains of soluble bovine striated muscle collagen. Biochim. Biophy. Acta 310:469.

McClain, P. E. 1976. Chemistry of collagen crosslinking. Proc. 29th Recip. Meat. Conf., p. 350.

McClain, P. E., Creed, G. J., Wiley, E. R., and Hornstein, I. 1970. Effect of postmortem aging on isolation of intramuscular connective tissue. J. Food Sci. 35:258.

McIntosh, E. N. 1967. Effect of postmortem aging and enzyme tenderizer on mucoprotein of bovine skeletal muscle. J. Food Sci. 32:210.

Miller, E. J. 1972. Structural studies on cartilage collagen employing limited cleavage and solubilization with pepsin. Biochem. 11:4903.

Miller, M. and Kastelic, J. 1956. Chemical responses of connective tissue of bovine skeletal muscle. J. Agr. Food Chem. 4: 537.

Miller, E. J. and Matukas, V. J. 1956. Chick cartilage collagen: A new type of α_1 chain not present in bone or skin of the species. Proc. Natl. Acad. Sci. 64:1265.

Miller, E. J. and Matukas, V. J. 1974. Biosynthesis of collagen: The biochemist's view. Fed. Proc. 33:1197.

Moeller, P. W., Fields, P. A., Dutson, T. R., Landmann, W. A., and Carpenter, Z. L. 1976. Effect of high temperature conditioning on subcellular distribution and levels of lysosomal enzymes. J. Food Sci. 41:216.

Moeller, P. W., Fields, P. A., Dutson, T.R., Landmann, W. A., and Carpenter, Z. L. 1977. High temperature effects on lysosomal enzyme distribution and fragmentation of bovine muscle. J. Food Sci. 42:510.

Morrison, R. I. G., Barrett, A. J., Dingle, J. T., and Prior, D. 1973. Cathepsins B_1 and D: Action on human cartilage proteoglycans. Biochim. Biophys. Acta 302:411.

Newman, R. E. and Logan, M. A. 1950. The determination of hydroxyproline. J. Biol. Chem. 184:299.

Nakamura, R. 1973. Factors associated with postmortem increase of extractable Ca in chicken breast muscle. J. Food Sci. 38:1113.

Ono, K. 1970. Lysosomal-type enzymes in beef longissimus dorsi muscle. J. Food Sci. 35:256.

Osebold, W. R. and Pedrini, V. 1976. Pepsin-solubilized collagen of human nucleus pulposus and annulus fibrosus. Biochim. Biophys. Acta. 434:390.

Parrish, F. C., Jr. and Bailey, M. E. 1967. Physicochemical properties of bovine muscle particulate cathepsin. J. Agr. Food Chem. 15:88.

Parrish, F. C., Jr., Rust, R. E., Popenhagen, G. R., and Miner, B. E. 1969. Effect of postmorten aging time and temperature on beef muscle attributes. J. Anim. Sci. 29:398.

Pfeiffer, N. E., Field, R. A., Varnell, T. R., Kruggel, W. G., and Kaiser, I. 1972. Effects of postmortem aging and stretching on the macromolecular properties of collagen. J. Food Sci. 37: 897.

Piez, K. A. 1966. Collagen. In "The Physiology and Biochemistry of Muscle as a Food." E. J. Briskey, R. G. Cassens, and J. C. Trautman (eds.), p. 315. University of Wisconsin Press, Madison.

Piez, K. A. 1967. Soluble collagen and the components resulting from its denaturation. In "Treatise on Collagen," Vol. 1. G. N. Ramachandran (ed.). Academic Press, New York.

Prockop, D. J., Kivirikko, K. I., Tuderman, L., and Guzman, N. A. 1979. The biosynthesis of collagen and its disorders. N. Engl. J. Med. 301:13,77.

Ramachandran, G. N. 1967. Structure of collagen at the molecular level. In "Treatise on Collagen," Vol. 1. G. N. Ramachandran (ed.). Academic Press, New York.

