THE INTERACTION OF PLANT POLYSACCHARIDES WITH COLLAGEN

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SUMMARY

This thesis examines the interaction of several plant polysaccharides with bovine collagen and the effect of these interactions on the physical characteristics of biopolymer wound dressings. Recent research in the field of wound healing has led to the development of biological wound dressings such as Fibracol ™, Sorbsan ™, E.Z.Derm™, Biobrane™ which contain protein and/or polysaccharides. When these materials are placed on an open wound the potential exists for soluble polysaccharide to diffuse from the dressing into the wound environment. This may alter the rate and quality of wound healing. Therefore, it is important to understand how the protein / polysaccharide interactions and other physical characteristics affect this diffusion.

Initially the rheology and co-precipitation of collagen / polysaccharide solutions were studied to determine any polysaccharides which were interacting with collagen. Polysaccharides of interest were then studied in an insoluble system by forming polysaccharide/ fibrous collagen wound dressing materials. The effect of different polysaccharides, their charge density, degree of polymerisation, chemical composition, and ionic crosslinking, on the diffusion of molecules from the wound dressing materials were studied. The tensile strength and the absorbency of these materials were also studied. In addition, the effect of different collagen fibres (soluble, insoluble, denatured and chemically modified) were investigated. It was found that the degree of polymerisation and chemical composition of the polysaccharide, and the form of collagen used to produce the materials influenced the amount of polysaccharide that was eluted from the material. Calcium ions were also shown to significantly reduce the level of diffusion in materials containing alginate.

It appears that the rate and extent of diffusion is controlled by a combination of polymer entrapment and specific polymer-polymer interactions.

Different polysaccharides were shown to have no significant effect on either the absorbency or tensile strength of the materials, therefore, it can be concluded that most of the physical characteristics are imparted by the collagen matrix.

In summary the results obtained from this work have led to a greater understanding of the factors which control the release of polysaccharides from collagen/polysaccharide composite materials and have given some insight into how the rate of diffusion may be altered to suit a particular application.

ACKNOWLEDGEMENTS

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Last and most definitely least I would like to thank Andy and Stuart for being good friends, letting me win at badminton and for teaching me that when things get bad, someone else is always worse.

TABLE OF ABBREVIATIONS

BSA	bovine serum albumin
CD	circular dichroism
CMC	carboxy-methylcellulose
CNBr	cyanogen bromide
CS	chondroitin sulphate
CS-PG	chondroitin sulphate proteoglycan
DMAPN	di-methyl-aminopropionitrile
DP	degree of polymerisation
G	guluronic acid
GAG	glycosaminoglycans
Gdn HCl	guanidine hydrochloride
HA	hyaluronic acid
HPC	hydroxypropyl-cellulose
HPMC	hydroxypropylmethyl-cellulose
IEP	isoelectric point
KS	keratan sulphate
LBG	locust bean gum
M	mannuronic acid
MC	methyl-cellulose
PG	proteoglycan
Pr-ap	protein - anionic polysaccharide interaction
Pr-p	protein polysaccharide interaction
PSC	pepsin solubilised collagen
r.u.	repeating unit
SDS	sodium dodecyl sulphate
TCA	tri-chloroacetic acid

ABSTRACT

1 INTRODUCTION

CHAPTER 1-INTRODUCTION

Polysaccharides and proteins are widely distributed in nature where they perform a range of biological and functional roles. As well as functioning as enzymes, antibodies, hormones, membrane components and the basis of all animal movements, some proteins act in a structural role. Similarly, many polysaccharides are responsible for the maintenance of structural integrity in the plant and animal kingdom (e.g. cellulose, chitin), as well as providing an energy reserve (starch, glycogen). Glycosaminoglycans (GAGs) and some proteins, e.g. fibronectin and laminin, play an important role in the interaction between cells and the extracellular matrix.

In animals the structural protein collagen is the major component of the connective tissues e.g. skin, tendon and cartilage (Grant and Jackson 1976) and is also the main fibrillar component of bone and teeth.

However, both proteins and polysaccharides are capable of interacting to produce an even greater range of functional characteristics. Of many protein /polysaccharide interactions in animals, possibly the most biologically conserved, and, therefore, presumably one of the most important in evolutionary terms is the interaction of collagen molecules with proteoglycans and glycosaminoglycans (GAGs) in the connective tissue of higher animals.

The GAGs found in animals are a family of heterogeneous polysaccharides, of which seven types are commonly recognised, six of which are structurally related. In general these molecules are composed of repeating disaccharide units consisting of a hexosamine, (usually N-acetyl glucosamine or N-acetyl galactosamine) glycosidically bound to either uronic (glucuronic or iduronic) acid or galactose. The monomer units of the seven glycosaminoglycans are shown in Table 1.1.

It was suggested as early as the 1950's (Gross and Kirk 1958) that for a range of physical characteristics such as that found in the connective tissues,

Table 1.1 The carbohydrate monomers of proteoglycans

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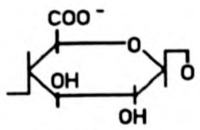
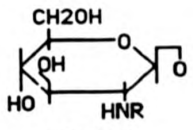
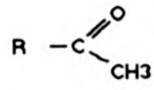
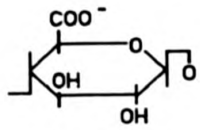
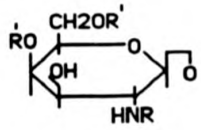
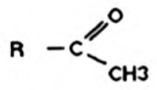
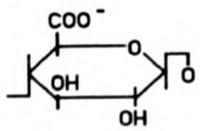
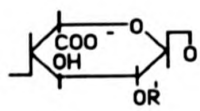
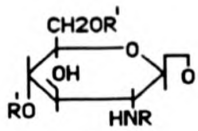
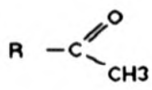
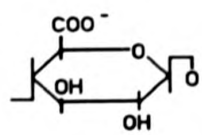
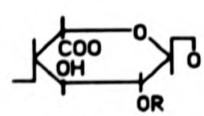
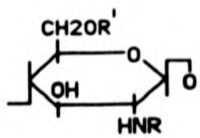
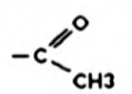
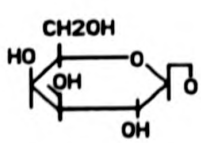
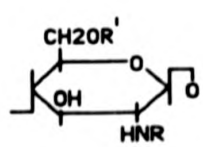
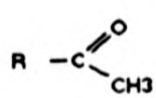
Hyaluronate	 β-D-GlcUA	 β-D-GlcN	
Chondroitin 4 and 6 sulphate	 β-D-GlcUA	 β-D-GalN	 R' –H or $-SO_3^-$
Dermatan sulphate	 β-D-GlcUA  α-L-IdUA	 β-D-GalN	 R' –H or $-SO_3^-$
Heparin, heparan sulphate	 β-D-GlcUA  α-L-IdUA	 β-D-GlcN	R = SO_3^- or  R' = –H or SO_3^-
Keratan sulphate	 β-D-Gal	 -D-GlcN	 R' –H or $-SO_3^-$

to occur, interactions between the GAGs and other molecules in the extracellular matrix were likely.

1.2-Polysaccharides

There are many different types and species of polysaccharide. It would not be possible to carryout a comprehensive review of the structures of all the major polysaccharides. Therefore, this overview will concentrate on those macromolecules selected for study in the present work

1.2.1-Alginate

Alginates are polysaccharides obtained from brown seaweed. Man has used seaweed as a source of food since early historical times. With the onset of the industrial revolution and its demand for raw materials, seaweeds were used for their chemical content.

The first record of alginates being isolated was by Stanford in 1883, who prepared them by destructive distillation, followed by a lixivation process (McDowell 1977). In the first description of his product, Stanford referred to 'soluble and insoluble algin', and soon realised that insoluble algin was an acid, whilst soluble algin was the sodium salt.

Compounds now available under the name alginates include alginic acid, its compounds with some metals and organic bases, and some organic derivatives.

Alginic acid and its salts of most di- and poly-valent metals are insoluble, but the alkali metal salts are water soluble. Sodium alginate is one of the most commonly used alginates and several types with differing structures, purities and degree of polymerisation are available.

Although alginate was purified as early as 1896, the first report on its structure was in 1930 by Nelson and Gretcher, who claimed it was a D-mannuronic

acid polymer. Likewise, Hirst *et al* (1939) determined alginic acid to be composed of ß1-4 linked mannuronic acid (Fig 1.1).

The first report of guluronic acid (Fig 1.1) present in alginate was by Fisher and Dorfel (1955), when it was discovered to be a major component.

Improvements in chemical techniques and analysis led to the finding that there are three kinds of polymer segments in the alginate polysaccharide chain (Haug *et al* . 1967). The first segment consists essentially of D-mannuronic acid (Fig 1.2 A), the second region is essentially an area of L-guluronic acid monomers (Fig 1.2 B), whilst the third is a region comprising alternating D-mannuronic and L-guluronic acid residues (Fig 1.2C)

Penman and Sanderson (1972) analysed alginate purified from a variety of different seaweeds, and calculated the relative proportions of the guluronic and mannuronic acids, (Table 1.2).

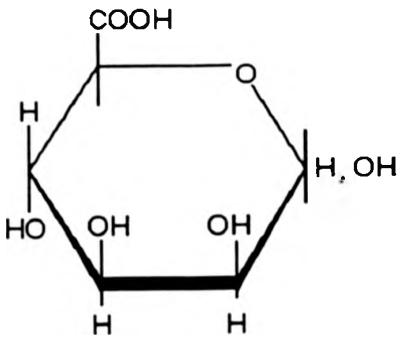
The molecular weight of alginate, like most polysaccharides, is not a definite value, since polysaccharides are of variable chain length; therefore their molecular size is usually defined as a range and mean value. Commercially-available grades of alginate are obtainable from mean molecular weights of greater than 450000 Da, which corresponds to a degree of polymerisation (DP) of 2250 units, down to alginates with a DP of 80.

Highly-polymerised alginic acid powder depolymerises slowly at room temperature to give alginic acid of low molecular weight; however these smaller molecular weight units (DP 40) are very stable and show no appreciable breakdown after years at 10-20 °C. The salts of alginate such as sodium alginate are generally more stable, and those with a DP 500 can be stored for 3 years with no degradation occurring. However, degradation can be observed with more highly polymerised alginates and can become serious if the temperature of storage is above 50° C.

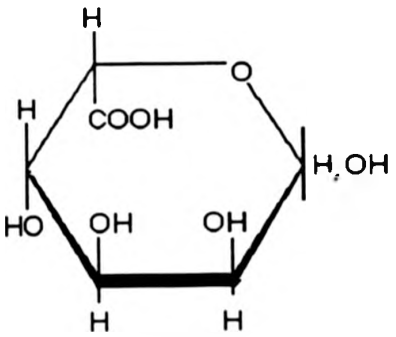
One of the most important and useful properties of alginates is the ability to form gels by reaction with divalent and multivalent cations, of which calcium is

Table 1.2 Chemical heterogeneity in alginate from different sources.

Source	% Guluronic acid	% mannuronic acid
A. Nodosum	43	57
M. pyrifera	42	58
L. digitata	40	60
L. hyperborea	68	32



D-mannuronic acid



L-guluronic acid

Figure 1.1 Haworth representation of the structures of D-mannuronic and L-guluronic acid

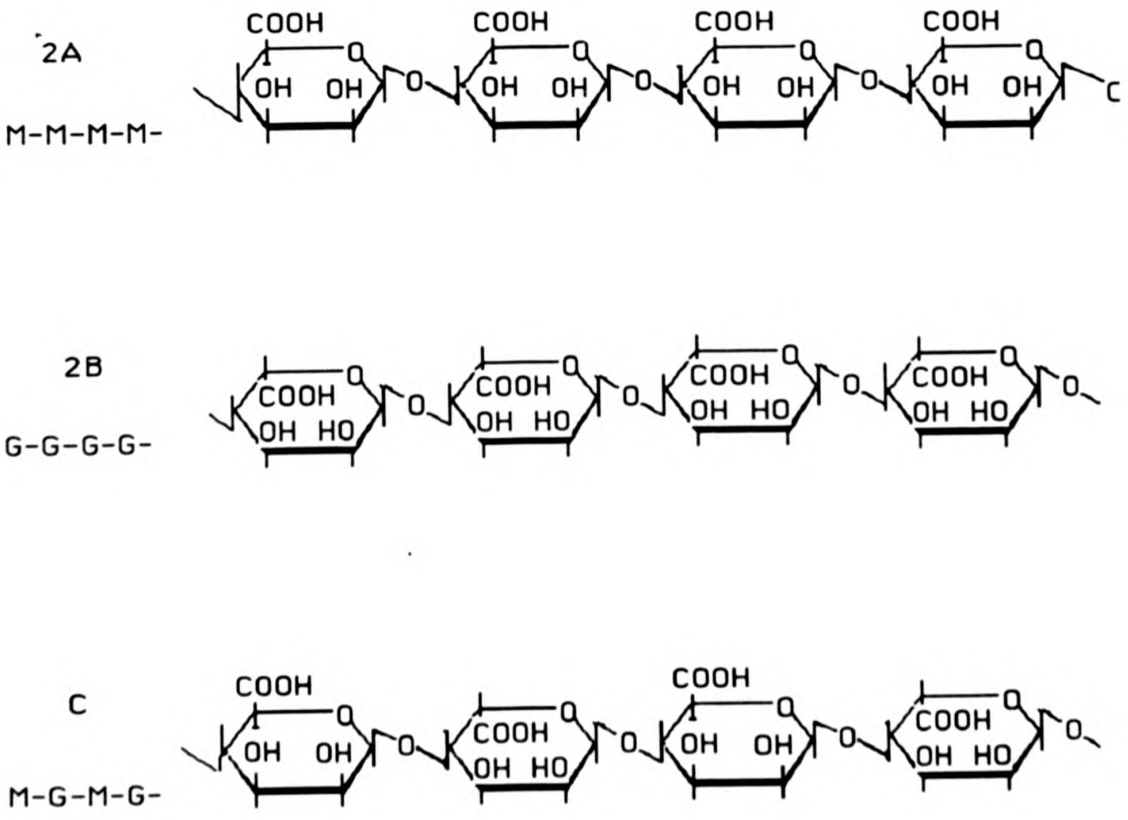


Figure 1.2 Examples of the monomer repeating structure found in alginate

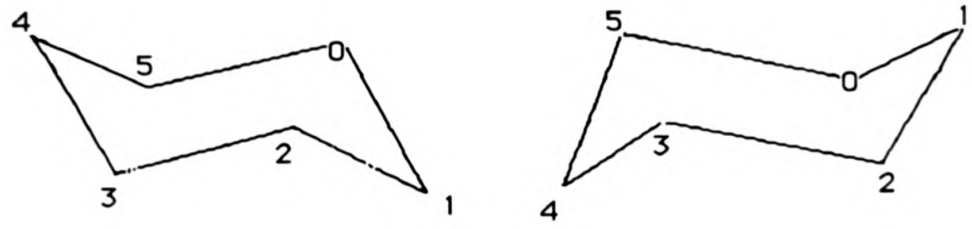
the most commonly used. The gels that are formed can resemble a solid in their ability to resist stress and deformation of shape. Typically the gels used in the food industry consist of 99-99.5% water and alginate. Applications in the food industry include milk desserts, table jellies and animal foods. In all of these systems alginate is used because of its excellent gelling and binding properties.

A gel may be defined as owing its physical properties to a system of polymers cross-linked into a network, which form at the gel point (Hermanns 1949). Much work has been done to elucidate the structure of the gel formed by calcium and alginate, and to determine how the individual polymer molecules interact with each other. Originally it had been suggested that the cross-links were simply a result of ionic bridging between carboxyl groups in adjacent polymer chains by the calcium ions (Rees 1969). This was however shown to be energetically unfavourable and unlikely to provide enough stability to form a gel. Later studies in this field centred on determining the secondary and tertiary structure of the polymers.

X-ray diffraction of fibres of alginate and polarised light IR spectroscopy have resulted in the nature of some secondary structure being determined. The shape of polymannuronic acid (poly M) was shown to be similar to that found in other ß1-4 linked hexosans, such as cellulose. The mannuronic acid hexose ring was shown to be in the C1 conformation (Fig 1.3)(Atkins *et al* 1971). This conformation results in the polymannuronic acid (poly M) being a flat-ribbon like molecule.

Polyguluronic acid (poly G) is different from that of the poly M, in that the sugar rings are in a 1C conformation, which results in a more buckled ribbon shape (Atkins *et al* 1971). Both the 1C and the C1 sugar conformation are shown in Fig 1.3.

On the basis of the fibre diffraction data, it was suggested that since both the poly M and the poly G segments of the polymer were ribbon-like in structure,



C1 1C

Figure 1.3 The different conformations of the hexose rings in alginate

that these ribbons would be capable of stacking together in sheets (Atkins et al 1971). Rees (1969) suggested that co-operative association of either the poly M or the poly G segment was involved in the formation of the cross-links. Circular dichroism studies have shown that the calcium ions react preferentially with poly G segments of the chain (Morris *et al* 1973). The nature of the poly G interactions has since been defined and the most recently proposed model is the 'egg-box' model (Morris and Welsh 1982). The rigid G blocks of the alginate chain are aligned in such a way as to form a cavity between two adjacent G monomers, where the cavity is the correct size to allow a divalent cation such as calcium to fit in. Once a calcium ion has located between the G blocks a second alginate polymer can align on top of the first, to encapsulate the cation. In this manner the calcium acts as a bridge between the two chains and allows junction zones to form, which leads to gelling. The model is illustrated in Fig 1.4.

1.2.2-Galactomannans

Galactomannans are neutral polysaccharides and as such would not be expected to interact strongly with proteins since there can be no charge-interactions. However as discussed in section 1.4.2 locust bean gum has been reported to prevent casein aggregation in milk. In addition galactomannans are widely reported as interacting with xanthan gum, which, like collagen, is a long rigid molecule. It is for this reason that possible galactomannan collagen interactions were investigated in the present study.

Galactomannans are derived from two major sources (a) the endosperm of plant seeds, principally in the Leguminoseae and (b) microbial products.

Microbial galactomannans are usually allergenic and are common to a number of microbes associated with skin diseases, such as the dermatophytes (Bishop *et al* 1965). The galactomannans may even be the causative agents of the symptoms (Barker *et al* 1967) since they are

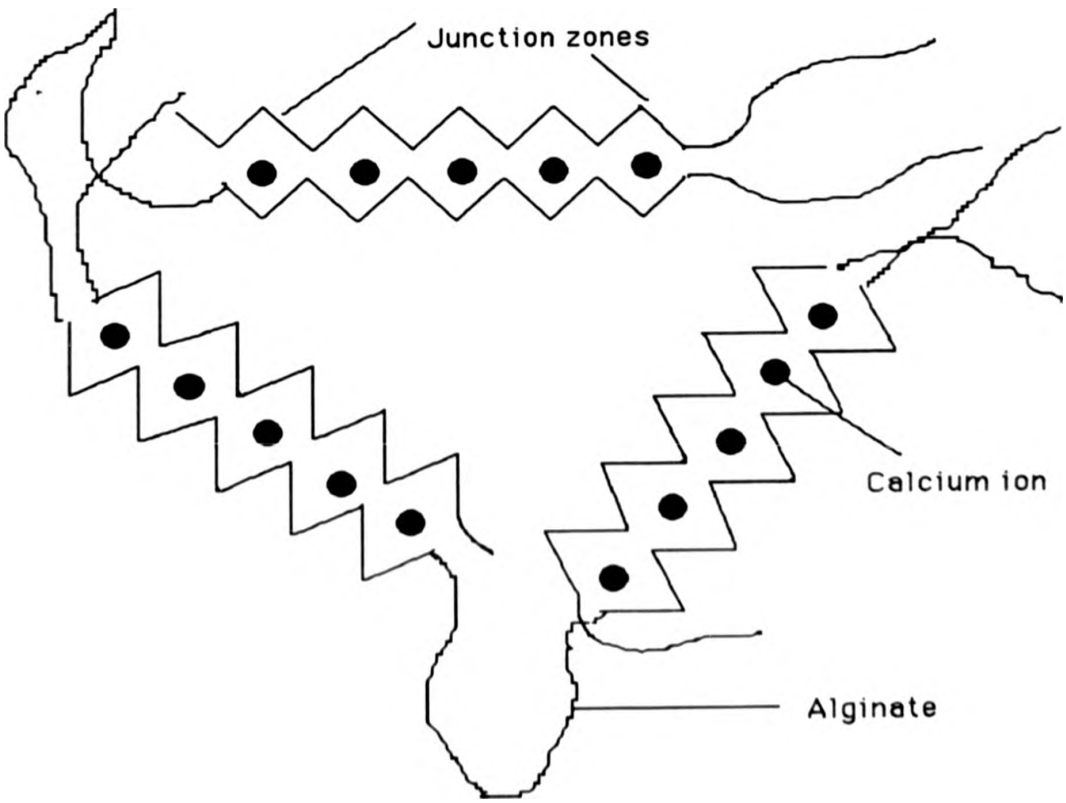


Figure 1.4 Alginate junction zones formed by calcium ion bridging

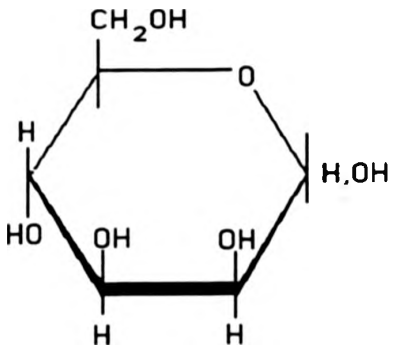
immunogenic (Sakaguchi *et al* 1969) and provoke hypersensitive reactions (Barker *et al* 1962).

Of the plant galactomannans, two types are the most commonly used commercially, and so are readily available. These are guar gum, and locust bean gum isolated from seeds which grow in pods on guar plants and carob trees, respectively.

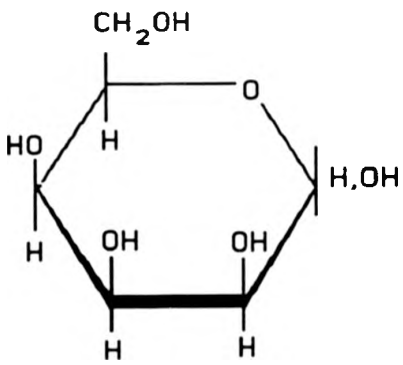
A few of the plants that produce galactomannan polysaccharides have been known and cultivated for many hundreds, and even thousands of years. The best known example is the carob tree (*Certonia siliqua*) which was originally native to Southern Europe and the Near East, but has since been transported to Australia and the U.S.A. (Binder *et al* , 1959). Carob pods have a long history of use by man for both animal and human food sources. In the latter case however this was usually in times of hardship rather than through choice. The synonym of 'St. Johns bread' reveals the biblical association with the carob tree, since the "locusts" eaten by John (Matthew 3:4) in the wilderness are thought to have been locust bean pods. Today guar and locust bean gum are used mainly for their capacity to thicken solutions that remain virtually unaffected by ions, pH and heating and cooling cycles.

During World War II the supply of locust bean gum (LBG) from the Mediterranean countries was severely restricted, and so a search for an alternative was conducted. The plant which was finally selected as a rival was guar (*Cyamopsis tetragonoloba*). Like LBG, the guar is obtained by extraction from the beans that grow in pods on the plants.

Galactomannans were first examined chemically in 1897, when Effront investigated the carbohydrate from the Locust bean pod. Two years later Bourquelot and Herrissey reported that the gum was composed of 83.5% mannose and 16.5% galactose. The structure of these sugar monomers is shown in Fig 1.5.



D-mannose



D-galactose

Figure 1.5 Haworth representation of D-mannose and D-galactose

Despite the fact that the chemical composition of galactomannan was known at the beginning of the 20 th century, it was not until the 1940's and 50's that the structure of the gums was investigated. Most of the structural studies have been restricted to LBG and guar gum, because of their industrial importance. The pioneering work indicated that the galactomannans contain a ß-D-1-4 linked mannan backbone to which single α-D galactosyl residues are attached at the O_6 position of some of the mannose residues. Analysis has shown that guar gum has approximately twice as many galactose residues as LBG. This information was based on polymer methylation analysis (Whistler and Stein 1951; Ahmed and Whistler 1950). Possible structures of guar and Locust bean gum are shown in Fig 1.6

It should be noted that the polymers do not have a regular repeating structure and that the distribution of substituents along the mannan chain is not known with certainty.

1.2.3-Carrageenan

Carrageenan is obtained by extraction with water or alkaline water of certain species of the class Rhodophyceae (red seaweed). It is a linear polymer consisting of galactose units linked with alternating α(1-3)and β (1-4) linkages. In addition, the galactose units linked α 1-3 in the structure often occur as 3,6-anhydro-D-galactose units, and sulphate ester groups may be present on some or all galactose units. Since carrageenan is a large molecule containing up to 1000 residues, the possibility for structural variations are enormous. Three idealised types of carrageenan have been proposed (Fig 1.7), however, commercially available samples contain a mixture of the three. Carrageenans are used extensively in the food industry as both gelling agents and thickeners.

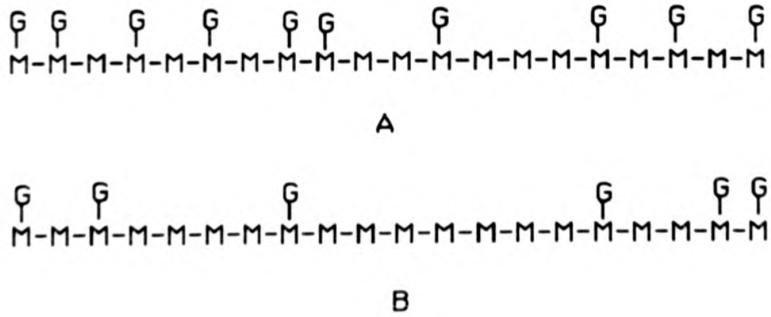
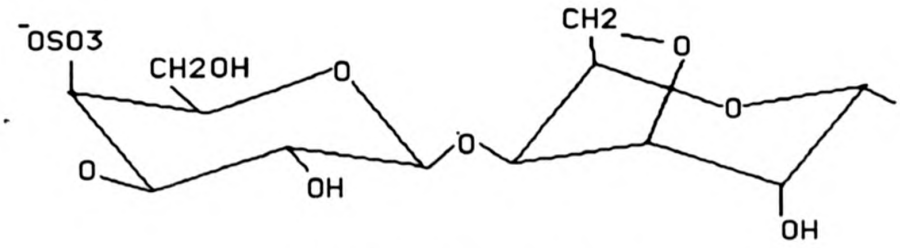
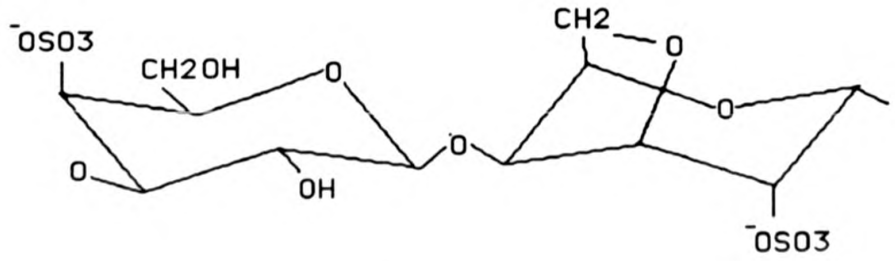


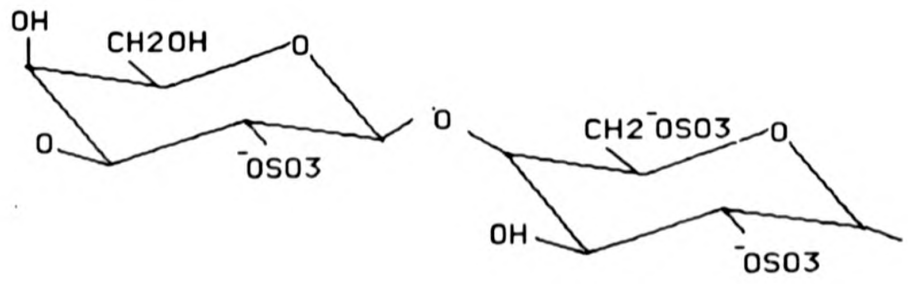
Figure 1.6 Possible structures for (A) guar gum having a D-mannose (M) :D-galactose (G) ratio of 1.8:1 and (B) locust bean gum with a M:G ratio of 3.5:1



KAPPA CARRAGEENAN



IOTA CARRAGEENAN



LAMDA CARRAGEENAN

Figure 1.7 The idealised structure of carrageenen

1.3-Collagen

Collagen in one or more of its many forms can be found in all groups of animals, with the possible exception of the protozoans (Fraser *et al*. 1987). In mammals collagen constitutes approximately one third of the total body protein. Its abundance can be traced to both its prime function of determining the form and structure of an organism, and its versatility, in fulfilling many different biological roles.

Collagen derives its name from two different Greek words, Kolla, meaning glue and Gennan, to produce; hence collagen means 'glue producer'. This is due to the fact that, upon boiling animal tissue, collagen is denatured to produce gelatin.

Before 1970 all vertebrate collagen was regarded as being a simple class of molecule consisting of two $\alpha 1$ chains and one $\alpha 2$ chain, with only slight heterogeneity between species. Since the discovery that collagen was polymorphic the number of recognised vertebrate collagens has grown to at least sixteen, which differ genetically, chemically and immunologically (Ayad et al 1994). The tissue distribution and functional characteristics of the collagens are shown in Table 3. For the purpose of this thesis only the fibrous collagens (types I and III) will be discussed. The structures of these collagen molecules has been extensively reviewed (Piez 1976, Fietzek and Kuhn 1976, Miller 1976, and Kuhn and Glanville 1980, Van der Rest and Garrone 1992) and so only a summary of the major structural features will be presented.

1.3.1-Biosynthesis of collagen

All the fibrillar collagens are initially synthesised in a precursor form called pro-collagen. The production of a procollagen molecule (consisting of three pro α chains) is a complex biosynthetic process, where diversity of the protein product arises from post-transcriptional and post-translational events.

Table 1.3 Collagens: types and distributions.

Type	Chains	Molecules	Representative Tissues
I	α1(I), α2(I)	$[\alpha1(I)]_2\ \alpha2(I)$ $[\alpha1(I)]_3$	Skin, bone, tendon, dentine, etcDentin, skin(minor form)
II	α1(II)	$[\alpha1(II)]_3$	Hyaline cartilage, vitreous body
III	α1(III)	$[\alpha1(III)]_3$	Skin, vessels
IV	α1(IV), α2(IV)	$[1(VI)]_2\ \alpha2(VI)$	Basement membrane
V	α1(V), α2(V), α3(V)	$[\alpha1(V)]_3$ $[\alpha1(V)]_2\ \alpha2(V)$ α1(V) α2(V) α3(V)	Hamster lung cell cultures Fetal membranes, skin, bone Placenta, synovial membranes
VI	α1(VI), α2(VI),α3(VI)	α1(VI)α2(VI)α3(VI)	vessels, skin, intervertebral disc
VII	α1(VII)	$[\alpha1(VII)]_3$	Dermoepidermal junction
VIII	α1(VIII), α2(VIII)	(?)	Descemet's membrane
IX	α1(IX), α2(IX), α3(IX)	α1(IX),α2(IX)α3(IX)	Hyaline cartilage, vitreous humour
X	α1(X)	$[\alpha1(X)]_3$	Growth plate
XI	α1(XI), α2(XI), α3(XI)	α1(XI)α2(XI)α3(XI)	Hyaline cartilage
XII	α1(XII)	$[\alpha1(XII)]_3$	Embryonic tendon and skin periodontal ligament
XIII	α1(XIII)	(?)	Endothelial cells
XIV	α1(XIV)	$[\alpha1(XIV)]_3$	Fetal skin and tendon

Following synthesis of pre-pro- α chains, cleavage of the signal peptide and insertion into cisternae of the rough endoplasmic reticulum, the pre-pro α chains align in the correct stoichiometry. The chains are cross-linked in the C peptide region, the triple helix is then formed in a zipper-like fashion from the C to the N terminus. Simultaneously the triple helical region of the molecule is cross-linked. The newly formed pro-collagen molecules are transported through the golgi apparatus and are secreted into the extracellular matrix where they are modified by a number of enzymes. At this stage the C-pro-peptide and the N pro-peptide are removed to form a collagen molecule.

1.3.2-Structure features of collagen

Type I Collagen is one of the largest of all protein molecules and is composed of three α polypeptides, each having a molecular weight of around 95000 daltons. Each of the α chains is wound in a left handed helix which contains approximately three amino acids per turn. Three of the collagen polypeptides are wound in a rope like structure to form a trimer known as collagen. This molecule is 280 nm in length and 1.4 nm in diameter. The collagen molecule is a right handed helix (Piez *et al* 1963). At each end of the helix there are non-helical domains. In type I collagen at the N-terminus there are around 24 non-helical amino acids, whilst at the C-terminus there are about 16.

The two types of alpha chains which make up the type I collagen molecule have been sequenced and found to contain 1052 amino acids (Hulmes 1973). The helical domain of the collagen has 338 repetitions of the sequence Gly-X-Y which is a triple helix forming sequence. This sequence is common to all types of collagen. However the distribution of amino acids at the X and Y positions varies (Fietzek and Kuhn 1976). Approximately one third of the X and Y positions are occupied by proline and hydroxyproline. The repeating sequence stabilises the triple helix conformation, with the glycine residues located in the inside of the helix due to their small side chain. Proline and

hydroxyproline stiffen the helix by preventing rotation around the C-N bond in the polypeptide backbone.

1.3.3-Fibrillogenesis

As well as the assembly of collagen monomers to form a triple helical collagen molecule, collagen is polymerised extracellularly into fibrils. The formation of fibrils is initiated by the removal of both the C and N terminal pro-peptides (discussed above), which allows the collagen molecules to align. The fibrils are generated as the collagen molecules self-assemble side by side in a quarter-stagger array, and have a wide range of diameters (10-300 nm). Once formed, the fibrils are stabilised by inter- and intra- molecular bonding. The fibrils can then associate to form fibres, which further associate into fibre bundles. When viewed under the electron microscope the fibrils have a striated appearance which can be explained by the "quarter stagger " arrangement of the collagen molecules (Fig 1.8).

Thus by arrangement of collagen molecules, large bundles of collagen fibres can be formed which are unique, stable and in the case of type I and type III collagen have a high tensile strength.

1.3.4-Commercial processing of collagen

Animal skins contain up to 85% collagen, thus by-products from tanneries can be used as a commercial source of fibrous collagen. In leather production the top layer of cow hide (the epidermis and upper dermis) is removed to be processed into leather, the lower half of the hide (the corium) can then be used as the starting material for fibrous collagen production.

Many different processes are carried out to extract collagen from hide in forms suitable for its use as a raw material from a mass of insoluble hide. The first step is dehairing of the hide, which is done under alkaline conditions. Following this, the corium is defatted and any residual pieces of flesh are

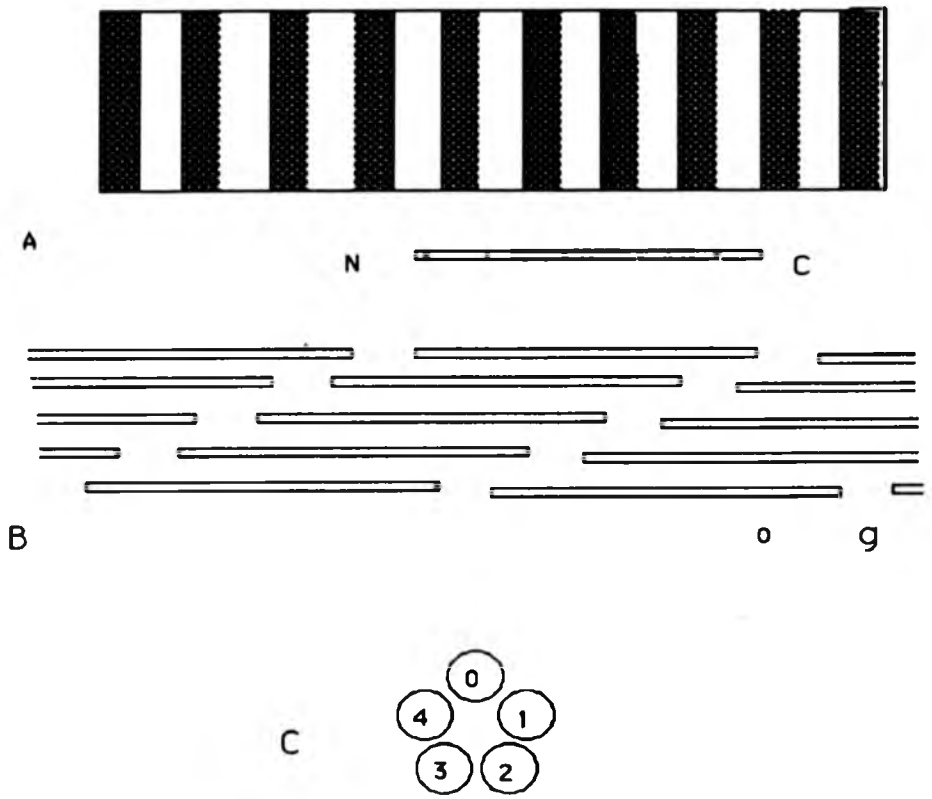


Figure 1.8 Staggered packing arrangement of molecules in the fibril

A Electron micrograph image of negatively stained fibre

B Top diagramatic representation of a single molecule

Bottom , illustration of an axial stagger between molecules that produces the striated effect seen in A. the overlap area is labelled o, the gap ,g.

C cross section of the overlap region in B

removed by liming, this process deaminates the glutamine and the asparagine residues (effectively reducing the IEP from over 9 to below 5), and breaks some of the cross-links. The cleaned and limed hides can then be buffered to a pH value close to the IEP, and chopped and homogenised to yield a dispersion of collagen fibres. Since this process does not denature the collagen, materials produced from the fibres can retain a high tensile strength. Other methods can be used to "clean" the collagen as a fibrous industrial product including salt and enzyme washing, but the liming process is perhaps the predominant method in use today. The resulting high tensile strength of the collagen and the fact that it can be reconstituted into a number of physical forms such as films, tubes, sponges and powders has led to its use in an number of industries including medical practice (Chvapil 1973, Leipivert *et al* 1985).

1.3.5-Soluble collagen

There are several methods of isolating soluble collagen which are commonly used. The method of choice depends on what the collagen will be used for, e.g. to study fibrillogenesis, acid solubilised collagen is most commonly used. However, to prepare collagen for use as an emulsifier, large yields are more important, hence, pepsin solubilisation of cow hide is commonly used.

1.3.6-Pro-collagen

As outlined above, collagen is synthesised as a triple helical pro-collagen molecule. Cleavage of the non-helical amino and carboxyl terminals produces a collagen molecule that rapidly polymerises to form collagen fibrils. The fibrils are stabilised by covalent cross-links which render the collagen molecules insoluble.

It is possible to isolate small amounts of pro-collagen from collagen containing tissue. However, type I and III pro-collagen molecules are processed to

collagen rapidly and so only exist in very small concentrations, particularly in mature animals. The preferred tissue for pro-collagen isolation is foetal skin or placenta. Simple washing of the tissue in Tris buffered saline in the presence of protease inhibitors will extract pro-collagen in a soluble form.

1.3.7-Neutral and Acid soluble collagen

A mixture of covalent cross-links have been shown to stabilise collagen fibrils through a combination of inter and intra molecular cross-links. Soluble collagen can be isolated from tissues that contain only an aldimine-acid labile intermolecular linkage by extraction with 0.5M acetic acid. Foetal calf skin or placenta have the highest percentage of this type of fibril, however, small amounts can be isolated from the tendons of young animals. Acid solubilised collagen obtained in this way is predominately type I, but is 15 amino acids shorter than native type I due to cleavage of an acid labile bond in the C-terminal non-helical region. Collagen prepared in this manner was used extensively as a model for studying fibrillogenesis (Na 1988).