Ratcliff, D., Bouton, P. E., Ford, A. L., Harris, P. V., Macfarlane J. J., and O'Shea, J. M. 1977. Pressure-heat treatment of post-rigor muscle: Objective-subjective measurements. J. Food Sci. 42:857.

Rigby, B. J. 1964. The effect of mechanical extension upon the thermal stability of collagen. Biochim. Biophys. Acta 79:634.

Roberts, J. D., Stewart, R., and Caserio, M. C. 1971 "Organic Chemistry: Methane to Macromolecules," 2nd ed., p. 311. W. A. Benjamin, Inc., Menlo Park, California.

Rowe, R. W. D. 1974. Collagen fibre arrangement in intramuscular connective tissue. Changes associated with muscle shortening and their possible relevance to raw meat toughness measurements. J. Food Technol. 9:501.

Sachar, L. A., Winter, K. W., Sicher, N., and Frankel, S. 1955. Photometric method for estimation of elastase activity. Proc. Soc. Exptl. Biol. Med. 90:323.

Sawant, P. L., Desai, I. D., and Tappel, A. L. 1964. Factors affecting the lysosomal membrane and availability of enzymes. Arch. Biochem. Biophys. 105:247.

Scott, P. G. and Veis, A. 1976. The cyanogen bromide peptides of bovine soluble and insoluble collagens. II. Tissue specific crosslinked peptides of insoluble skin and dentin collagen. Connective Tissue Res. 4:117.

Scott, P. G., Telser, A. G., and Veis, A. 1976. Semiquantitative determination of cyanogen bromide peptides of collagen in SDS-polyacrylamide gels. Anal. Biochem. 70:251.

Sharp, J. G. 1963. Aseptic autolysis in rabbit and bovine muscle during storage at 37°C. J. Sci. Food Agr. 14:468.

Shubert, M. and Hamerman. D. 1968. The chemistry of the fibers of connective tissue. In "A Primer on Connective Tissue Biochemistry." M. Shubert and D. Hamerman (eds.), p. 5. Lea & Febiger, Philadelphia.

Shimokomaki, M., Elsden, D. F. and Bailey, A. J. 1972. Meat tenderness: Age-related changes in bovine intramuscular collagen. J. Food Sci. 37:892.

Smith, G. C., Arango, T. C., and Carpenter, Z. L. 1971. Effect of physical and mechanical treatments on the tenderness of the beef longissimus. J. Food Sci. 36:445.

Spiro, R. G. 1969. Characterization and quantitative determination of the hydroxylysine-linked carbohydrate units of several collagens. J. Biol. Chem. 244:602.

Spiro, R. G. and Spiro, M. J. 1971a. Studies on the biosynthesis of the hydroxylysine-linked disaccaride unit of basement membranes and collagens. I. Kidney glucosyltransferase. J. Biol. Chem. 246:4899.

Spiro, M. J. and Spiro, R. G. 1971b. Studies on the biosynthesis of the hydroxylysine-linked disaccharide unit of membranes and collagens. II. Kidney galactosyltransferase. J. Biol. Chem. 246:4910.

Stagni, N. and DeBernard, B. 1968. Lysosomal enzyme activity in rat and beef skeletal muscle. Biochim. Biophys. Acta 170:129.

Steel, R. G. D. and Torrie, J. H. 1960. "Principles and Procedures of Statistics." McGraw-Hill Book Co., Inc., New York.

Tanzer, M. L. 1973. Crosslinking of collagen. Science 180:561.

Tanzer, M. L., Church, R. L., Yaeger, J. A., Wampler, D. E., and Park, E. D. 1974. Procollagen: Intermediate forms containing several types of peptide chains and noncollagen peptide extensions at the NH_2 and COOH ends. Proc. Natl. Acad. Sci. 71:3009.

Tanzer, M. L., Fairweather, R., and Gallop, P. M. 1973. Isolation of the crosslink, hydroxymerodesmosine, from borohydride-reduced collagen. Biochim. Biophys. Acta 310:130.