1.3.8-Pepsin soluble collagen

Collagen can be extracted from most connective tissue (including mature hide) by treatment with pepsin. The source tissue is cut into small cubes and suspended in 0.5M acetic acid, with pepsin, usually in a ratio of 1:10 enzyme: substrate, and allowed to digest at 4 °C. Residual insoluble collagen can be removed by centrifugation, and the resulting soluble collagen can be purified by salt precipitation and resuspension. The pepsin does not cleave the triple helix but digests the non-helical cross-linking domain thereby liberating the whole triple helix as a soluble entity. As a result the collagen which is obtained lacks the non-helical region and so has a slightly shorter chain length than native collagen. The collagen obtained in this way is heterogeneous since the

covalent cross-links that stabilise the molecule are intact, this is illustrated in Fig 1.9.

1.4-The nature of protein polysaccharide interactions

In order to understand protein/polysaccharide interactions, it is important to be able to determine how the macromolecules associate with one another. There are several types of interaction possible between macromolecules, and these can be divided into five categories, discussed below.

1.4.1-Electrostatic interaction

Most of the work carried out on protein polysaccharide (pr-p) interactions has been concerned with electrostatic interactions. This is because the insoluble nature of the protein anionic polysaccharide (pr-ap) complexes makes their detection easier.

At pH values below the isoelectric point (IEP) of a protein, the protein and an anionic polysaccharide will carry opposite net charges. Therefore it is apparent that a protein and polysaccharide interacting under these conditions is simply a case of two polyions of opposite charge interacting. At a pH below the IEP of the protein, insoluble complexes can be formed even at very low concentrations e.g. 10 µg/ml (Tolstoguzov 1986), provided that the ionic strength of the mixture is sufficiently low (generally less than 0.1-0.2)

When proteins and anionic polysaccharides are mixed at an ionic strength which prevents the formation of insoluble complexes, the solution divides into two phases: one phase contains a high concentration of the two reagents, and this has been termed the coacervate phase. The second phase is a dilute solution of the two reagents which is called the equilibrium liquid. The pioneering studies on this phenomenon were carried out in the early 20 th century on the interactions of gelatin and gum arabic (Tiebacks 1910) and later by Bungeberg de Jong(1938).

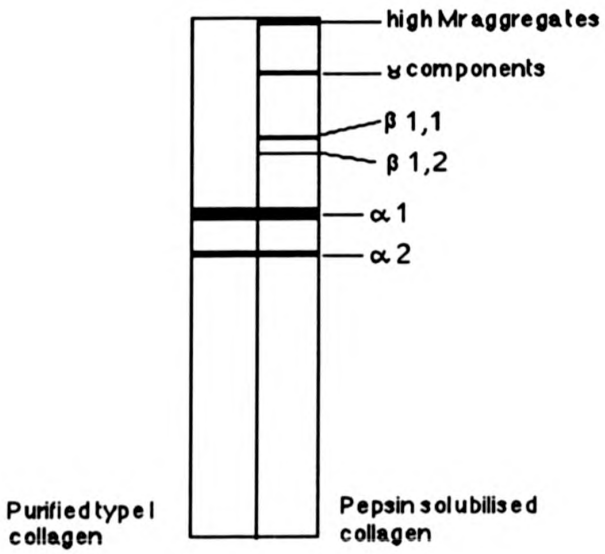


Figure 1.9 SDS polyacrylamide gel banding pattern of pepsin solubilised, and non-crosslinked, Type 1 collagen

As well as complex coacervation, much attention has been paid to soluble pr-ap complexes, which can form between opposite or similarly charged macromolecules, where the complexes remain soluble indefinitely.

The process of electrostatic complex formation can be considered at the molecular level, as a gradual attachment of protein chains to a polyanionic polysaccharide chain. In this manner the protein acts as a ligand and the polysaccharide is the nucleus of the complex. In this way as each successive ligand is attached, the net charge on the polysaccharide decreases (Wajermann *et al* 1972, Tolstoguzov and Wajermann 1975). Therefore the final charge on the pr-ap complex will depend on the folding of the protein and the availability of the charged groups.

For proteins that have a disordered conformation (e.g. gelatin or an unfolded globular protein), the composition of the complex coacervate phase, formed with the anionic polysaccharide is electrically neutral. This is because all the polar amino acid residues will be exposed on the protein, and the polypeptide chain will be flexible enough to allow interaction with the polysaccharide. In this manner the ratio of the charges carried by the macromolecules will determine the protein/ polysaccharide ratio in the complex.

Insoluble complexes of globular proteins with anionic polysaccharides generally contain an excess of protein and therefore possess a net positive charge at a low pH. In contrast, soluble complexes of globular proteins with anionic polysaccharides are generally far from being saturated and so have a net negative charge.

Polysaccharides with carboxyl groups are incapable of complexing with proteins above the protein IEP, due to the net negative charge on the protein. The forces of repulsion between the macromolecules are therefore greater than the forces of attraction. Sulphated polysaccharides on the other hand are capable of interacting above the IEP. This is because they have a higher charge density and so they form weaker, soluble complexes. An example of

this is the interaction between serum albumin and dextran sulphate, where the complex formed can remain in solution up to pH 8.5 even though the serum albumin IEP is 5-5.3 (Noguchi 1960 ;Thomson and McKernan 1961).

As discussed earlier protein-polysaccharide interactions illustrate only one case of an interaction between oppositely charged macromolecules in solution. This area of interactions has been reviewed by Zezin and Rogatsheva (1973), and Bektrov and Bimedina (1977). However mixtures of proteins and polysaccharides show some unusual properties, such as the non-equilibrium nature of Pr-ap complexes (Gurov *et al* 1977), which are discussed below.

The structure and properties of soluble complexes have been investigated by Gurov and his colleagues (1974, 1978 and 1981). The non-equilibrium nature of the complexes was shown by the dependence of the solubility, and other properties of the complexes, on the physical conditions during formation. This is illustrated in Figure 1.10.

If a protein and anionic polysaccharide are mixed below the protein IEP, then an insoluble complex is formed (point A). This insoluble complex is called a mixing complex (M complex). If the same solutions are mixed at a pH above the protein IEP (point B) and then the pH is lowered by dialysis, a soluble complex called a titration complex (T-complex) is formed (point C). A similar effect can be achieved by changing the ionic strength of the solution. It is apparent that by mixing the same protein and polysaccharide at the same pr/ap ratio, but under different physical conditions, complexes which differ in their physical characteristics can be formed.

Surprisingly the two types of complex are very stable, since both T and M-complexes can be stored for months without precipitation or dissolving, respectively.

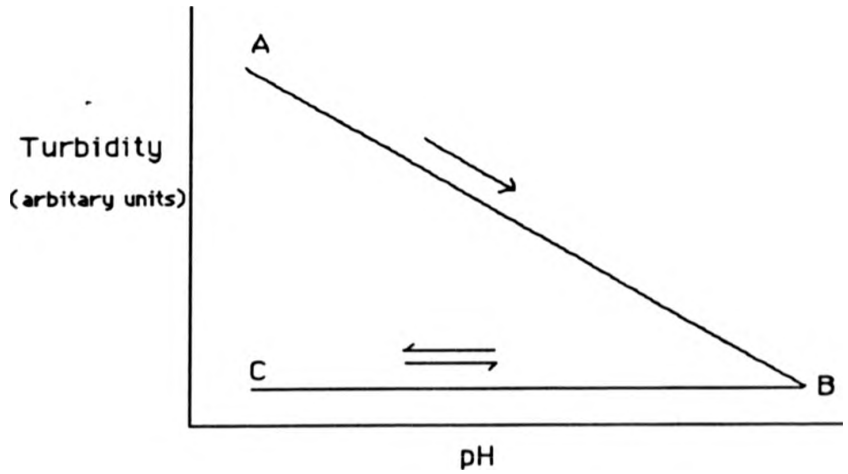


Figure 1.10 Schematic diagram of an acid base titration of a mixture of protein and dextran sulphate solution

1.4.2-Hydrogen bonding.

Hydrogen bonds can be formed between uncharged molecules as well as charged ones. In a hydrogen bond, a hydrogen atom is shared by two other atoms. The atom to which the hydrogen is more tightly linked is called the hydrogen donor, whilst the other atom is the hydrogen acceptor. The donor in a hydrogen bond in a biological system is either an oxygen or a nitrogen atom that has a covalently attached hydrogen atom. The acceptor is either oxygen or nitrogen

Although there is much documented evidence of both inter- and intra-molecular hydrogen bonding in proteins and also between polysaccharides, there is considerably less information on bonding between proteins and polysaccharides.

In theory there is no reason why proteins should not be capable of hydrogen bonding to polysaccharides, assuming that steric positioning of the molecules is such that the two macromolecular chains can come into close contact.

Within the extracellular matrix hydrogen bonding of the GAGs to collagen cannot be ruled out, but any investigations into this have been largely inconclusive.

One example where protein-polysaccharide hydrogen bonding has been indicated is the interaction between casein and locust bean gum (LBG). Locust bean gum is a galactomannan, i.e. it has a linear polymeric backbone of ß 1-4 linked mannose units, which are substituted with single galactose units (section 1.2.2). The areas of the backbone which are unsubstituted are called 'smooth' regions, whilst those with galactose branches are called 'hairy' region. If milk is heat-treated the protein casein is denatured and self-aggregates to produce small protein bodies; addition of LBG to the milk can prevent this aggregation. The proposed model to explain this effect is shown in Figure 1.11. It was suggested that the casein interacts with the LBG through hydrogen bonding with the galactose residues of the LBG; the areas

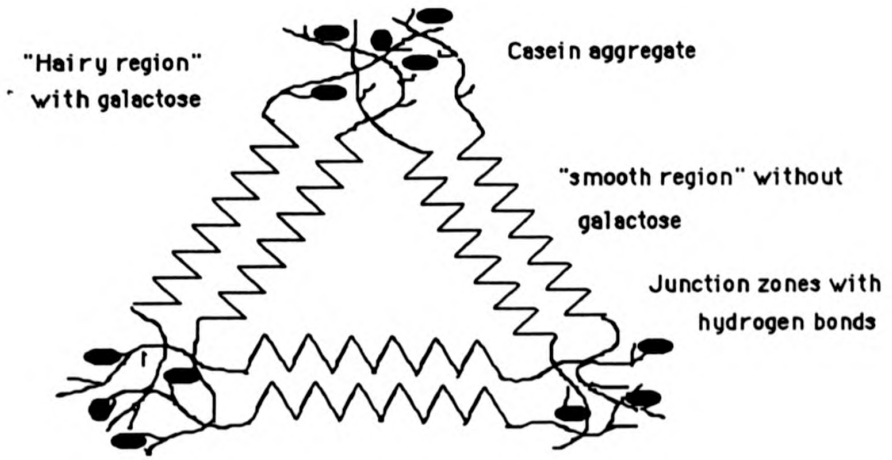


Figure 1.11 A model for LBG/ casein interaction in milk proposed by B.Jud

of the mannose backbone which are substituted with galactose. However, it is also possible that simple entanglement is occurring (unpublished results B. Jud, Unipectin Industries Ltd)

There are also instances of more specific interactions between proteins and polysaccharides, for instance in the immune system: where the binding of an antibody to a polysaccharide antigen is by a combination of electrostatic and hydrogen bonding.

A second example of highly specific hydrogen bonding is in enzyme-substrate specificity, when a polysaccharide-degrading enzyme comes into contact with its substrate. The precise recognition of the substrate is facilitated by a combination of non-covalent bonds, these are usually a mixture of hydrogen and ionic bonding. In this way a substrate is not only delivered to the active site of the enzyme, but through hydrogen bonding is orientated in the precise position necessary for the catalysis to occur. Several examples of this type of interaction have been reported by workers studying enzymes that act upon polysaccharides; these include lysozyme, hexokinase and taka-amylase. In the case of lysozyme there are a total of six hydrogen bonds distributed between three enzyme sub-binding sites and three acetyl glucosamine residues (Clarke and Wilson 1988), whilst the interaction of arabinose binding protein with arabinose is facilitated by 10 hydrogen bonds (Quinocho 1986). This subject has been reviewed recently (Sharon and Lis 1990) and will not be discussed in detail in this thesis

1.4.3-Covalent bonding

In the extracellular matrix of animals GAGs, with the exception of hyaluronic acid, are covalently attached to a protein core to form proteoglycan (Brandt 1981). Of the proteoglycans, the most studied and best characterised are those present in cartilage, particularly chondroitin sulphate proteoglycan.

Cartilage proteoglycans consist of a central core protein with up to 80 GAGs; which are covalently linked to serine residues of the protein (see section 1.6). As well as being bound to protein cores, there is evidence that GAGs are covalently linked to collagen. One example that has been well defined is the covalent attachment of GAGs to type IX collagen (McCormick *et al* 1987).

The covalent attachment of heparin, a GAG, to proteins has been investigated as a mechanism of retaining GAG molecules in prosthetic devices. Narrow diameter vascular prostheses made of collagen should have a uthrombogenic surface. This has been achieved by incubation of the device in a solution of heparin (Rubin and Stenzel, 1969), however, it was feared that the ionic interaction would allow the GAG to leach into the blood stream. Raghunath *et al* (1983) have shown that the heparin can be cross-linked to the collagen, by carbodiimide, to 'lock' it into the prosthesis.

In food systems it would often be advantageous to link a protein covalently to a polysaccharide in order to stabilise the complex over a wide range of conditions. An example of this is in the production of ice cream. When alginate is treated with propylene oxide at 70-85 C, propylene glycol alginate is produced; if the pH is then raised in the presence of gelatin the two become covalently linked via a lysine group (conference presentation G. Stainsby, Proctor Department of Food Science, University of Leeds). This produces an emulsifying agent that is very stable and resists 'creaming' (coalescence of the oil droplets).

A second example of the importance of covalently bound protein/ polysaccharide complexes is illustrated with the naturally occurring biopolymer gum arabic. This is a hetereopolymeric substance consisting of galactose, arabinose, rhamose and glucuronic acid residues. In addition there is a small amount of protein present which forms an integral part of the structure (Vandevelde and Fenyo 1985). This complex is one of the world's most commonly used gums in the food industry, owing to its excellent ability

to thicken and stabilise emulsions. Despite this, its mode of action is not understood but is thought to be due to the mixture of protein and polysaccharide.

1.4.4-Hydrophobic and Van Der Waals Forces

Although polysaccharides are by nature hydrophilic molecules, the steric positioning of the hydroxyl groups due to the carbon ring can lead to hydrophobic patches on the surface of the sugar (Lemieux 1988). It has been suggested that these hydrophobic areas could form contacts with hydrophobic regions of proteins, possibly by stacking between aromatic rings of tyrosine and tryptophan amino acids.

Van der Waals forces are the attractive forces between atoms other than those due to hydrogen or ionic bonding. It has been reported that in the interaction between lysozyme and its substrate there are 141 van der Waals contacts (Clarke and Wilson 1988).

Although both of these types of interaction are possible between many polysaccharides and proteins, there are very few documented cases due to the lack of information on the complexes which is required to establish the nature of these contacts.

1.4.5-Physical entanglement

When long chain polymers are placed in a dilute solution they will rotate freely with only minimal interactions with other molecules, (Fig 1.12a). However, as the concentration of the polymers increases, a point will be reached where the number of molecules is such that they will be forced to interpenetrate, or "entangle" due to the volume constraints (Fig 1.12b). The concentration at which the polymer chains interact is called the coil overlap concentration (Gidley and Robinson 1990). Although, in theory, there will be a single concentration of interaction, effects appear gradually with increasing

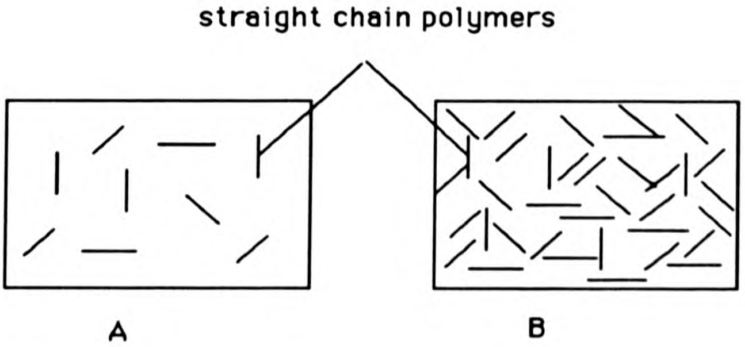


Figure 1.12 Schematic representation of (A) a dilute polymer solution , and (B) a polymer system beyond its coil overlap concentration

concentration. At concentrations above the coil overlap, viscosity increases more rapidly as the polymer chains are forced to interact to a greater extent This area of physical interpenetration is termed the semi-dilute region. A logarithmic plot of specific viscosity/concentration against relative viscosity/concentration can be extrapolated to zero to determine the intrinsic viscosity of a solution. A second graph of log specific viscosity against log intrinsic viscosity x concentration provides a linear plot. The theoretical gradient of this slope can be calculated (De Gennes 1979) and compared to the measured value.

If both are similar, then only a physical entanglement of the polymers is occurring. If, on the other hand, the two values are significantly different, then the increase in viscosity can be attributed to more specific polymer interactions such as hydrogen bonding or electrostatic interactions described earlier.

1.5-The interaction of collagen with glycosaminoglycans

Many methods have been used in an attempt to understand the interactions of collagen and GAGs These have included electrophoresis (Mathews 1965), affinity chromatography (Obrink and Wasteson 1971), precipitation (Toole and Lowther 1968), light scattering (Mathews and Decker 1968), circular dichroism (Gelman and Blackwell 1974), equilibrium binding (Obrink *et al*1975) and agglutination of collagen coated erythrocytes (Conochie *et al*1975).

Initial investigations demonstrated that the GAG/collagen interactions were at least partially electrostatic in nature since raising the ionic strength prevented association. This meant that any hydrogen bonding that may have been occurring was not sufficient to cause complex formation. The strength of binding was also shown to be dependent on the length of the polysaccharide chain and also its linear charge density (Mathews 1965) (Obrink and Wasteson 1971). Frannson (1970) showed by binding studies that GAGs

containing iduronic acid interacted more strongly than those with glucuronic acid. This suggests that the shape of the GAG, as well as the charge upon it, is important in the interaction with collagen

Typical binding ratios for GAG/collagen complexes are 2:1, 4:1, and 5:1 for chondroitin sulphate, depending on the size of GAG used in the study (Obrink and Surdelof 1973).

GAGs are capable of binding many more than one collagen molecule as can be demonstrated by mixing soluble collagen and GAGs which results in precipitation. Both the collagen and the GAGs are long chain repeating structures and therefore a number of interaction sites would be expected. However, the potential number of sites available for interaction is relatively small when one considers that only 8% of the collagen molecule is composed of polar amino acids. In addition, these tend to be found clustered together in small groups on the collagen molecule. Therefore the actual number of sites that a polyanion can bind to a collagen molecule is significantly reduced. Another consideration is that *in vivo* these interactions occur at physiological pH, which is above the isoelectric point of collagen and so the number of positive charges on the collagen will be further reduced. As a consequence a GAG chain may have to align itself parallel to a collagen molecule, or coil around it, in order to interact at enough sites to stabilise the complex (Mathews 1965).

The theory of GAGs having to align or entwine, may explain why GAGs that have iduronic acid bind more strongly to collagen than their glucuronic counterparts. Perhaps the iduronic acid with its sterically different structure is capable of aligning more closely to the triple helix. Interestingly similar results have been observed by groups studying the binding of lipoproteins, and factor 4 to GAGs (Iverius 1972), In each of these cases it was found that Dermatan sulphate (DS) proteoglycan showed tighter binding than the Chondroitin sulphate proteoglycan (CS-PG). No obvious explanation could be given for

this since both molecules have a similar charge density. This recurring phenomenon highlights the need for information regarding the spatial arrangement of charged groups on polysaccharides. The possibility of the GAGs having to entwine also provides an explanation as to why long chain GAGs bind more tightly, since they would be capable of interacting with more binding sites on the collagen.

1.6-Interaction with proteoglycan

In general GAGs do not occur as free chains *in vivo*, but exist covalently bound to a protein with many GAG chains bound to a single protein core. In these complexes the protein is the smallest proportion of the complex, typically accounting for 5-15%. The protein cores of these complexes are very diverse but can in general be divided into three classes determined by their morphology, i.e. small, large and very large.

The salient physical features of the proteoglycans have been determined by rotary shadowing electron microscopy (Wiedmann *et al* 1984). Schematic diagrams of each of these classes are shown in Fig 1.13.

It was suggested that if free GAG chains were capable of binding to soluble collagen, then the proteoglycan molecules which contain many more GAG units should be capable of binding several collagen molecules, leading to high molecular weight aggregates. This was indeed shown to be the case by Mathews (1965).

1.7-The effect of proteoglycan binding on collagen in development and fibrillogenesis

The structural features exhibited by the wide variety of tissues in which collagen is the major component stem from the way that the collagen molecules arrange themselves within the tissues. The 'interstitial' collagens (types I,II and III) form fibres by parallel alignment of collagen molecules

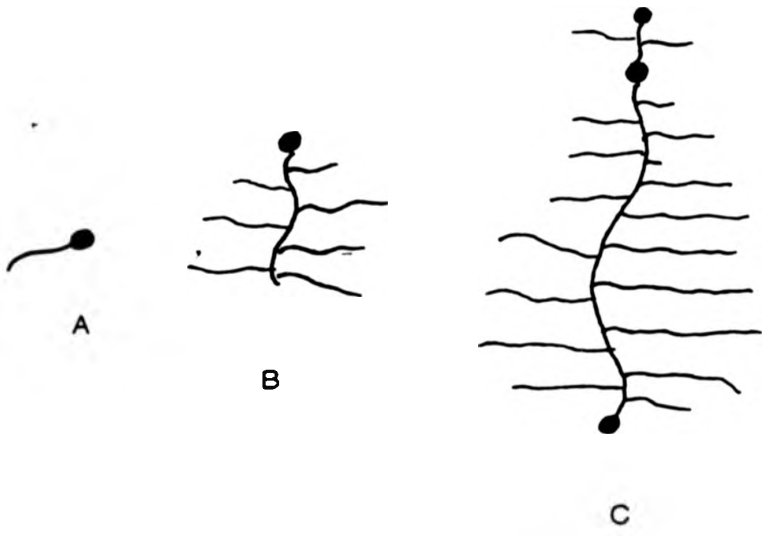


Figure 1.13 Diagrammatic illustration of the appearence of (A) the small, (B) the large, and (C) the very large proteoglycans when viewed by rotary shadowing.

during fibrillogenesis. In tendon the collagen forms large diameter fibres all aligned in the same direction. This provides unidirectional strength to allow muscle attachment and the transmisson of energy and movement to the bone. In skin however the fibres are much smaller and are arranged randomly to provide strength in all directions. In development it is the processes which control this fibrillogenesis that ultimately determine the form that collagen will take in the fully developed tissue. It has been suggested by many authors that GAGs and proteoglycans are responsible for controlling collagen fibril growth, (Toole and Lowther 1968, Lowther *et al* 1970, Mathews and Decker 1968, and Obrink 1973a).

Much of the original work carried out on this subject was invalidated due to the heterogeneous nature of the two species being studied. Sajdera and Hascall's (1969) work on proteoglycan isolation and that of Miller (1971) on collagen other than type I were considerable breakthroughs in that they founded methods for separating heterogeneous preparations of the macromolecules. Work since has shown that chondroitin sulphate (CS) and keratan sulphate (KS) have little effect on fibrillogenesis (Snowdon and Swann 1980, Oegona *et al* 1975 and Obrink 1973a).

Hyaluronic acid was shown to accelerate fibre formation (Obrink 1973a) and initially this was thought to be an exclusion volume effect. Where the collagen molecules can not move close to the large bulky HA chains and are effectively concentrated into a smaller volume. However neither CS or KS showed a similar effect even at a higher concentration. It is more likely therefore, that a more subtle and specific interaction is occurring

In general GAGs which interact strongly with collagen at low temperatures (i.e. dermatan sulphate, heparan sulphate (Obrink 1973a) and heparin (Obrink 1973b) were shown to accelerate the initial step in fibrillogenesis.

Proteoglycans do not behave according to the simple scheme above, and in the case of CS -proteoglycan, although having no effect on fibrillogenesis

(Snowdon and Swann 1980, Obrink 1973a) or even slowing it down (Obrink 1973b; Lowther and Natarajan 1972), the CS-proteoglycan (CS-PG) was incorporated into the collagen fibrils. This contrasts with free CS where the polysaccharide was not incorporated.

In embryonic tissue collagen fibrils grow in an environment rich in hyaluronic acid and PG (Scott *et al* 1981, Toole 1982). Fibril development occurs in two phases: the first is where the fibrils remain thin and any newly synthesised collagen tends to be laid down in the form of new fibrils. This is followed by a second phase where the fibrils increase in diameter due to fibril fusion and aggregation of new molecules of collagen (Wood and Keech 1960). It was observed that phase two coincided with the decline in the proteoglycan/collagen ratio, apparently due to the decline in the amount of CS-PG being produced. Therefore phase two may be dependent on low levels of CS-PG. CS-PG has been reported to be associated with collagen fibrils in several tissues (Scott 1980) and it is possible that the binding of CS to the fibres restricts their growth. It is possible that once the level of CS-PG has dropped to a critical level larger fibrils can begin to form by the displacement of the CS-PG with newly synthesised collagen molecules. Thus by affecting the fibril size, proteoglycans can alter the physical characteristics of the collagen. This indicates that collagen-polysaccharide interactions are very important in development and may be an important force in the direction of the major and subtle differences in connective tissue structure and function

1.8-Food systems

Since the interaction of proteins and polysaccharides have led to such a wide range of rheologies *in vivo*, it is not surprising than food scientists have attempted to exploit such interactions in food systems. Many food products use protein polysaccharide interactions to control the texture, mouth-feel, stability and taste of many processed foods. The major difference between

studying processed food systems and *in vivo* systems, is that in many cases food systems are simpler, contain fewer components and are fabricated by man.

Proteins are commonly used as functional components in food systems in two main areas: as emulsifiers for oil-in-water or water-in-oil emulsions, where their surface active properties are critical, and as gelling agents, relying on lattice networks or fibres. In both respects gelatin (denatured and partially degraded collagen) is widely used. Polysaccharides are not generally surface active but can give rise to high viscosity solutions and gels (such as those formed with alginates) which are used to modify the physical properties of food. However, when proteins and polysaccharides are added in combination, interactions may occur which effect the functionality of the mixture. This change in functionality can often be exploited to produce foods with novel physical properties.

Two areas in food production where protein polysaccharide interactions have been utilised are in the production of emulsions and whipping agents. Bovine serum albumin (BSA) is used as an emulsifying agent. It has been reported that the binding of the polysaccharide dextran to BSA modifies the surface active properties and the emulsifying activity of the protein (Gurov and Tolstoguzov1988). It has been shown that a given quantity of oil can be emulsified by using much smaller amounts of protein-polysaccharide complexed than protein alone. Similar results have been published for a variety of mixed food systems, for instance corn oil-in-water emulsions have greater stability and less tendency to cream and coalesce when prepared using casein-pectin complexes rather than casein alone. This is thought to be due to precipitation of casein-pectin complexes and the subsequent absorption of the complexes onto the surface of the oil/water interface, leading to an encapsulation and stabilisation of the oil droplets (Tokaev *et al* 1987).

The stabilisation of foams by the protective adsorption of protein-polysaccharide complexes at the interface between air and water has also been investigated in order to enhance the encapsulation of air bubbles. It has been shown that the foaming properties of proteins can be greatly improved by interaction with anionic polysaccharides (Poole *et al* 1984) or cationic polysaccharides (Poole 1988). Interactions between gelatin and anionic polysaccharides (AP) below the IEP of gelatin are known to produce foam structures, which are used extensively in mousse products. The precise method of interaction has not been determined, however it is thought that the gelatin-AP complex has advantages in terms of texture, compared with gelatin alone. The polysaccharides which have successfully been used in this regard are carrageenan, alginates, pectins and carboxymethyl cellulose.

As well as using protein polysaccharide complexes to alter the functionality of a food system, there are many reported cases of using the complexes as a food source, or to replace another ingredient, such as fat, within a food. A typical example of this is described by Wen-sherng and his colleagues (1989) who mixed protein and polysaccharide to form complexes which were then homogenised. The homogenised dispersion of the complex had a fat-like texture and was suitable as a fat substitute in food products such as ice cream, salad dressing, dips, spreads and sauces. In a case such as this (and many similar cases in the food industry) the designed functionality can be achieved with a range of protein and polysaccharides components, for instance polysaccharides with poly-carboxylic acid or sulphate groups, such as CMC, pectin, alginates, gellan, xanthan gum and carrageenan, whilst the protein source can be vegetable or animal, such as soy protein, casein, egg protein, peanut protein, cottonseed protein, sunflower protein or pea protein or, indeed a mixture of these. The chosen polysaccharide and protein source are then blended together at a pH below the IEP of the protein, until insoluble

complexes form. The complexes are then simply homogenised until a smooth creamy texture is achieved.

The fibrous nature of some protein-polysaccharide complexes has been exploited in several areas of the food industry, for instance to produce fibres that resemble torn meat fragments in both texture and appearance (Okada 1989). The fibres are formed from an insoluble complex of alginate and a heat coagulable protein such as soy protein, or egg albumin. The precipitated fibres can be formed by mixing soluble sodium alginate with the protein, and introducing a calcium salt. Co-precipitation of calcium alginate-protein complexes occurs giving an insoluble protein-polysaccharide complex. This material can then be flavoured, cooked and compressed to yield meat analogues such as 'crab sticks'.

Many food related patents involving protein-polysaccharide interactions are non-specific with respect to the precise protein source used, since, as long as the protein is food approved and imparts the correct functionality, the only other consideration is the cost. In some cases however, more specific interactions are used, such as in meat reconstitution. Once the prime cuts of meat have been taken from an animal carcass, some off-cuts that are left are still good quality meat but are lacking texture. Several methods have been proposed to eliminate this area of wastage, by reconstituting the meat into large pieces.

One such system involving protein-polysaccharide interactions uses alginates (Richardson 1988). Alginate is mixed with 80% good quality meat off cuts and 20% fat, with calcium ions added to form a gel. This method has been shown to work for lamb, beef, pork and chicken. Although gelling of the alginate is an important step, it is also necessary to allow the alginate to bind to the meat proteins before it gels, to achieve the desired texture. This illustrates that polysaccharides are capable of interaction with insoluble protein, such as the muscle fibres in meat, as well as soluble protein.

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Cold desserts are a further area of food production where protein and polysaccharides are utilised. Many cold desserts are made from gelled milk products. In the majority of these cases the polysaccharide used is carrageenan, a linear sulphated polysaccharide, which interacts with milk proteins. Interestingly the amount of carrageenan required to gel milk was found to be only 20% of that needed to gel the equivalent amount of water (Personal communication: Jens Roesen). This can be explained by the fact that carrageenan interacts with casein in milk. In order for gelling to occur in a system, linear polymers must associate. In water, carrageenan chains will interact to form junction zones which consist of two molecules intertwined in a double helix (Rees 1972), once a sufficient number of zones have formed gelation will occur. The same is also true in milk. However, because casein is capable of forming junction zones with carrageenan, the number of junction zones is increased through casein-carrageenan interaction. This results in a lower concentration of carrageenan being necessary to form sufficient junction zones, and cause gelation. A diagram of kappa-casein and carrageenan chains interacting is shown in Figure 1.14. A synergistic effect on gelling, such as that observed with carrageenan and casein, is what has led to protein-polysaccharide systems becoming so popular in the food industry. Often a gel can be formed that requires very small amounts of solid; this is more economical in the use of raw materials and can improve the palatability of the product.

1.9-Methods of investigating protein anionic polysaccharides interactions

1.9.1-Circular dichroism

Optically active compounds are capable of rotating plane polarised light. Optical rotatory dispersion, measures how much polarised light is rotated on passing through a solution. Circular dichroism is based on a similar principle; however instead of measuring rotation of polarised light, the difference in the

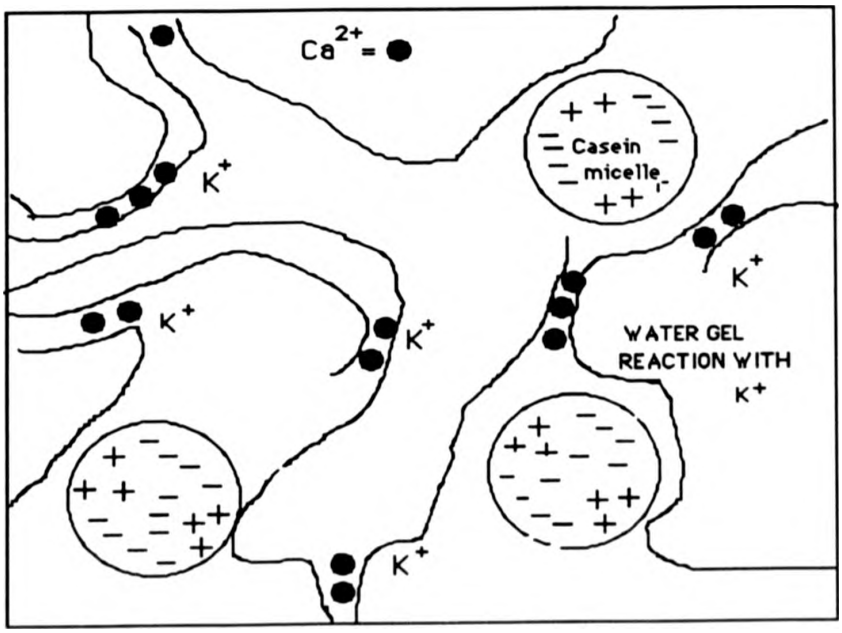


Figure 1.14 Diagramatic interaction of carrageenan polymers with α casein at the interface of an emulsion particle in milk

absorption of left and right polarised light is measured at different wavelengths.

In protein molecules many of the carbon atoms along the polypeptide backbone are optically active The precise spectra obtained from CD depends on the spatial arrangement of these atoms in relation to one another, in this manner any change in protein structure will involve the rotation of bond angles, and a resultant change in the CD spectra. CD is therefore capable of determining changes in secondary and tertiary structure. Collagen is a particularly good molecule to study because of its highly repetitive structure. Gelman and Blackwell (1974) measured CD spectra on mixtures of soluble collagen and polysaccharides, using the CD to detect the melting point (Tm) of the collagen helix, and then adding the GAGs to see if they had any effect on the melting point. In all cases the GAGs had the effect of raising the Tm, leading to the conclusion that they were stabilising the collagen molecule in some way.

1.9.2-Precipitation

Work has been carried out on the precipitation of protein polysaccharide complexes by a number of researchers e.g. Toole and Lowther 1968: Toole 1976: Oegema *et al* . 1975). Toole used radio-labelled ^{35}S proteoglycans and added them to a suspension of collagen. The resulting precipitate was centrifuged and the amount of radiolabel incorporated into the precipitate was measured. Similar investigations were carried out by Oegema *et al* , but in this case the rate of precipitation (fibre formation) was measured as well as the composition of the precipitate. This method of analysis has the advantage of being easy to carry out, and allows the binding ratio of the protein polysaccharide complex to be calculated.

1.9.3-Affinity chromatography

Affinity chromatography was used by Greenwald *et al* (1975) and Obrink (1973a), to investigate the interaction of proteoglycans with collagen. Soluble collagen was covalently bound to CNBr activated sepharose 4B, which was placed in a chromatography column. Proteoglycans were applied to the column at physiological pH and ionic strength to measure any binding that was occurring. This technique is well understood and has the advantage that once the column is packed, many different polysaccharides can be applied, allowing a large number of samples to be screened for an interaction. By altering the elution conditions of the column, the range of physical conditions over which interactions occur can be determined.

1.9.4-Electrophoresis.

Interactions between polysaccharides and proteins can be detected using electrophoresis. This technique was popular in early studies (Mathews 1965), however, it has not been widely used since, due to the development of alternative techniques, such as chromatography, that are easier to use.

1.9.5-Viscosity

The development of more reliable and accurate viscometers has meant that the detection of small transient interactions, between bioploymers, is possible using viscometry. Simple chain entanglement as described in section 1.4.5 can be detected using rotational viscometers. However, for smaller, but permanent interactions, this method is unsuitable since the rotational movement of the viscometer plates destroys any delicate structure that may have formed. Clearly, small deformation techniques are required which preserve the structure of an interaction, this can be achieved by oscillatory movements of the viscometer plates as opposed to rotational. The theory behind these measurements is complex and very mathematical in nature, but

nonetheless can provide important information on biopolymers interacting in solutions and gels (Gidley and Robinson 1990).

1.10 Aim of this work

The aim of this work was to investigate the interaction of a range of polysaccharides with collagen. Initially any interactions of interest were found by studying mixtures of polysaccharides and pepsin solubilised collagen in solution. Once characterised in solution, the interactions were then investigated in biomedical wound healing devices prepared from fibrous insoluble collagen. By using this system it was hoped to determine the effect of different polysaccharides, molecular distribution, degree of polymerisation and ion concentration on the release of the polysaccharides, from the wound healing devices, into solution.

CHAPTER 2 MATERIALS AND METHODS

2.1. PREPARATION OF BIOPOLYMERS

2.1.1. Fibrous collagen

Fibrous collagen was prepared from limed bovine hide that had been chopped, and swollen in acetic acid (obtained from Johnson and Johnson MBG Bellshill, Scotland). This collagen 'paste' was freeze dried in trays 25x 500x 1000 mm. The dry collagen was then broken up by hand and passed through a Pfiffer mill with a 1 mm plate. During this operation the mill was kept below 25°C by pre-cooling the removable parts in a -18°C freezer, to reduce the possibility of heat denaturation of the collagen. The resulting fibres are shown in Plate 1

2.1.1.2 Methylation of fibrous collagen

Methylation of fibrous collagen was carried out in dehydrated methanol containing 0.1M HCl according to the method of Wang *et al* (1978). Collagen fibres (2g) were suspended in 2l methanol and HCl and stirred by overhead mechanical stirrer for 7 days. The fibres were then filtered and washed with pure methanol and air dried.

2.1.2. Pepsin solubilised collagen

Pepsin solubilised collagen (PSC) was prepared from limed calf hide obtained from Devro Limited (Moodiesburn, Scotland). The hide was cut into small cubes (2-3 mm) and (500g) added to 10 litres of acetic acid (0.05M). The cubes had a 70% moisture content and using this value, the amount of collagen in the hide was calculated, pepsin (Sigma chemical Co) was then added in a 1:50 (pepsin:collagen) ratio. The solution was then stirred at 4°C for 72 hours to allow digestion to occur, and the mixture was centrifuged (12000g for 20 minutes) to remove the undigested hide. The supernatant was collected and the pH raised to 7 by the addition of sodium hydroxide (10M).