Tappel, A. L. 1966. The Physiology and Biochemistry of Muscle as a Food. E. J. Briskley, R. G. Cassen, and J. C. Trautman (eds.), p. 237. University of Wisconsin Press, Madison.

Tappel, A. L. 1969. Lysosomal enzymes and other components. In "Lysosomes in Biology and Pathology." J. T. Dingle and H. B. Fell (eds.), p. 207. North Holland Publ., Amsterdam.

Traub, W. and Piez, K. A. 1971. The chemistry and structure of collagen. Adv. Protein Chem. 25:243.

Trelstad, R. L. 1974. Human aorta collagens: Evidence for three distinct species. Biochem. Biophys. Res. Comm. 57:717.

Trelstad, R. L., Catanese, V. M., and Rubin, D. F. 1976. Collagen fractionation: Separation of native types I, II and III by differential precipitation. Anal. Biochem. 71:114.

Trelstad, R. L., Kang, A. H., Igarashi, S., and Gross, J. 1970. Isolation of two distinct collagen from chick cartilage. Biochem. 9:4993.

Trelstad, R. L., Kang, A. H., Toole, B. P., and Gross, J. 1972. Collagen heterogeneity high resolution separation of native $[\alpha_1(I)]_2$ and $[\alpha_1(II)]_3$ and their component α chains. J. Biol. Chem. 247:6469.

Van Den Berg, L. 1964. Physiochemical changes in some frozen foods. J. Food Sci. 29:540.

Viidik, A. and Vuust, J. 1980. Molecular structure and higher organization of different collagens. In "Biology of Collagen." A. Viidik and J. Vuust (eds.), p. 1. Academic Press, New York.

Weber, K. and Osborn, M. 1969. The reliability of molecular weight and determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406.

Whitaker, J. R. 1964. Postmorten proteolytic changes affecting myofibrillar proteins. Proc. 17th Recip. Meat Conf., p. 153.

Wierbicki, E., Cahill, V. R., and Deatherage, F. E. 1957. Effects of added sodium chloride, potassium chloride, calcium chloride, magnesium chloride, and citric acid on drip losses after freezing and thawing. Food Technol. 11:74.

Wray, W., Boulikas, T., Wray, V. P., and Hancock, R. 1981. Silver staining of proteins in polyacrylamide gels. Biochem. 118:97.

Wu, J. J., Dutson, T. R., and Carpenter, Z. L. 1981. Effect of postmortem time and temperature on the release of lysosomal enzymes and their possible effect on bovine connective tissue components of muscle. J. Food Sci. 46:1132.

APPENDICES

APPENDIX A

Isolation and Purification of Intramuscular Connective Tissue

A. Reagents:

1. 1.1 M KCl in 0.1 M K_2HPO_4-K H_2PO_4 buffer (pH 7.4)
2. 1 M KCl solution
3. 0.9% NaCl

B. Procedures:

1. Free the muscle of fat and epimysial tissue.
2. Mince the meat.
3. Place 50 g of sample in a Waring blender with 200 ml of cold deionized-distilled water and blend for 10 sec at full speed.
4. Let stand 2 min and blend at additional 10 sec.
5. Filter through 2 layers of cheese cloth or stainless steel screen to remove sarcoplasmic proteins and water.
6. Repeat steps 3,4 and 5 four times. For the last 3 times, use 1.1 M KCl-0.1 M K_2H PO_4-KH_2PO_4 buffer solution (pH 7.4) in place of water. This solubilizes and removes myofibrillar proteins and any remaining sarcoplasmic proteins.
7. Remove crude intramuscular connective tissue (IMCT) from blender cup by screening and retain the precipitate.

8. To the crude IMCT, add 100 ml of 1 M KCl and agitate for 24 hr. Change the KCl solution every 12 hr by centrifuging at 3000 x G, 5°C, for 10 min and save the precipitate. This step will remove traces of mysoin and actin.
9. To each IMCT sample, add 100 ml of 0.9% NaCl and agitate for 12 hr. Centrifuge the precipitate and add new NaCl to and agitate 12 more hr followed by centrifugation.
10. Wash precipitate two times with 100 ml deionized-distilled water to remove salt from sample.
11. After centrifugation, the isolated collagen is recovered and frozen at -4°C.
12. Store frozen at -4°C until used.
13. Samples can be dried for optional storage.