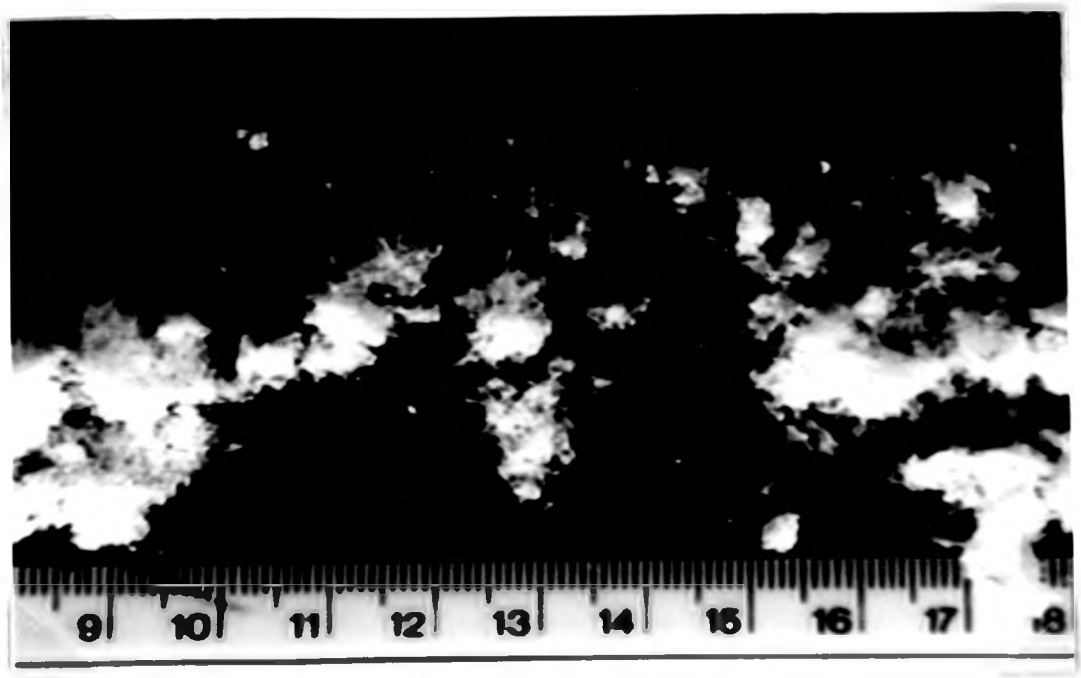


Plate 1 (a) Digital image of fibrous collagen (scale cm)

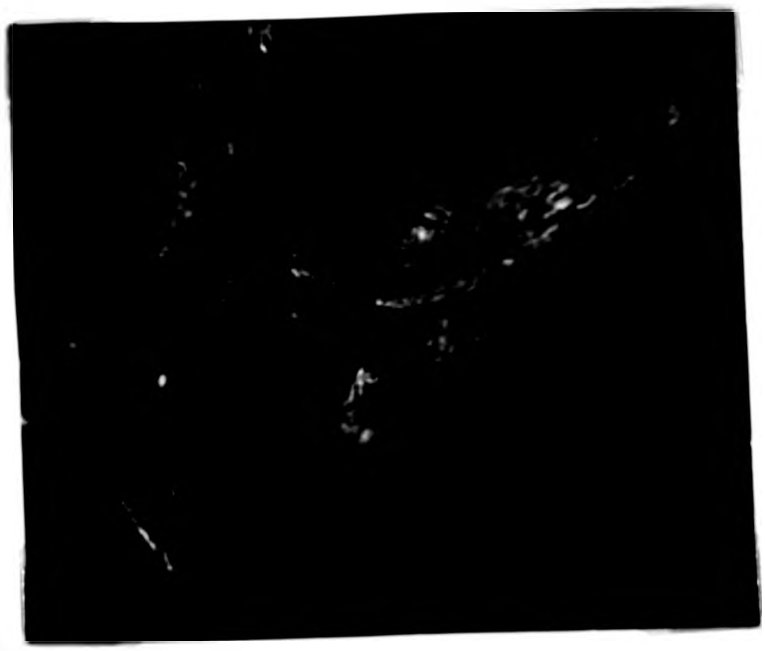


Plate 1 (b) Collagen fibre visualised under phase contrast (104x magnification)

The solution was left for 24 hours to inactivate the pepsin. After this time the pH was lowered to 3 by the addition of glacial acetic acid. At this stage an assay for pepsin activity was performed to ensure that none remained (section 2.2.12). If measurable pepsin activity was found, the pH was again raised to 7 and left for further a 24 hours prior to acidification. Sodium chloride was then added to a final concentration of 5% and left overnight to allow collagen precipitation to occur. The precipitate was collected by passing the solution through a sieve (1 mm mesh) and further concentrated by centrifugation (12000 g for 10 minutes). The pellet obtained was redissolved overnight in 10 litres of acetic acid (0.05M). The salt precipitation step was then repeated and the pellet redissolved as before. The solubilised collagen obtained in this way was then assayed for protein (see sections 2.2.10 and 2.2.11) and stored at 4°C.

2.1.3 Gelatin

Gelatin was prepared by denaturation of fibrous collagen (prepared according to method 2.1.1). The denaturation was achieved by suspending fibrous collagen in 0.05M acetic acid and heating to 60°C for 15 minutes. The suspension was then cooled to room temperature and stored at 4°C.

2.1.4. Polysaccharides

Six alginate preparations, varying in molecular size and ratio of mannuronic /guluronic acid (M/G) were obtained from Protan Ltd (Drammen, Norway). The technical data on these samples are shown in Table 2.1. Guar and locust bean gum were donated by Meyhall chemicals (Kreuzlingen, Switzerland.). All the polysaccharides were supplied in a powder form, and were prepared as 1% stock solutions (w/v) in deionised water unless indicated otherwise. This was done by adding the powder slowly to water stirred to a vortex by a mechanical overhead stirrer (Janke and Kunkel GMBH), to prevent

Table 2.1 Alginate technical information

Manufacturers Code No	Viscosity (centipoise)	Molecular weight (kDa)	Guluronic acid residues (%)
LF 120	50-150	150-250	40
SF60	400-600	350-450	65
HF 60	600-1200	450-600	65
LAM LV	45-55	150-180	35
LF1060	40-70	120-160	65
LF1040	40-70	130-170	45

aggregation of the particles and allow good dispersion. Any large aggregations were dispersed by passing the solution through a Silverston mixer (Chesham) for 30 seconds. The solutions were then heated to 90°C for 1 minute to promote and standardise the development of viscosity, then allowed to cool to room temperature before being stored at 4°C for up to two weeks.

2.1.5. Biopolymer sponges

Collagen/alginate sponges were prepared by making the collagen fibre slurry and the alginate premix separately and then mixing the two. The collagen slurry was prepared by stirring 24.58g of collagen fibres in 5 litres of 0.05M acetic acid at 4°C. The mixture was then transferred to a Waring blender (capacity 4l) and homogenised in two batches of 2.5l, for 30 seconds three times.

The calcium alginate premix was prepared by taking 600 ml of a 1% alginate solution in 0.05M acetic acid, adding 300 ml of $CaCl_2$ H_2O (3.025 g In 300 ml of 0.05M acetic acid). The amount of calcium added was such that 50% of the calcium binding sites on the alginate polymer chains were filled (based on a titration of sodium alginate with Ca^{2+} ions, personal communication Dr P. Watt). The calcium alginate mixture was then mixed in a Waring blender for 15 seconds three times.

The two components were then mixed: for 10% alginate sponges 698.66g of collagen slurry was mixed with 51.34g of calcium alginate premix. The mixing was done by adding the alginate to the blender cup over a 15 second homogenisation. One further 15 second homogenisation was carried out before the mixture was placed in a vacuum chamber (Gallenkamp) to degas. Solutions and suspensions that required degassing were placed in a beaker 10 times the volume of the sample, in the vacuum oven at room temperature. A vacuum of not less than 10^{-1} mbar was then applied (using an Edwards two

stage pump) until no further bubbling occurred in the slurry. If upon removal from the oven there were still air bubbles present, the sample was returned to the chamber for a further 10 minutes

Once degassed, 600 ml of the mixture was poured into a freeze drying tray (500x250x35mm to a depth of 5 mm) and placed in a blast freezer (Foster) at - 30°C until frozen. The samples were then freeze dried.

2.1.6. Biopolymer Films

Biopolymer films were prepared by adding 3.6g of collagen powder to 500 ml of 0.05M acetic acid and mixing in a Waring blender for 30 seconds three times. The calcium alginate premix was prepared as for sponges (section 2.1.5). Collagen slurry (500 ml) was homogenised in a Waring blender with 1 g of glycerol, for 15 seconds during which 60.6 ml of the calcium alginate was added. The mixture was homogenised for a further 15 seconds then degassed. The degassed slurry was poured into plastic trays (520 x 330 x 15 mm) to give a total solids content of 5g per tray. The trays were then placed in a drying cupboard and allowed to air dry at room temperature. Once dried the films were carefully peeled from the tray.

2.1.7. Freeze drying

Samples to be freeze-dried were frozen with a temperature probe placed in the sample at the side of each tray and loaded into the freeze drier (Virtis 1000l, USA). Before loading, the freeze drier shelves were chilled to -25°C. Over the 48hrs duration of freeze drying the shelf temperature was raised in 10°C increments to room temperature. The process was then complete and the samples were removed.

2.2. ASSAYS

2.2.1. Alginate release from collagen-alginate composites

2.2.1.1. Extraction with stirring

To measure the amount and the rate of alginate release, a sample of sponge or film was taken, weighed and placed in 10 ml 0.9% saline. The sample was cut using a cork borer (15 mm diameter) to ensure size uniformity.

The composite sponge and saline were placed in a 15 ml sterilin container and shaken on a rotary shaker (120 rpm). To investigate the rate of release, samples of 0.25 ml were removed from the container at intervals up to 24 hours, and replaced with the same volume of fresh saline at each time-point.

The amount of alginate in these samples was determined using the carbazole uronic acid assay (section 2.2.11.1). The total amount of alginate released was determined by analysis of the supernatant after 24 hours.

The molecular weight distribution of the alginates released from the collagen alginate sponges was determined by taking three collagen alginate sponges weighing approximately 600 mg and placing them in a 165 ml sterilin container with 100 ml of 0.9% saline. The samples were shaken on an orbital shaker (Jankle and Kunkel) for 4, 24 and 72 hours, and at each time point the saline was decanted and replaced with fresh solution. The three extracts were dried at 35°C by rotary evaporation, and re-dissolved in 10 ml of deionised water. The salt was removed by 24 hour dialysis against deionised water and the dialysate was chromatographed on the Sepharose 4B™ column (650x22 mm).

To extract residual insoluble alginate from the composite, it was necessary to remove the collagen from the insoluble collagen-alginate complexes. The sponges were placed in 100 ml of water and the pH adjusted to 3 by the addition of acetic acid. Pepsin was added (0.5% w/v, 1590 units mg^{-1} and the sponges incubated overnight at 35°C. The mixture was then heated to 60° C for 15 minutes, cooled to room temperature and a further 0.25g of pepsin was

added. The sponges were then incubated at 35°C for a further 4 hours. Sodium hydroxide was then added to raise the pH to 8 and the solution was dialysed overnight against 10 litres of 0.01M sodium hydroxide with 1 mM EDTA. The contents of the dialysis tubing were centrifuged (10000g for 15 minutes) and any insoluble material discarded. The supernatant was then dialysed against 10 litres of water for 24 hours. The contents of the tubing were evaporated to dryness and the residue was redissolved in 40 ml of deionised water. Two volumes of ethanol (at -18°C) were then added to precipitate the alginate, and the solution held at this temperature for 4 hours. The solution was again centrifuged (200g for 15 minutes) the supernatant discarded and the pellet redissolved in 10 ml of 0.1M sodium sulphate prior to chromatography on Sepharose™ 4B.

2.2.1.2 Extraction in phosphate buffered saline

A second method was used to extract insoluble alginate from the collagen-alginate complex. This consisted of extraction in 0.1M PBS containing 10 mM EDTA at room temperature overnight. The solution was centrifuged at 10000 g for 10 minutes, the pellet was discarded, and the supernatant rotary evaporated to dryness (35°C). The residue was redissolved in 10 ml of deionised water, dialysed overnight against 20 litres of deionised water, and applied to the Sepharose™ 4B column.

2.2.1.3. Extraction by passive diffusion

Initially it was planned to construct a wound healing model which mimicked the environment of a biomedical sponge placed on a wound. The requirements were for the sponge to be supported in contact with a limited volume of liquid rather than immersed in a large volume to give "sink" conditions. The support medium had to allow simple sampling of saline

solution without interference with the carbazole assay for uronic acids. Several supports were tested but found to be unsuitable; these included polyurethane foam, Sepharose™ beads and polyacrylamide. Finally, glass beads (diameter 1 mm) were chosen since they were inert, easily separated from the liquid, and recyclable. The arrangement used for the model is illustrated in Figure 2.1.

To assess how effective the beads were in allowing passive diffusion of molecules, small circles of sponge were dipped in methylene blue dye and placed on the wound model. It was found that the dye diffused uniformly from the sponge. This was taken as evidence that the beads did not inhibit the diffusion of small molecules into the 'interstitial fluid.

To construct the wound model 7 g of glass beads were placed in a petri dish (diameter 90 mm), with 7 ml of saline solution (0.9%). This volume was sufficient to cover the beads without an excess on the surface. The sponge sample was then placed on top of the beads and left for 24 hours at room temperature. After this time the sponge was removed and the saline was decanted from the beads, the alginate content was determined using the carbazole uronic acid assay.

2.2.2. Permeability of collagen-polysaccharide films

Permeability studies were carried out on collagen polysaccharide films by measuring the weight of saline lost from a vessel through a film clamped over the opening (see Fig 2.2). A 20 ml Sterilin container was adapted to measure permeability by removing the centre of the lid and clamping the film over the mouth of the container. A 5 mm diameter hole was drilled in the bottom of the container through which 10 ml of saline was introduced. The initial weight was noted and the containers were placed on a mesh support in a sealed incubator and weighed at set time intervals. The incubator was set to a temperature of 37°C.

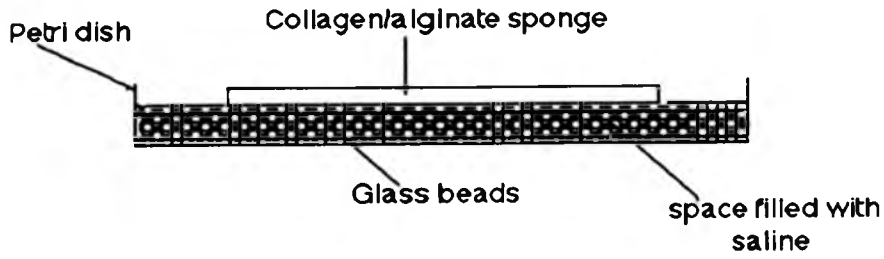


Figure 2.1 Schematic representation of the "wound healing " model

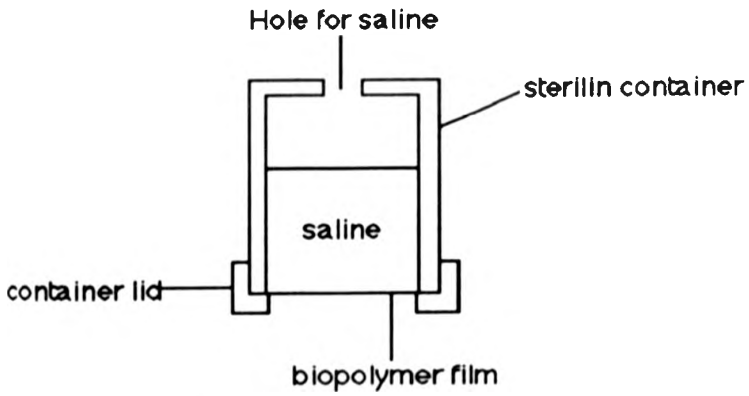


Figure 2.2 Cross-sectional view of the film permeation apparatus

2.2.3. Tensile strength of sponges and films

The tensile strength of samples was measured on a Lloyd tensile tester (500 series). Samples were cut using a dumbell shaped die (Fig 2.3). The sample was then clamped in the jaws of the instrument using tissue paper to prevent the samples being damaged (Fig 2.4). The distance between the clamps was 100 mm and the loading rate was 10 cm/min applied until the sample broke. The tensile strength of the sample was taken to be the point of maximum strain and was measured in Newtons.

2.2.4. Absorbance of saline by sponges.

To measure the swelling and water-binding characteristics of the sponge composites, samples were placed in a small volume of saline (typically 10 mg of sponge placed in 5 ml of saline). At regular time intervals the sponges were removed from the saline, excess fluid drained and the sample weighed. The rate and total amount of saline absorbed was then calculated and expressed as ml of saline per mg of sponge.

2.2.5. Rheology

2.2.5.1. Bulb Viscometry

The relative and specific viscosities of the Protan alginates were measured on an Ubbelhode suspended level viscometer (Fisons). The viscometer is used to compare the viscosity of a solution with that of its solvent, at a constant temperature.

The viscometer was clamped in a water bath, half submerged in water at 25°C. 1.5 mM phosphate buffered saline pH 7.3 was added to the viscometer (through tube A, Fig 2.5) and equilibrated for 10 minutes. The PBS was

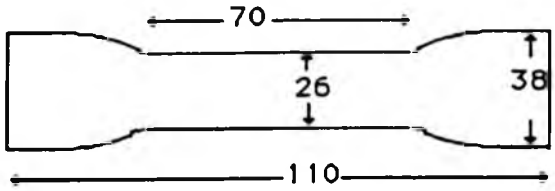


Figure 2.3 Shape and dimensions (mm) of the samples used for tensile testing

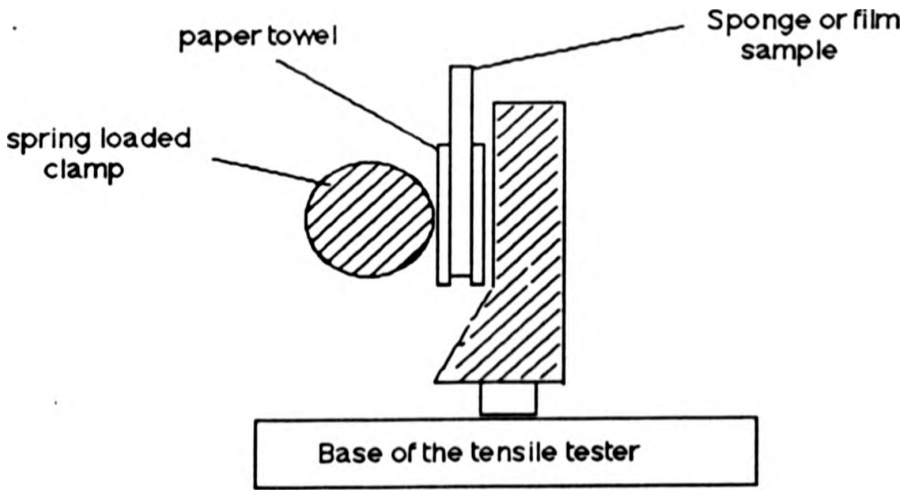


Figure 2.4 Cross sectional view of clamping mechanism with sample in place

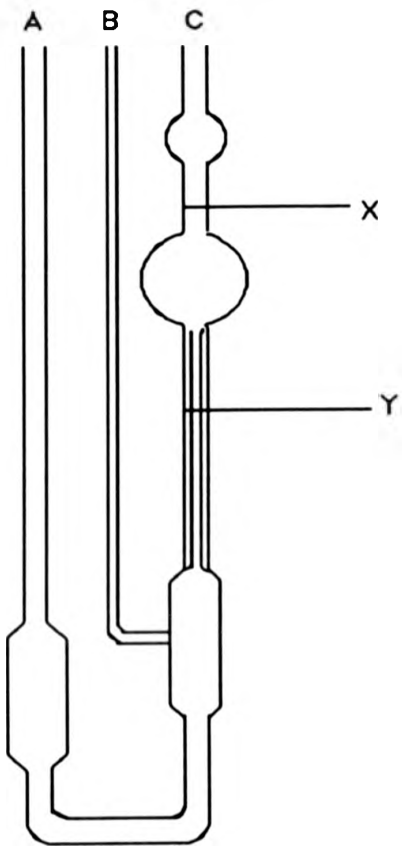


Figure 2.5 Ubbelhode suspended level viscometer. The solution being measured is drawn into arm C and the time taken for the level to drop between points X and Y is recorded.

sucked by pipette filler into the top bulb in tube C. The time taken for the level of the solution to fall from X to Y was then measured three times. Alginate was added to tube A and mixed by blowing air through tube B. The solution was drawn into tube C and the time taken for the level to fall from line X to Y was recorded three times. If the average time was less than 140 seconds more alginate was added to the solution and the measurements repeated, until an average greater than 140 seconds was achieved. The concentrations of the alginate samples were determined by the carbazole uronic acid assay (Section 2.3.11.1). The formula used to calculate the molecular weight of the samples is that of Fermentech Limited (unpublished results, given below.)

T_0 = average time of buffer T_1 = average time of sample

c = concentration of sample (g/100 ml)

$$n_{sp} = \frac{T1}{T0} - 1 \qquad \ln n_{rel} = \ln \frac{T1}{T0}$$

$$[n] = \frac{\sqrt{2\ (n_{sp} - n_{rel})}}{c}$$

$$Mwt = \text{Antilog}\ \frac{\log[n] + 3.244}{0.75}$$

2.2.5.2. Controlled stress rheometry

Rheology measurements were made on two controlled stress rheometers. The Deer rheometer was a manually adjustable cone and plate rheometer, the details of which are given in Table 2.2. The plate which was used in each case depended on the viscosity of the sample being measured. The plate was chosen so that the shear rate was between 1 and 50 radians/sec (1<shear rate<50 rad/s), since at shear rates in excess of 50 rad/sec, the sample was forced from between the plates, Whereas, shear rates below 1 were in the region of high shear thinning and were largely inconsistent and unrepeatable.

The second Rheometer was a computer-operated controlled stress rheometer (Carri-Med); the cone and plate assembly used was 6 cm diameter with a 0.5 degree angle and 12 micron gap.

The preparation of PSC and polysaccharides was carried out as described in section 2.1.2 and 2.1.4. The required amount of each component was weighed out to give a final volume of 50 ml and added to a 60 ml sterilin container. The container was then shaken gently by hand whilst repeatedly inverting for 15 seconds, and the samples were then stored overnight at 4°C.

When applying a sample to the Deer rheometer, a defined amount of sample was added according to Table 2.2. This was done by spooning the sample onto the base plate which had been tared on a digital balance.

With the Carri-med rheometer an exact amount of sample was unnecessary, since approximately 1 ml was placed on the plate and, once the cone was positioned, the excess sample that extruded at the side was carefully removed with a tissue.

2.2.6. Gel electrophoresis

2.2.6.1 SDS polyacrylamide electrophoresis of collagens

The solutions used in the preparation of SDS-PAGE are shown in Table 2.3. The procedure is based on a method devised by Laemmli (1970). The samples were prepared by mixing 1:1 (v/v) with sample buffer containing 10% β–mercaptoethanol. This mixture was placed in a boiling water bath for two minutes. Upon cooling 5% (v/v) of 1:1, 1% bromophenol blue/β–mercaptoethanol was added. The components for 6 and 12 % gels are shown in Table 2.4. Electrophoresis was carried out at 12 mA until the bromophenol blue dye front was 5 mm from the bottom of the gel. The gels were stained for 30 minutes in 0.1% Coomassie blue in 10% acetic acid/methanol and de-stained in 10% acetic acid overnight.

Table 2.2 The parameters for the Deer rheometer measuring system

Plate number	Plate diameter cm	Cone angle °	sample gap mm	Max stress dyne/cm	Max shear sec-1	Range of viscosity cp	Sample volume (ml)
MS-1	5	2	0.07	1500	1050	1 to 50	1.2
MS-2	5	0.5	0.018	1500	4200	1 to 20	0.3
MS-3	4	4	0.14	3000	3000	10 to 500	1.2
MS-4	2	2	0.07	45000	1050	75 to 1500	0.1

Table 2.4 Composition of 6 and 12 % PAGE gels

concentration of gel % acrylamide	6%	12%
separating gel buffer (ml)	10	10
Distilled water (ml)	22	14
Acrylamide stock soln (ml)	8	16
TEMED (µl)	15	30
10% NH4- persulphate (µl)	140	140

2.2.6.2 Electrophoresis of alginates

Gels were prepared to a final concentration of 1% polyacrylamide/1% agarose. The solutions used in gel preparation are given in Table 2.3 and Table 2.5. The agarose solution was prepared and kept in a water bath at 45°C, the acrylamide solution (minus the ammonium persulphate) was also heated to 45°C and then added to the agarose. Finally, the ammonium persulphate was added and the mixture was poured between two glass electrophoresis plates. The gels were cast in slabs (140 x 160 mm x0.8 mm) and run on Hoefer scientific apparatus, at 200 volts for 1.5 hours. The samples were dissolved in an equal volume of sample buffer (table 2.3); 5% volume of 1% bromophenol blue was added and the samples were loaded onto the gel. The gels were stained in 1% methylene blue in 10% acetic acid for 10 minutes, then destained overnight in 20% acetic acid

2.2.7. Gel filtration chromatography

Chromatography of alginates was performed using Sepharose 4B (Pharmacia) in a column (650 x 22 mm Wright, England) which was equilibrated with 0.1 M sodium sulphate. The void volume was determined using blue dextran (Sigma International) at a concentration of 10mg/ml. The column was calibrated with a protein mixture: amylase (200 kDa), LDH (140 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and Aprotinin (6.5 kDa).

The six samples of Protan alginates were applied to the column in 4 ml of the eluting buffer (0.5 mg/ml alginate in 0.1M $Na_2 SO_4$) and eluted at 50 ml/hr and 5 ml fractions collected.

A second column designed for semipreparative use was also packed with Sepharose 4B. This column (400 x 40 mm, Wright, England) was loaded with 100 mg of alginate (20 ml at 5 mg/ml), and 5 ml fractions were collected.

Table 2.3 Solutions required for SDS-PAGE

1	Separating gel buffer	
	1.5M Tris	91.0g in 500ml
	0.4% SDS	2.0g in 500ml
2	Stacking gel buffer	
	0.5M Tris	6.05g in 100ml
	0.4% SDS	0.4g in 100ml
3	Sample buffer x2	
	30ml of 10% SDS	
	12.5 ml of stacking gel buffer	
	10ml glycerol	
	Adjusted to pH 6.8	
4	Reservoir buffer x5	
	0.125M Tris	15.1g in 1 l
	0.96M glycine	72.0g in 1 l
	0.5% SDS	5.0g in 1 l
	The pH is correct when diluted to normal concentration (ie 300ml made up to 1500ml)	
5	TEMED	
	N,N,N´,N´- tetra methylethylenediamine	
6	Ammonium persulphate	
	A 10% w/v solution in water made up just before use	
7	Acrylamide stock solution	
	30% acrylamide	30g in 100ml
	0.8% bisacrylamide	0.8g in 100ml
8	Staining solution	
	0.1% coomasie blue R250 in acetic acid/methanol/water	
9	De-staining solution	
	10% acetic acid in water, 200ml are made up to 2 litres with water.	

Table 2.5 Composition of an acrylamide/agarose gel

	1% acrylamide/1% agarose
Agarose	0.8g
Water	56.5ml
separating gel buffer	8ml
DMAPN (6.4%)	5ml
30% acrylamide stock	2.67ml
water	7.5ml
10% ammonium persulphate	0.4ml

2.2.8. High Pressure Liquid Chromatography (HPLC)

2.2.8.1 Molecular weight analysis of commercial alginates

A Kontron HPLC system was used (models 450 mtz, 422, 425, 430). Two gel permeation columns were run in series: the first was a 10 micron 'Aqua Gel' (Jones Chromatography, Strathaven, Scotland) and the second a TSK gel G300pwxl (Anachem, Luton, England). The columns were eluted with 0.5M NaCl at a flow rate of 1 ml min^{-1}.

Calibration of the column was performed using dextrans of known molecular weight (Crawford Scientific, Strathaven, Scotland), with detection of the polysaccharide by U.V. absorbance at 210 nm.

2.2.8.2 Amino acid analysis of methylated collagen by HPLC

The collagen (50 mg) to be analysed was added to 2 ml of 6M HCl in an airtight vial and hydrolysed for 16 hours at 105°C. The pH of the hydrolysate was corrected to pH 8-9 by the addition of 6M NaOH and the total volume was adjusted to 10 ml by the addition of water. 50 µl of this solution was added to 200 µl of 0.5M lithium borate buffer pH 9.5 with 100µl of dansyl chloride in acetonitrile (6 mg ml^{-1}). The mixture was vortexed and placed in the dark for 24 hours at room temperature. 70% Phosphoric acid (15 µl) and 0.5M lithium borate buffer pH 9.5 (500 µl) were added, the sample was centrifuged at 13000g for 5 minutes and placed in an HPLC vial for analysis.

The HPLC column used was Spherisorb ODS, 3µ, (25 cm x 4.6 mm), the mobile phase was 86% (25mM Acetic acid + 25mM sodium dihydrogen orthophosphate) and 14% acetonitrile. The amino acids were eluted from the column and detected by UV absorbance at 254 nm.

2.2.9. Soluble collagen /polysaccharide titrations

The 1% stock solutions of sodium alginate were diluted to give 20 ml of a 2 mg/ml alginate solution in each case. PSC was diluted with 0.05M acetic acid

to a final concentration of 0.75 mg/ml. The pH of the alginate and the collagen was adjusted by the addition of acetic acid or sodium hydroxide. The 20 ml of alginate was placed in a 60 ml sterilin container on a magnetic stirrer. The solution was stirred and aliquots of 0.8 ml PSC were added. After each addition of collagen the solution was allowed to mix for 30 seconds prior to removal of 0.8 ml for analysis. When removing the sample, care was taken not to include any the collagen alginate precipitate that may have formed. Once removed, the samples for collagen analysis were centrifuged for 5 minutes at 900g. The supernatant was removed by pipette and 500µl mixed 1:1 with concentrated hydrochloric acid for hydrolysis and hydroxyproline content (section 2.2.10.1)

The alginate content of the supernatant was also assayed as follows. Samples were centifuged (12,900 for 5 minutes) and 2 volumes of 95% ethanol (cooled to -18°C) was added to each sample, followed by centrifugation at (12900 g for 5 min). The ethanol was then removed and the pellet resuspended in 0.5 ml of water for subsequent carbazole assay.

The ratio of each biopolymer in the precipitate could have been calculated on a w/w basis but that was not considered accurate owing to the heterogeneity of the alginate samples.

The stoichiometry was calculated on the basis of repeating units (r.u.) of each of the biopolymer chains. A repeating unit of the alginate was taken as being one single sugar unit with an average molecular weight of 176 Da. In the case of collagen the unit of repeat was taken to be one full turn of the triple helix. A single alpha chain has three amino acids per turn of the helix, and so in a turn of the triple helix there will be nine amino acids. Taking the average molecular weight of the amino acids to be 100, the repeat unit of the helix has a molecular weight of 900 Da. By using these molecular weights and the mass of each component in the precipitate, the molar equivalent of repeating units could be calculated.

Sample calculation

$$\text{(moles of collagen) repeating units} = \frac{\text{weight of collagen}}{900}$$

$$\text{(moles of alginate) repeating units} = \frac{2\ \text{mg}}{176}$$

$$\text{therefore the ratio of collagen /alginate} = \frac{\text{ru of collagen}}{\text{ru of alginate}}$$

2.2.10. Protein determination assays

2.2.10.1 Hydroxyproline analysis

Collagen concentrations were estimated by hydroxyproline analysis. This was performed on a Bukhard auto analyser, and the reagents are shown in Table 2.6.

Samples were prepared by complete acid hydrolysis: insoluble collagen samples were suspended in 6M HCl, and soluble samples were mixed 1:1 with concentrated HCl. The samples were then placed in disposable screw-cap test tubes, sealed, and left overnight at 105°C in a heating block (Grant, England). Samples were neutralised by adding 3.2 ml of alkali diluent (Table 2.6), until a salmon pink colour was achieved. If the colour of the mixture was not salmon pink then the pH was adjusted using 6M NaOH or 6M HCl. The sample was then made up to 10 ml in a volumetric flask and 1 ml of sample placed in the autoanalyser sample cup.

TABLE 2.6 Solutions for hydroxyproline analysis

1 Propanol diluent

2 volumes of propan-2-ol diluted with 1 volume of water

2 Stock buffer

57.0g of sodium acetate trihydrate
37.5g of trisodium citrate
5.5g of citric acid
dissolved in 500ml of water, when dissolved add 400ml of propan-2-ol and dilute to 1 litre with water

3 Chloramine T

7.0g of chloramine T in 100mls of water, diluted with 500mls of stock buffer, prepare daily.

4 Colour reagent

30g of 4-Dimethyl aminobenzaldehyde in 45ml of 60% perchloric acid. Diluted by the addition of 250 ml of propan-2-ol.

5 Stock standard

500g of hydroxyproline dissolved in 1litre of 0.01M HCl

6 Working standard

Prepare a range of working standards from 1-5mg/litre by diluting the stock standard with 0.01M HCl.

Standard solutions were prepared by adding hydroxyproline to 0.06M HCl to give a stock solution of 100µg/ml which was used to prepare standard solutions of 1-5µg/ml fresh each day.

The autoanalyser was allowed to run for 30 minutes or until a steady baseline was obtained before the samples were analysed. Samples were run at a sensitivity of 1.7 which was the value that caused a maximum chart recorder reading for a 5 µg/ml sample.

Hydroxyproline contents were calculated from a standard curve taking into account the sample dilutions. To convert hydroxyproline values to collagen, a factor of 7 was used, based on a value of 13% hydroxyproline in collagen.

2.2.10.2. Biuret protein assay

The Biuret method of soluble protein determination was used to calculate the concentration of collagen present in PSC. The Biuret reagent was prepared by dissolving NaOH (29g) in deionised water (200 ml) adding Benedicts solution (43 ml) and making up to 1 litre with deionised water. The PSC samples were diluted 1:1 with 0.05M acetic acid and centrifuged (at 12000 g for 10 minutes). 1 ml of the supernatant was then added to 4 ml of the Biuret reagent, the solution was mixed vigorously and the absorbance measured at 550 nm. The values were measured against a 2 mg/ml purified PSC standard.

2.2.10.3 Lowry protein determination assay

The reagents used in the Lowry assay are given in table 2.7. In each case 0.5ml of the sample to be tested was place in a test tube and 2.5 ml of reagent C (table 2.7) was added and left to stand for 10 minutes. 0.25 ml of reagent D was then added with mixing and left for 30 minutes. After this time the absorbance was read at 650 nm on a spectrophotometer.

Table 2.7 Solutions for the Lowry protein determination assay.

Reagent A	0.5g $CuSO_4.5H_2O$ and 1g of tri-sodium citrate dissolved in 100ml of distilled H_2O
Reagent B	20g of Na_3 CO_3 and 4g NaOH dissolved in 1l H_2O
Reagent C	To 50ml of reagent B add 1ml of reagent A
Reagent D	To 10ml of Folin and Ciocalteu's reagent add 10 ml distilled H_2O

Reagents B and C were made up daily, reagent D was prepared immediately before use.

2.2.10.4. Assay for pepsin activity

The pepsin assay was used in the preparation of PSC as a final step to ensure that all pepsin had been inactivated to prevent the possibility of further digestion of the PSC upon storage. Pepsin activity was measured by the liberation of TCA-soluble peptides from Haemoglobin. Haemoglobin (2g) was dissolved in 100 ml of 0.06M HCl and filtered. 2.5 ml of haemoglobin solution was incubated in a water bath at 35.5°C with 0.5 ml of PSC sample or standard pepsin solution for 10 minutes. 5 ml of 5% TCA solution was then added to precipitate any polypeptides, and this precipitate was removed by filtration. Absorbance of the filtrate was measured at 280 nm against a blank of haemoglobin and water. If the PSC sample had a similar absorbance to that of the blank it was concluded that no pepsin activity was present.

2.2.11. Polysaccharide assays

2.2.11.1. Uronic acid determination

The uronic acid assay was essentially the carbazole method of Bitter and Muir (1962). The two reagents were 98% sulphuric acid with 25mM di-sodium tetraborate and 0.125% carbazole in absolute ethanol. The assay was carried out by adding 1.5 ml of the sulphuric acid reagent to 0.25 ml of sample. The mixture was heated at 100°C for 10 minutes. The tubes were then cooled in ice and 50µl of carbazole reagent added. The samples were mixed and heated at 100°C for a further 15 minutes. After this time the tubes were cooled to room temperature and the absorbance measured at 530 nm. The standards used for this assay were alginates made up in water over the concentration range 0-100µg/ml. The standard curve obtained is shown in Figure 2.6.

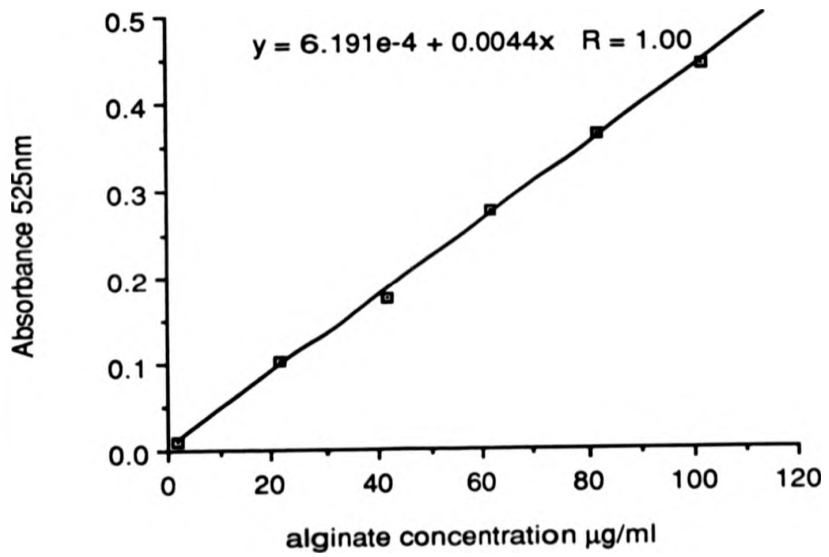


Figure 2.6 Standard curve of colour development versus alginate concentration, using the carbazole acid assay

2.2.11.2. Phenol sulphuric acid assay for total carbohydrate

This assay is based on the formation of a chromophore with phenol after hydrolysis of the carbohydrate. The reagents were concentrated sulphuric acid and 5% (w/v) phenol in water.

200µl of sample was placed in the bottom of a clean test tube, and 1 ml of concentrated sulphuric acid was added without touching the side of the tube. The tubes were left for 5 minutes and 200µl of 5% phenol was added and mixed. The absorbance was measured at 490 nm. The assay was linear in the range 0-200 µg of glucose equivalents per ml.

2.2.12. Circular dichroism

Circular dichroism is the measure of the ability of a molecule to differentially absorb circularly polarised light. This ability is dependent on the molecule being optically active. Unfortunately, the precise structure of a molecule cannot be predicted from its CD spectra,since the rules for determining the structure are largely empirical. Despite this, CD can be a powerful tool for measuring any change in the structure of a molecule.

Proteins often have large areas of α-helix or ß sheet, these absorb left and right polarised light to different extents, resulting in characteristic CD spectra. The triple helical structure of collagen makes it particularly suitable for investigation by CD, since the highly repetitive helical structure gives a strong signal.

The effects of guar and locust bean gum on the secondary structure, and the stability of the secondary structure, of collagen were investigated. In addition, the effect of pH on the secondary structure of collagen was determined.

Samples of PSC and galactomannan gums were prepared as described previously (section 2.1.2 and 2.1.4). The samples were centrifuged at 15000g for 30 minutes, to remove any insoluble material, and dialysed against dilute

acetic acid at the required pH, overnight. The PSC and galactomannans were diluted to 0.1% w/v for analysis on the Perkin Elmer CD instrument.

To determine the effects of the galactomannans on the structure of the collagen, the two were mixed 10 minutes before being placed in the cell holders. The mixture was equilibrated to 25°C and the spectra measured from 190 nm to 260 nm.

The samples for stability of the collagen triple helix were prepared in an identical manner. The cuvette of sample was equilibrated for 10 minutes before the spectra were measured at 220 nm and 2 °C intervals between 20 and 42°C.

CHAPTER 3:-RESULTS

3. COLLAGEN-POLYSACCHARIDE INTERACTIONS IN SOLUTION

3.1. Effect of pH on the viscosity of pepsin solubilised collagen

Since collagen fibres swell when the pH is raised or lowered below the pI of the protein, it was expected that the pH of a solution of collagen would also determine its viscosity. In order to test this, the viscosity of a solution of PSC was measured at a range of pH values between 2.5 and 4.5. The viscosity profiles of the solution are shown in Figure 3.1.