APPENDIX B

Collagen Solubility (Heat Labile)

A. Reagents:

1. Ringer's stock solution

   8.6 g NaCl

   0.33 g $CaCl_2$

   0.3 g KCl

   Make to 1 liter with deionized water. Dilute 25 ml of stock solution to 100 ml with water for 1/4-strength Ringer's solution.

B. Procedures:

1. Trim muscle free of fat and epimysial tissue.
2. Mince the sample.
3. Weigh 4.0 g of minced sample into a 40 ml centrifuge tube.
4. Add 12.0 ml of 1/4-strength Ringer's solution.
5. Homogenize the sample in a Virtis blender at full speed.
6. Heat sample for 70 min at 77°C with constant agitation.
7. Centrifuge the sample at 3000 x G for 10 min at 5°C.
8. Decant supernatant into test tube. Wash residue with 8.0 ml of 1/4 strength Ringer's solution.

9. Centrifuge the washed residue at 3000 x G for 10 min at 5°C.
10. Decant supernatant into same test tube used in step 8. Transfer residue into separate test tube and retain. Rinse centrifuge tube with 10.0 ml of water and add to residue tube of step 10.
11. Determine the hydroxyproline content according to procedure described in Appendix C.

APPENDIX C

Hydroxyproline Determination

A. Reagents:

1. 0.02 % Methyl red

   0.04 g Methyl red powder

   Make to 200 ml with 95% ethyl alcohol.

2. 0.01 M Copper sulfate

   2.495 g $CuSo_4 \cdot 5H_2O$

   Make to 1 liter with distilled water and store in the dark.

3. 2.5 N NaOH

   100 g NaOH

   Make to 1 liter with distilled water

4. Stock hydroxyproline

   0.025 g Hydroxyproline

   Make to 250 ml with 0.001 N HCl. Make standards 1 to 30 μg/ml.

5. 6% Hydrogen peroxide

   Dilute 100 ml of 30% hydrogen peroxide to 500 ml with distilled water (prepare daily)

6. 3.0 N Sulfuric acid

   Add 767 ml of concentrated sulfuric acid to a 1 liter volumetric flask, make to volume with distilled water.

6. 5% p-DABA (p-dimethylaminobenzaldehyde)

   Dissolve 25 g of p-DABA in N-propanol and make to 500 ml.

   Note: Prepare prior to use.

7. 12 N hydrochloric acid

8. N-Propanol 95%

B. Procedure:

1. To the supernatant obtained in Appendix B add 20.0 ml of 12 N HCl. To the residues add 10.0 ml of 12 N HCl. Final concentration of HCl for hydrolysis is 6 N.

2. Hydrolyze all samples in autoclave for 16 hours at 108°C and 800 mm Hg (15 lb) pressure.

3. Remove samples from autoclave, cool, add approximately 3 g of activated 60-mesh charcoal to each bottle and shake thoroughly (to remove humin).

4. Filter contents of each test tube through Whatman No. 1 paper into a 300 ml flat bottom evaporation flask. Rinse bottle three times and filter paper once with distilled water.

5. Evaporate to dryness under vacuum with rotary evaporator at 70°C.

6. Add 10 ml water to each flask plus three drops of 0.02% methyl red and titrate to a milky color with 1.0 N NaOH.
7. Filter samples into a volumetric flask using Whatman No. 1 paper. Rinse flask and paper with distilled water.
8. Dilute samples with water to contain between 1-30 μg/ml of hydroxyproline per sample.
9. Blanks, standards and samples are run in duplicate. Tubes are set up as follows:

Tube	Water	Sample	Standard
Blank	1.0 ml	0	0
Standard	0	0	1.0 ml
Sample	0	1.0 ml	0

10. With pipettes place in each tube, in succession, 1 ml each of 0.01 M copper sulfate solution, 2.5 N sodium hydroxide, and 6% hydrogen peroxide (shake tubes gently after each addition).
11. Mix solutions and shake occasionally during 5 min period, then place in a water bath at 80°C for 5 min with frequent vigorous shaking.
12. Chill tubes in an ice bath and add 4 ml of 3.0 N sulfuric acid. Agitating at the same time, then add 2 ml

of p-dimethylaminobenzaldehyde and mix thoroughly.