The viscosity of the collagen decreased as the pH of the solution increased. The curved nature of the lines showed an inverse relationship between viscosity and shear stress, which is characteristic of a shear thinning solution, i.e. a solution which exhibits a loss of viscosity as a greater force is applied. When the force applied was increased above 0.6 dynes cm^{-2} the viscosity curve began to plateau and the curves approach a single value.

The collagen solution measured at pH 4 appeared to have a higher initial viscosity but returned to a lower value as the force applied was increased. Precipitation of the collagen close to the isoelectric point may have resulted in small masses of precipitate fouling the cone and plate arrangement at low shear rates leading to the apparent higher initial viscosity. This was supported by turbidity measurements of the collagen solutions (Table 3.1) and the fact that at pH 5 the sample of PSC was fully precipitated from solution. Measurement of the viscosity at pH 5 was not possible due to obstruction of the plates by the precipitate.

Since the viscosity of the collagen solutions was shown to be dependent on the pH, all further measurements were made at pH 3 where the viscosity is high and there was no detectable precipitation.

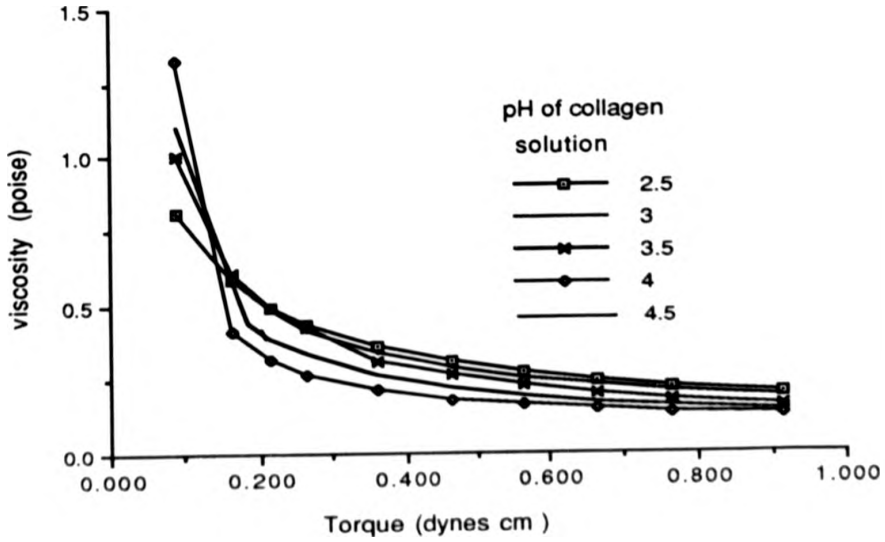


Figure 3.1 The viscosity of PSC at different pH values measured using the Deer rheometer

All viscosity measurements were performed on a 5cm plate with a 2° angle at 25°C. The collagen solutions were at a concentration of 1.25mg/ml and samples were left at each shear stress for 30 seconds to stabilise before viscosity measurements were made. The pH of the solutions were adjusted by adding 1M NaOH until the required value was obtained.

Table 3.1 Turbidity of PSC solutions over the pH range 2-5

	pH2	pH3	pH4	pH5
PSC	0.121	0.133	0.146	0.220

The absorbance measurements were performed on PSC solutions of 1.25 mg/ml, at 720 nm

3.2 Viscosity measurements of collagen/polysaccharide mixtures

Viscosity measurements of polysaccharide/collagen samples were performed on the Deer rheometer. Samples of the polysaccharide and collagen solutions alone were diluted to give the same concentration of each component as in the mixture. In this way the viscosity of the mixture was expected to be the sum of the viscosities of the two individual components if no interaction occurred.

3.2.1 Modified celluloses

Three modified celluloses were screened for an interaction with soluble collagen: hydroxypropyl cellulose (HPC), hydroxypropylmethyl cellulose (HPMC) and methylcellulose (MC). The results are shown in Figures 3.2, 3.3 and 3.4. In all three cases the concentration of the polysaccharide was standardised at 0.1% because the viscosity at this concentration was so low that upon mixing, the contribution of the polysaccharide to the final viscosity would be minimal. Solutions of MC, HPMC and HPC at concentrations of 0.1% had viscosities below 0.03 poise. In each case the mixture of PSC with the celluloses had a slightly lower viscosity than the PSC alone. This could be due to a complex forming and lowering the viscosity due to ordering of the system or undetectable precipitation. The difference was, however, so small that it was considered insignificant. At an applied torque of 20 Dynes cm^{-2} there was no difference between the PSC and the mixtures with HPC.

3.2.2 Modified Starch

Modified starch was investigated since it is a highly soluble simple polysaccharide with a short chain length, which should reduce any interaction with the collagen due to entanglement. The results of the viscosity measurements (Fig 3.5) show very similar curves for the starch and the

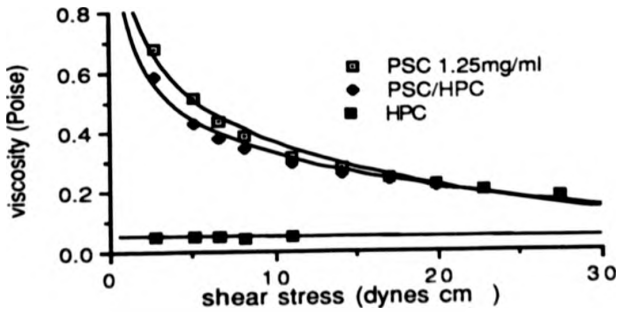


Figure 3.2 Viscosity profile of HP cellulose mixed with PSC

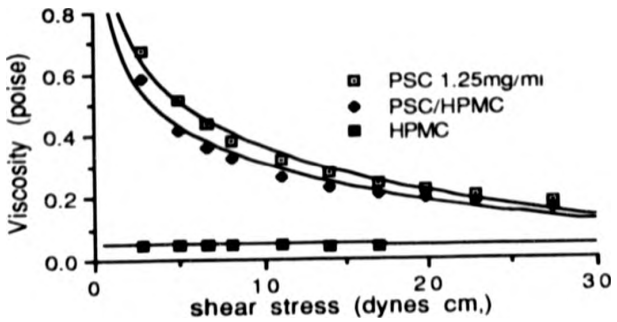


Figure 3.3 Viscosity profile of HPMC mixed with PSC

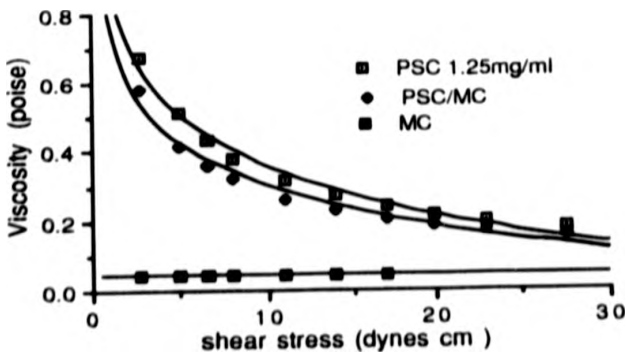


Figure 3.4 Viscosity profile of Mcellulose mixed with PSC

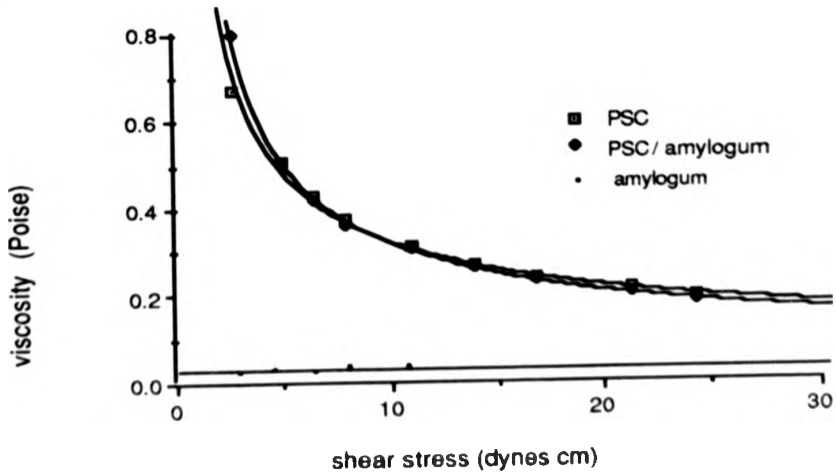


Figure 3.5 Viscosity profile of modified starch with PSC

Measurement systems and conditions are as described in Figure 3.1. The starch was measured at 0.1%w/v ,PSC at 1.25 mg/ml. The mixture of amylogum and PSC had final concentrations of 1.25mg/ml PSC and 0.1% amylogum.

PSC/starch mixture. Apart from the initial readings at low shear stress, the curves coincide exactly, suggesting that there is no interaction between the two components.

3.2.3 Galactomannans

Two galactomannans were screened for possible interactions with PSC:- guar gum and locust bean gum. Each had a polymannan backbone linked β 1-4, with single galactose units branched along the backbone. The structures are thus similar, varying only in the degree of galactose substitution, (Fig 1.6).

3.2.4 Guar Gum

Guar gum was the first galactomannan to be screened, and the viscosity profile is shown in Fig 3.6. Surprisingly, the curves showed that the viscosity of the mixture of guar and PSC was considerably greater than the sum of the individual components. This was unexpected since guar gum is a neutral polysaccharide and obviously is incapable of interacting electrostatically with collagen. In an attempt to confirm that an interaction did occur, a second experiment was carried out where aliquots of PSC were added to water or guar gum, with the viscosity of the resulting solution measured after each addition. The viscosity was then plotted against the number of additions of PSC (Fig 3.7). As well as showing a different initial viscosity the slope of the two lines was also significantly different. If no interaction was occurring then the addition of collagen would result in a similar viscosity change for both solutions. As this was not the case, it appears that an interaction or entanglement of the biopolymers was occurring.

The pH dependence of the interaction was investigated over a pH range of 2.5 to 6. The results show that the viscosity of the mixtures decreased as the pH increased (Fig 3.8). This is most probably due to the degree of swelling of the collagen since it has already been shown that PSC viscosity is pH

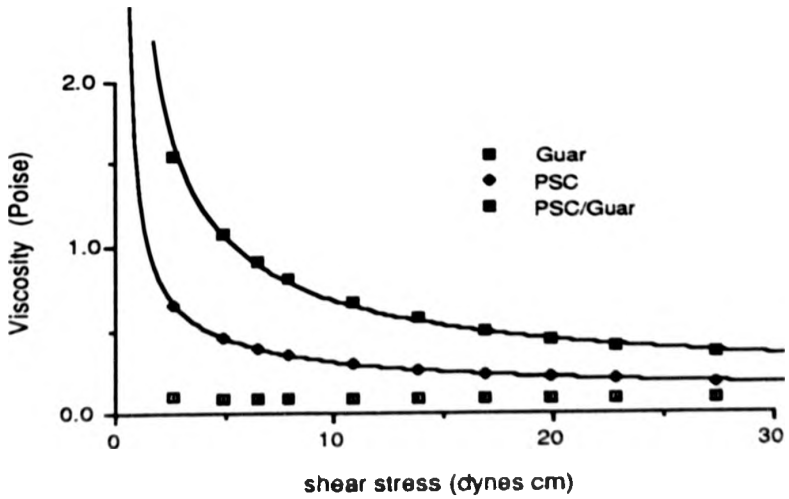


Figure 3.6 Viscosity of PSC and Guar gum mixtures measured at different shear stresses

The guar gum was measured at 0.1% w/v, and PSC at 1.25 mg/ml. The mixture of PSC with guar had final concentrations of 0.1% guar and 1.25 mg/ml collagen. The rheology was performed on a Deer rheometer MS-2, at 25°C and pH3

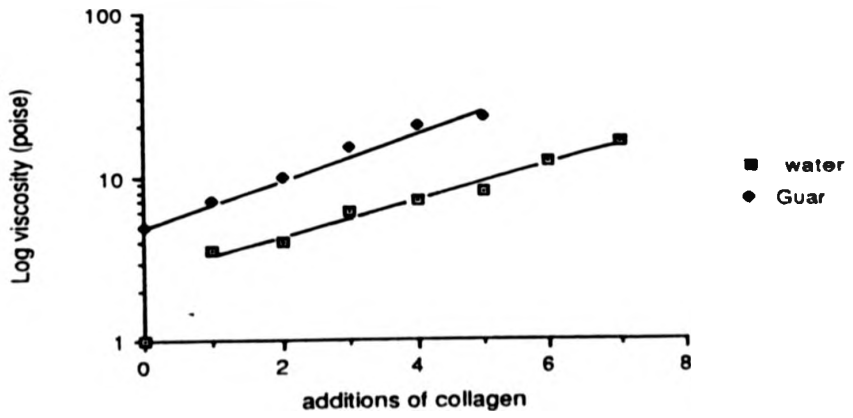


Figure 3.7 Titration of water and guar gum with PSC

Sequential additions of 1.2ml PSC (2.5mg/ml) were made to a starting volume of 20ml water After each addition, and thorough mixing a sample of 1.2 ml was withdrawn and the viscosity was measured at 4.47 dynes cm A similar experiment was performed adding PSC to 20 ml of Guar gum at a concentration of 0.2%

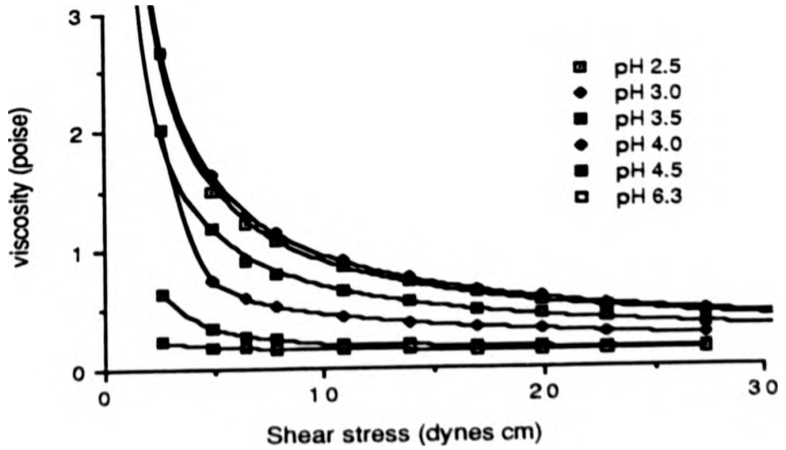


Figure 3.8 The effect of pH on the viscosity of PSC/Guar mixtures

All solutions had final concentrations of 0.1% guar, 1.25 mg/ml PSC. The solutions were adjusted to the correct pH by small additions of 5M NaOH.

dependent. The viscosity of guar gum alone was largely unaffected by the pH of the solution (illustrated in Fig 3.9).

Figure 3.8 shows further evidence of an interaction since, in contrast with the precipitation of PSC at a pH of 4.5 and above, no precipitation of collagen occurred when the pH was raised to 6 in the presence of guar gum. This would suggest that the guar gum was interacting with the PSC in a manner which prevented or reduced reconstitution of the collagen fibres.

3.2.5 Locust bean gum

Since there was an unexpected interaction between guar and PSC it was considered relevant to investigate whether a similar interaction occurred between PSC and LBG, which is structurally related to guar. Viscosity measurements were made on the Deer rheometer and the results plotted in Figure 3.10. The results showed that locust bean gum also caused a significant increase in viscosity above the predicted value.

3.3 Mechanism of interaction between guar gum and PSC

Since the interaction of PSC with these neutral gums was unexpected, more detailed analysis was considered necessary. In order to perform this analysis a Carri-med controlled stress rheometer was used.

Collagen/guar gum solutions were prepared containing different proportions of each biopolymer. The proportions chosen ranged from 0 to 100 % in 10% increments. It was predicted that a plot of viscosity against concentration could result in three types of relationship shown in Figure 3.11. If no interaction occurs between the two polymers plot (a) would be expected because viscosity would be proportional to the ratio of the biopolymers in the mixture. If however an interaction was occurring then either plot (b) or (c) would be expected since such an interaction would lead to either an increase or decrease in the viscosity of the solution.

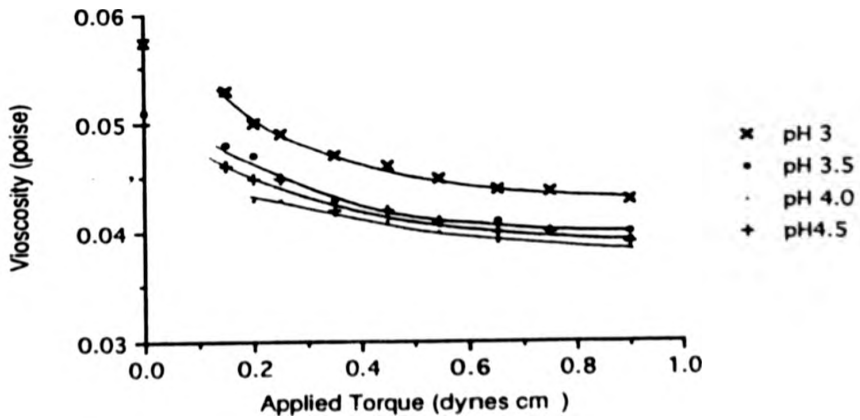


Figure 3.9 The effect of pH on the viscosity of guar gum solutions

The viscosity of a 0.1% solution was measured , after adjustment to the correct pH by the addition of 5M NaOH

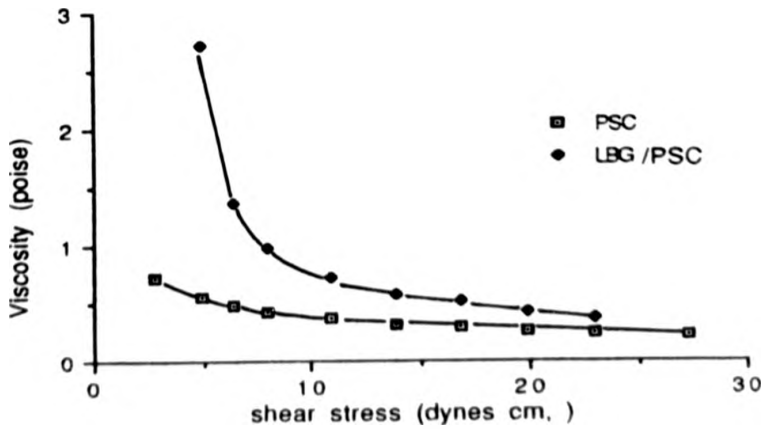


Figure 3.10 The viscosity of locust bean gum mixed with pepsin solubilised collagen, measured at 0.1% polysaccharide, 1.25mg/ml PSC. The samples were tested on deer rheometer measurement system, MS-2, at room temperature, pH3

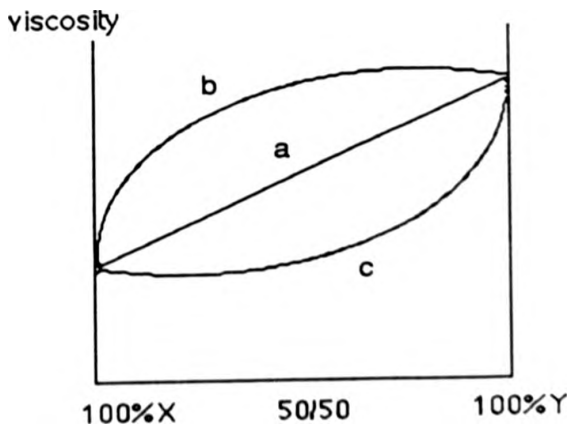


Figure 3.11 The curves typically observed when mixing two polymers in different proportions and measuring the viscometry of the mixture. Plot (a) signifies no interaction, whilst (b) and (c) are typical of two interacting polymers.

The viscosity measurements recorded for each of the guar /collagen solutions are shown in Figure 3.11. The results showed that 100% PSC was the most viscous solution and as the level of Guar increased, the viscosity decreased. It was not possible to determine whether an interaction was occurring from this type of plot, since a range of shear stresses were measured, and the mixtures were shear thinning over this range. The data from three shear stress values was chosen (20,30 and 50 dynes cm^{-2}) and plotted in Figure 3.12. In this format it is easier to compare the viscosity of the mixtures at the same shear stress, and any deviation from linearity is clearly shown.