13. Place tubes in a water bath at 70°C for 16 min, then cool in cold water.

14. Transfer contents to absorption tubes and read absorbance with a spectrophotometer at 540 m μ.

15. Prepare a curve with standard solutions and find the amount of hydroxyproline from this curve. The reading is micrograms per one ml of unknown sample.

16. Multiply μg/ml by total volume to which sample is diluted. This gives μg/4 grams. Divide by 4 to put it on a μg/gm basis of hydroxyproline reagents.

17. To convert hydroxyproline to collagen multiply by 7.25. This gives μg/gm collagen.

APPENDIX D

Assay for β-Galactosidase Activity

A. Reagents

1. 0.1 M Sodium phosphate buffer (Na H_2PO_4-Na-H PO_4), pH 7.3
2. β-Lactose solution

   Dissolve 10 mg of β-lactose in 1.0 ml sodium phosphate buffer, pH 7.3
3. O-Dianisidine

   Dissolve 2 mg in 1.0 ml water (prepare fresh for each run)
4. PGO enzymes (peroxidase/glucose oxidase)

   Dissolve 1 capsule (Sigma stock No. 510-6) in 100 ml water (prepare just prior to use)
5. Perchloric acid 4.2%

   Dilute 1.0 ml of 70% perchloric acid with 15.7 ml water.
6. Stock glucose

   0.1 g Glucose per ml

   Make standard solutions containing 1,2,3,4,5,6,7,8,9 and 10 μg respectively per ml

B. Procedures

1. Place 4.0 ml of β-lactose solution into appropriately labelled test tubes.
2. Add 1.0 ml of 0.1 M sodium phosphate buffer (NaH_2PO_4-$NaHPO_4$), pH 7.3.
3. To start reaction, add 0.5 ml of enzyme and incubate at 37°C for 30 min.
4. To stop reaction, add 1.0 ml of perchloric acid (4.2%). Centrifuge if turbid.
5. Incubate 0.2 ml of supernatant, 0.1 ml of O-dianisidine with 6.0 ml PGO solution at 37°C for 30 min.
6. Read absorbance (510 nm) of each tube against water as reference. Subtract the reading of the blank for the test and use this figure to calculate μ grams glucose from the standard curve.

APPENDIX E

Assay for Cathepsin C Activity

A. Reagents

1. 0.1 M Phosphate buffer (KH_2PO_4-Na_2HPO_4, pH 6.0) containing 50 mM β-mercaptoethylamine hydrochloride
2. 0.5 mM Gly-phe-2-naphthylamide (GPNA) freshly dissolved in potassium-sodium phosphate buffer, pH 6.0.
3. 10% Trichloroacetic acid (TCA)
4. 0.1 % Sodium nitrite
5. 0.5% Ammonium sulfamate
6. 0.5 mg N-(1-Naphthyl) ethylene-diamine dihydrochloride per 1 ml of 95% ethanol
7. Stock β-naphthylamide 1 mM

   Make standards with solution containing 0.2, 0.4, 0.6, 0.8, 1.2 and 1.6 mM respectively per ml.
8. Prepare a curve with standard β-naphthylamide solution.

B. Procedures

1. Pre-incubate 1.6 ml of enzyme sample with 0.4 ml β-mercaptoethylamine at 40°C for 5 min.
2. Add 2.0 ml of GPNA to the pre-incubated sample solution and incubate at 37°C for 90 min.
3. To stop the reaction, add 2.0 ml of 10% TCA.

4. Filter samples with Whatman No. 1 filter paper if turbid.
5. Transfer 1.0 ml of filtrate to clean tubes.
6. With precise timing, add 1.0 ml of 0.1% sodium nitrite solution.
7. After 3 min add 1.0 ml of ammonium sulfamate solution.
8. After 2 min add 2.0 ml of NED (N-(1-naphthyl)ethylene-diamine).
9. Allow color to develop for 10 min and read absorbance in a spectrophotometer at 520 nm.
10. Calculate cathespin C units and specific activity by using the naphthylamide standard curve.

APPENDIX F

Assay for Collagenase Activity

A. Reagents

1. 0.2 M Sodium formate buffer (pH 3.5) containing 10 mM cystine
2. 0.1 M Acetic acid - glycerol solution

   75 ml 0.1M Acetic acid is made to 100 ml with glycerol and adjusted to pH 3.5.
3. Collagen substrate

   Dissolve 10 mg bovine tendon collagen in 1.0 ml acetic acid-glycerol solution.

B. Procedures

1. Incubate 1.0 ml of enzyme solution, 2.0 ml of collagen substrate, and sodium formate buffer to make 6.0 ml sample solution and incubate for 3 hr at 40°C.
2. Remove residual collagen by centrifuging at 3000 x G for 10 min at 5°C.
3. Pipette 5.0 ml supernatant into a test tube. Add same amount of 12 N HCL and seal tube with a teflon-lined cap.
4. Determine the hydroxyproline content as described in Appendix C.
5. Calculate collagenase units and specific activity.

APPENDIX G

Assay for Elastase Activity

A. Reagents

1. Orcein-impregnated elastin (Sigma)
2. 0.2 M Tris HCl buffer, pH 8.8
3. 0.5 M Phosphate buffer (K_2HPO_4-KH_2PO_4), pH 6.0.
4. Elastase (pancreatopeptidase EEC 3.4.21.11) 2x crystallized (Sigma).

B. Procedures

1. Place 20 mg of orcein-impregnated elastin in a test tube.
2. Add 1 ml of 0.2 m Tris-HCl buffer, pH 8.8.
3. Add 1 ml of water, 1.0 ml of enzyme sample preparation and incubate tubes at 40°C for 180 min.
4. To stop the reaction, add 2.0 ml of 0.5 M phosphate buffer, pH 6.0.
5. Filter through Whatman No. 1 paper into a test tube.
6. Read absorbancy in a spectrophotometer at 590 nm against a Tris-HCl buffer blank, pH 8.8.
7. Calculate the elastin concentration of the sample solution from the standard curve.

APPENDIX H

Amino Acid Analysis of IMCT Sample

1. Weigh 5 mg of freeze-dried samples into a hydrolyzing vessel.
2. Add 2 ml of 6 N HCl to the vessel.
3. Flush the contents of the vessel with dry nitrogen and then vacuumize by water aspiration.
4. Hydrolyze samples in a solid heating block at 110°C for 24 hrs.
5. Remove samples from solid heating block, allow to cool.
6. Transfer hydrolysates to a 125 ml flat bottom evaporation flask. Rinse hydrolyzing vessel with small amount of distilled water.
7. Evaporate to dryness under vacuum with rotary evaporator at 37°C.
8. Bring to a concentration of approximately 100 μg/0.5 ml with diluting buffer (Na citrate) pH. 2.2.
9. Filter samples into a volumetric flask using Millipore (0.45 μ) to remove lipids and other impurities.
10. Run standard amino acid analysis.

APPENDIX I

Biuret Procedure

A. Reagents

1. 1.50 g $CuSO_4 \cdot 5\ H_2O$
2. 6.00 g $NaKC_4 H_4O_6 \cdot 4\ H_2O$
3. 500 ml Distilled water
4. 300 ml Carbonate-free 10% NaOH
5. Mix $CuSO_4 \cdot 5\ H_2O$ and $NaKC_4H_4O_6 \cdot 4H_2O$ into 500 ml distilled water.
6. Slowly add the 300 ml of 10% NaOH
7. Make to 1 liter with distilled water

B. Standard Curve

1. Prepare a solution of 10mg/ml crystalline bovine serum albumin in distilled water.
2. Make quantitative dilutions to obtain protein solutions containing 0, 1.0, 2.5, 5.0, 7.5 and 10.0 mg/ml.
3. Place 1.0 ml each of the protein solutions in separate test tubes.
4. Add 4.0 ml of biuret reagent.
5. Mix thoroughly and let stand 30 min at room temperature for color development.
6. Read absorbance at 540 nm.

7. Plot absorbance vs protein concentration in mg/ml.

C. Procedure

1. Add 4 ml of biuret reagent to 1 ml of unknown protein solution and to 1 ml H_2O blank.
2. Mix and allow to stand for 30 min.
3. Read absorbance at 540 nm against reagent blank.
4. Read unknown protein concentraton in mg/ml from standard curve.

APPENDIX J

Separation of IMCT Components by Lithium Dodecyl Sulfate/7.5-15% Polyacrylamide Gradient Gel Stabilized with 5-17% Sucrose Gradient

A. Reagents

1. Stock Acrylamide (Poly A)

Stock Acrylamide (Poly A)	500 ml	Final Conc.
Acrylamide	146.1 g	29.2%
Bis acrylamide	4.0 g	0.8%
Tetramethylethylene-diamine (TEMED)	0.75 ml	0.15%

2. Gel Buffer

Lower gel buffer	90.75g (pH 8.8)	1.5 M Tris-HCl
Upper gel buffer	30.25g (pH 6.8)	0.5 M Tris-HCl

3. Gradient Gel

Gradient Gel	35 ml each	
Light (7.5% acrylamide- 5.0% sucrose)		
Sucrose	1.75 g	5%
Lower gel buffer	8.75 ml	25% (0.375M Tris)
Poly A 30%	8.75 ml	7.5%
H_2O	16 ml	46%
Ammonium persulfate (APS), 10% solution	0.35 ml	0.1%
(TEMED)	———	(0.04%)

Heavy (15% acrylamide - 17.5% sucrose)

Sucrose	6.13 g	17.5%
Lower gel buffer	8.75	25% (0.375M Tris)
Poly A (30%)	17.5 ml	15%
H_2O	5.25 ml	15%
APS, 10% solution	0.35 ml	0.1%
(TEMED)	———	0.75%

4. Stacking Gel (5%)

Upper gel buffer	5 ml	25% (0.125 M Tris)
Poly A (30%)	3.34 ml	5%
H_2O	11.50 ml	57.5%
TEMED	0.015 ml	0.1%
APS, 10% solution	0.20 ml	0.1%

5. Electrolyte Buffers 2 liter

Lower buffer

Tris	5.04 g	0.021 M
Glycine	24.02 g	0.16 M

Upper buffer Add to 300 mls of above

Lithium dodecyl-sulfate	0.3 g	0.1%
EDTA - Na_2	0.124 g	1.2 mM

6. Sample Buffer — 100ml (pH 6.8)

Lithium dodecyl sulfate	2.0 g	2%
Sucrose	12.0 g	12%
Tris-HCl	0.605 g	50 mM
EDTA - Na_2	0.069 g	2 mM
Dithiothreitol	0.309 g	0.02 M

7. Standards: Dilute 1:20 with sample buffer to which has been added bromphenol blue, and heat at 65-75° for 15 min or boil for 2 min.

(Note: Filter all solutions through 0.45 μ Millipore filter membrane. Refrigerate all solutions).

8. Stain

   0.25% Coomassie Blue R-250 in methanol: acetic acid (HAc):H_2O (50:10:40). Stain several hours or overnight.

9. Destain

   Methanol: acetic acid: H_2O (50:10:40)

   Destain until background is clear.

B. Procedures

I. Preparation of gels

1. Place 35 ml of light gradient gel solution (7.5% acrylamide- 5.0% sucrose) and 35 ml of the heavy gradient gel solution (15% acrylamide - 17.5% sucrose) in the density gradient maker and

pump into electrophoretic assembly.

2. Polymerize gels for about 1 hr.
3. Place 7-10 ml of stacking gel solution on top of gradient gel.
4. Polymerize the stacking gel for about 30 min.

II. Preparation of collagen sample

1. Weigh 500 mg of dry collagen sample into a small test tube or flask.
2. Add 50 ml dispersion solution (0.1 M acetic acid)
3. Add 50 mg of enzyme pepsin and incubate for 24 hr at room temperature.
4. After inactivation of the enzyme by raising the pH to 8.0 determine the protein content of the solubilized collagen.
5. Place a known amount of solubilized collagen in sample buffer (sample buffer - collagen ratio 2:1). Use approximately 15 mg/ml of solubilized collagen.
6. Heat at 65-75°C for 15 min or boil for 2 min.

III. Addition of samples to gels

1. Overlay gels with running buffer until the sample column is completely full.
2. Apply 100 to 200 μg of collagen sample to the gel, using a microsyringe.

3. Pour approximately 400 ml of running buffer into the upper bath, pour the remaining buffer (approximately 1000 ml) into the lower bath.
4. Perform electrophoresis at a constant current of 10-12 mA for 24 hrs.

IV. Gel staining and destaining

1. Remove gels from slabs or chambers.
2. Stain gels in glass trays with Coomassie blue stain for 12 hr or overnight.
3. Pour off the stain and transfer gels to destaining trays. Rinse gels with distilled water and place in destaining solution.
4. Make repeated changes with destaining solution until the gel has been destained.
5. Soak gels in 50% MeOH-0.05% formaldehyde (2-3 hr) with three changes of solution.
6. Add new MeOH, leave overnight and then decant.
7. Soak in 2 changes of distilled H_2O for minimum of 1 hr and then decant.
8. Stain with solution C for 20 min with agitation:

   Solution A - Dissolve 4 g silver nitrate in 20 ml H_2O .

Solution B - Mix 105 ml 0.36% NaOH + 7 ml conc. NH_4OH.

Solution C - Add A to B dropwise with constant stirring and make up to 500 ml (use within 5 min).

9. Rinse with three 5-min washes of distilled H_2O with gentle agitation.
10. Develop the silver stain solution D until bands appear.

    Solution D - Mix 2.5 ml of 1% citric acid + 0.25 ml 38% formaldehyde in 500 ml H_2O (make fresh daily).
11. Rinse well with H_2O and return to 50% MeOH ($\pm$ 10% HAc) to stop stain development.
12. Destain in Kodak Rapid Fix ($\pm$ 5-10% MeOH to slow down destaining). Add 237 ml solution A to 500 ml H_2O. With agitation add 25 ml solution B plus 5-10 ml MeOH. Make up to 950 mls.
13. Rinse with distilled H_2O a couple of times and then soak in Kodak hypo clearing agent for 30 min.
14. Rinse with distilled H_2O a couple of times and place in 50% MeOH.

15. For destaining stabilized gels, rinse 30 min in distilled H_2O, and destain with the following: 100 ml 1.5 M sodium thiosulfate + 100 ml (0.15 M cupric sulfate + 0.6 M NaCl + 0.9M NH_4OH)
    Wash in distilled H_2O and equilibrate in 50% MeOH (restain as before if necessary).
    Notes:
    a) The gel can be stained with Coomassie Blue first and restained with silver by removing all traces of HAc with 50% MeOH.
    b) The gel can be stained with silver stain first, destained with Kodak Rapid Fix, cleared with hypoclearing agent and then restained with Coomassie Blue as usual.
    c) To prevent sticking to glass dishes while staining with silver, wash pans well with dichromate cleaning solution, rinse with distilled H_2O, rinse with EtOH, and coat with Sigmacote.
16. Wrap gels in clear plastic wrap, label, place in plastic bags, seal and store at 2-4°C.