The results showed that at 50 dynes cm^{-2} the relationships between 100% guar gum and 100% PSC was linear. At 30 dynes cm^{-2} the initial part of the graph (0% PSC to 60% PSC) was also linear, however towards the higher PSC ratio a small increase in the expected viscosity is noted. The plot of viscosity measured at 20 dynes cm^{-2} showed a considerable deviation from linearity in the same region as the 30 dynes cm^{-2} plot. To amplify this interaction more concentrated solutions of each of the biopolymers were prepared. The solutions were concentrated by freeze drying the collagen and guar gum, and re-dissolving in a smaller volume. Samples of freeze dried bioploymers were re-dissolved in 0.05M acetic acid at a concentration of 1%w/v. Mixtures ranging from 100% PSC to 100% guar were again prepared and analysed on the rheometer. Once more three values of shear stress were chosen and the viscosity of the solutions at each stress was plotted (Fig 3.13). There was a substantial divergence from linearity as the proportions of collagen and guar changed. At a stress value of 25 dynes cm^{-2} the peak occurred where the PSC proportion was 30-70%. The maximum enhancement of viscosity occurred at 45% PSC and was 42 poise. Expressed as a percentage, this was 58% higher than would be expected if no interaction was occurring. Increasing the shear stress to 35 dynes cm^{-2} also resulted in an increase of viscosity between 30-70% PSC. Again, the maximum value

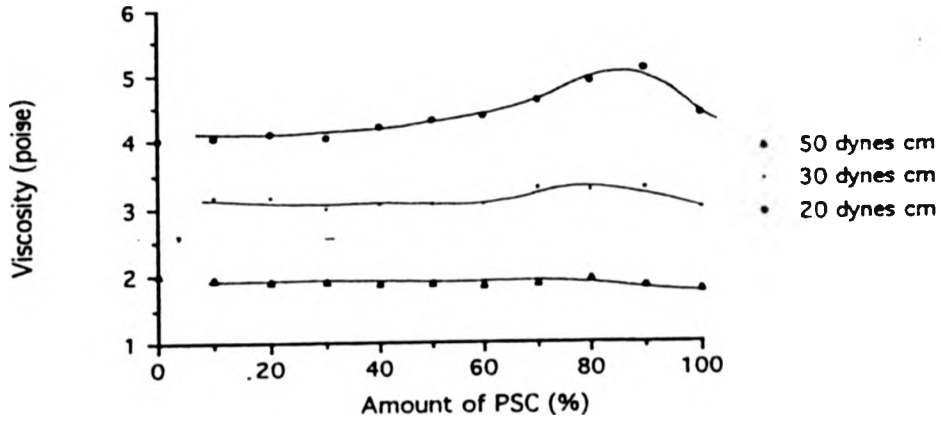


Figure 3.12 The viscosity of different mixtures of PSC/guar gum. The viscosity measurements of the samples were performed on a Carri-med rheometer. The measurment system was a 6cm plate with a 0.5° angle, and the measurements were made at 25°C. The viscosities were measured at three different shear stress values (20, 30 and 50 dynes cm^{-2}). The solutions used in the sample preparation were 7.5 mg/ml guar gum, and 4,7 mg/ml PSC

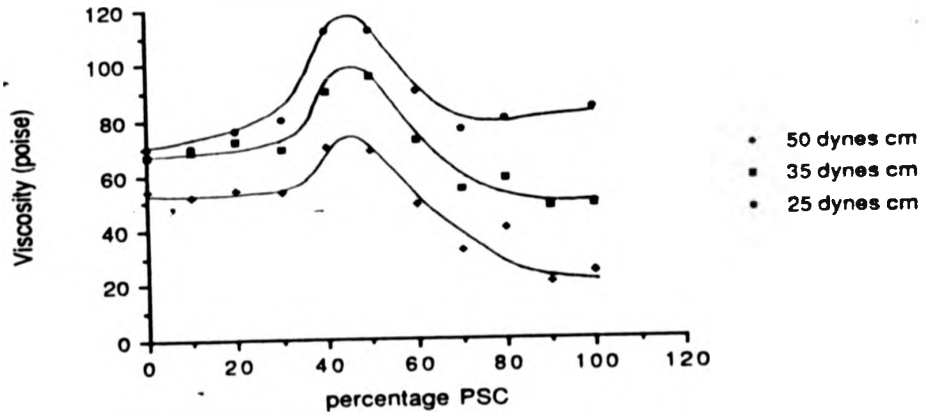


Figure 3.13 The viscosity of high concentration mixtures of PSC and guar gum. The PSC and guar were both 10mg/ml w/v solutions, measurements were performed under the same conditions as those in Figure 3.12

was at 45% PSC where the value was 36 poise greater (59% higher than expected). A similar pattern was seen at 50 dynes cm^{-2}, with the peak increase in viscosity again being at 45 % PSC and 22 poise. The level of enhancement over the predicted value in this case was found to be 55%. It has been shown that in each case there was an enhancement of the viscosity of the solutions. Although the size of the increase was different at each shear stress, i.e. 25 dynes cm^{-2}>35 dynes cm^{-2}>50 dynes cm^{-2} the percentage increase was similar in each case. The fact that the percentage increase remained constant whilst the actual increase became smaller with shear stress indicated that at higher shear stress values the peak would become smaller and almost undetectable.

3.4 Titrations of soluble collagen with anionic polysaccharide

When dilute solutions of soluble collagen and anionic polysaccharide are mixed, a white fibrous precipitate forms. Using this principle, titrations of collagen and polysaccharide were made in order to calculate the binding stoichiometry of the co-precipitate, and to determine the effect of pH on complex formation.

The initial results showed two important practical considerations; firstly that the soluble collagen became insoluble above pH 4.5; (section 3.1) limiting titrations to the pH range 2.5-4.5.

Secondly the colorometric analysis of hydroxyproline on the autoanalyser varied with the pH of the sample. It was considered necessary, therefore, to prepare hydroxyproline standards for each pH value tested.

Once it was apparent that the amount of collagen in solution was detectable, and that the pH range was 2.5- 4.5, an experiment was performed to optimise the time of mixing between the addition of collagen and the removal of a sample. Times of 10, 30 and 120 seconds were tested. The samples were then tested by the Lowry protein determination assay (methods 2.2.10.3). The

results (Fig 3.14) show there is no appreciable difference between the three mixing times. The mixing time did not therefore appear to be critical, so for consistency all further titrations were performed with 30 seconds mixing, prior to sampling.

Five different alginates were titrated with soluble collagen. Experiments to determine the effect of denaturation, denaturants and different polysaccharides were also performed.

3.4.1 Alginates

Figure 3.15 shows the results of the titrations of alginate LF1040 with pepsin solubilised collagen at pH values 2.5-4.5. No collagen was found in the supernatants after the initial additions of PSC to the alginate, indicating complete precipitation of the collagen. After a certain point (taken to be the end point of the titration) collagen appeared in solution corresponding with the disappearance of free alginate in the supernatant. Subsequent addition of collagen produced a linear increase in the amount of collagen in the supernatant. The end points of the titration range from 5-17 additions of collagen depending on the pH that the two biopolymers are mixed. As the pH of the mixture was increased from 2.5 to 4.0 the amount of collagen required to saturate the alginate increased. The final pH point of 4.5 had a lower end point than that of the pH 4 mixture(16.1 additions compared to 17)

Using the calculation detailed (section 2.2.9), the ratios of alginate / collagen in the precipitates were calculated and are shown in Table 3.2.

The ratio of alginate/collagen ranged from 1 : 0.54 to 1 : 1.65. This illustrates a significant effect of pH on the binding stoichiometry of the two macromolecules. To determine what effect the properties of the alginate have on its ability to bind collagen, four other alginates were titrated under the same conditions as alginate LF1040. The characteristics of these alginates are shown in Chapter 2, Table 2.1.

Table 3.2 The binding ratios of collagen and five different commercially available samples of alginate.

Expressed as Alginate:collagen

Titration	Protan Alginate				
pH	Lam LV	SF	HF	LF120	LF1040
2.5	1:0.43	1:0.63	1:0.58	1:0.53	1:0.54
3	1 0.64	1:0.64	1:0.85	1:0.67	1:1.29
3.5	1:0.97	1:1.08	1:0.87	1:0.97	1:1.62
4	1:1.4	1:1.62	1:1.72	1:1.55	1:1.65

Table 3.3 Binding ratios of carrageenan and hyaluronic acid with collagen.

Expressed as polysaccharide :collagen

Titration	polysaccharide	
pH	Carrageenan	Hyaluronic A.
2.5	1:1.17	1:0.54
3	1:1.27	1:0.75
3.5	1:1.42	1:1.31
4	1:1.72	1:1.30
4.5	1:1.38	1:1.29

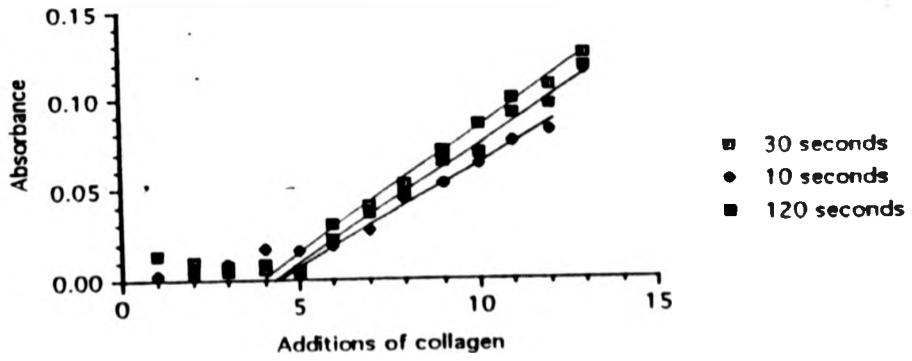


Figure 3.14 The effect of mixing time on the amount of collagen in solution during polysaccharide titrations. The polysaccharide is protan alginate LF1040 titrated at pH 2.5

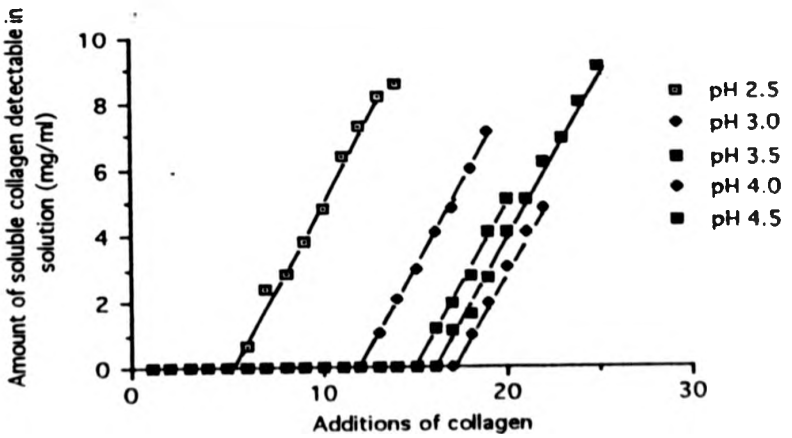


Figure 3.15 The titration of alginate (LF1040) with soluble collagen over the pH range 2.5-4.5. The collagen was added in 1.2 mg additions to a mixing solution of alginate (20mls at 0.1mg/ ml)

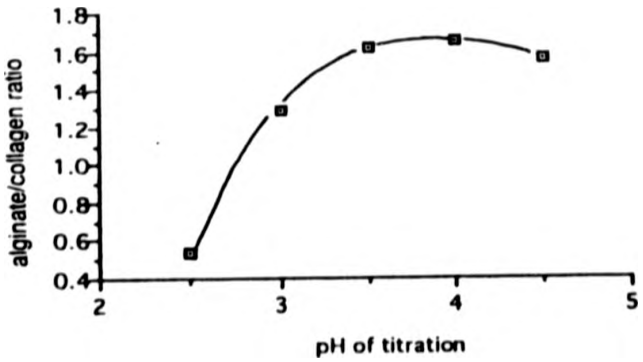


Figure 3.16 The effect of pH on the alginate binding ratio of alginate/collagen. Using Protan alginate LF1040 titrated with soluble collagen

The results of the titrations were similar to those obtained for LF1040 and are illustrated in Figure 3.17. In each case the initial additions of collagen resulted in no detectable collagen in the supernatant, and a linear increase after the end-point.

The number of moles of repeating units of each biopolymer in the precipitate were calculated and are shown in Table 3.2.

When titrated at pH 2.5 each of the alginates had very similar binding ratios, the average being 1 : 0.54. At pH 3.0 alginates LAM LV, SF, HF and LF120 had almost identical ratios, however the LF1040 was approximately twice that of the other alginates. This was also shown to be the case at pH 3.5. At a mixing pH of 4.0 all five alginates showed a similar amount of collagen binding.

As an alternative method of determining the end point of the titration, the amount of alginate remaining in solution was measured (Methods 2. 2.9). The purpose of this was to determine if all the fractions of the alginate molecular weights were interacting with the collagen. It was possible that small chains of alginate would not be capable of interaction, and may stay in solution. The results obtained from the initial experiments are shown in Figure 3.18. These showed that alginate was removed from solution at exactly the same point as collagen appears. The fact that after the end point no alginate is detectable suggests that all the alginate chains are capable of binding to collagen. This analysis of alginate remaining in solution also provided a second method of determining the end point of the titration, since both methods give the same result. The alginate assay was, however, more time consuming and so the remaining titrations were performed using the hydroxyproline assay for collagen determination.

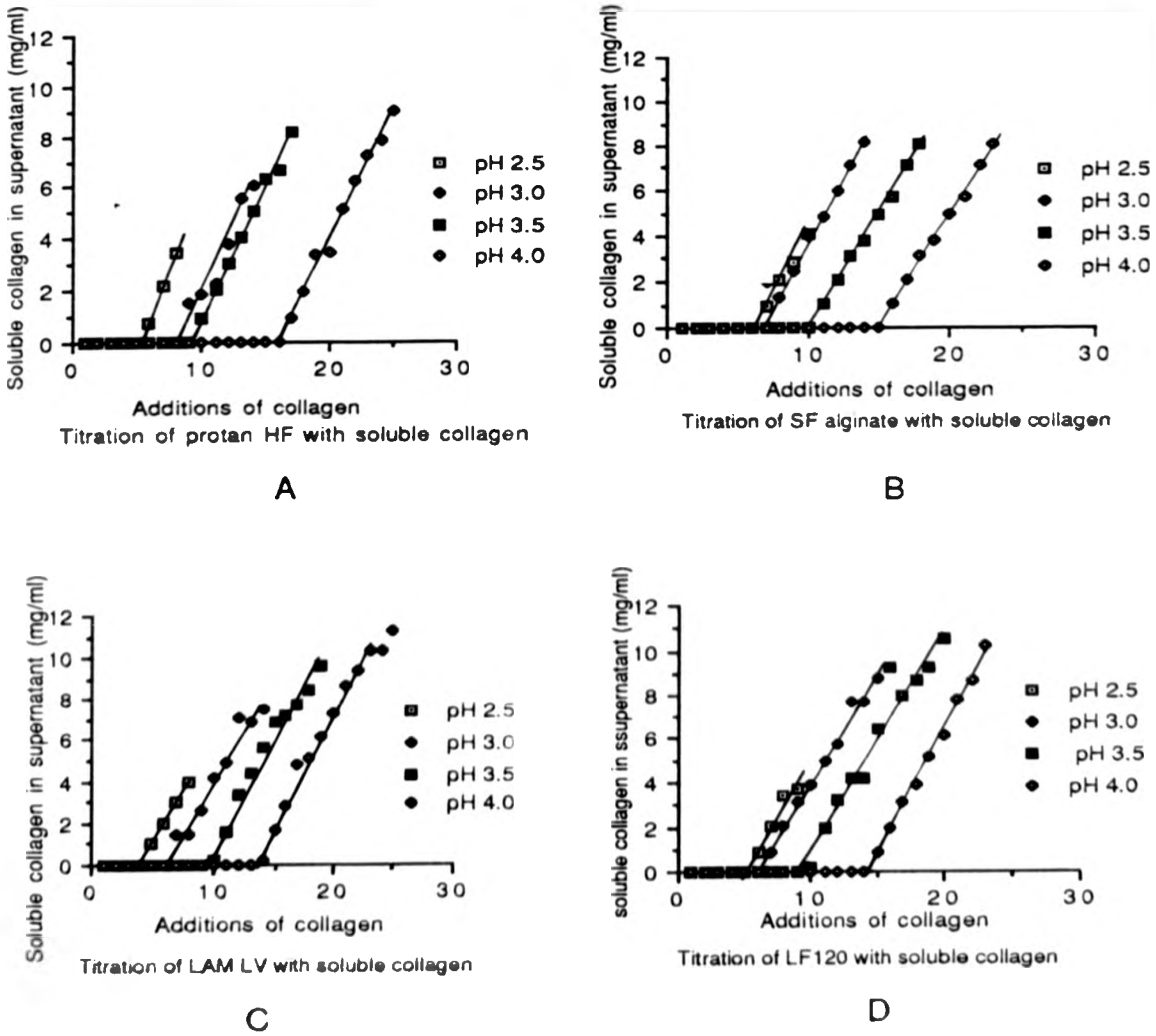


Figure 3.17 The titration of four Protan alginates with pepsin solubilised collagen. (A) Protan HF, (B) Protan SF, (C) LAM LV, and (D) LF 120. Over the pH range 2.5 -4.0

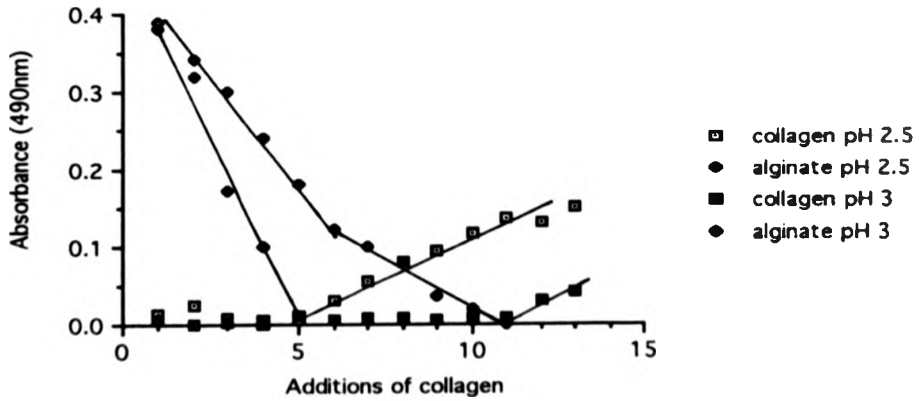


Figure 3.18 Titration of alginate LF1040 with pepsin solubilised collagen at pH 2.5 and 3.0

3.4.2 Hyaluronic acid

Structurally, hyaluronic acid is similar to alginate, the main difference is that HA is a larger molecule (ranging from approximately 10^6- 8x106 Da in mammalian tissues) and has a lower charge density. Titrations of HA with collagen were carried out to compare binding capacity with that of the alginates. The results are shown in Figure 3.19.

The end points ranged from 5-11 additions of collagen, which is a narrower range than that for the alginates. The binding ratios of HA to collagen are given in Table 3.3 and show that at pH 2.5 and 3.0 that the binding was very similar to that of the alginates. At pH 3.5, 4.0, and 4.5 the biopolymer ratio remains unchanged. At pH 4.0 the collagen /HA ratios were slightly lower than those of the corresponding collagen/alginate ratios. As in the case of alginate LF1040 the ratio increased from pH 2.7-4.0, and then fell at pH 4.5.

3.4.3 Carrageenan

Titrations of carrageenan were carried out to compare polysaccharides with carboxyl groups (such as alginate) to one containing sulphated residues (carrageenan). Titrations of carrageenan were carried out over the same pH ranges as the alginate experiments i.e. pH 2.7 -4.5. The results are presented in Figure 3.20 and Table 3.3. It is obvious that the range of end points was much narrower than with the uronic acid containing polysaccharides. As with alginate and HA the ratio of the polysaccharide to the collagen increased with pH up to 4.0, decreasing slightly at pH 4.5.

3.4.4 Titration of alginate with denatured collagen

The polysaccharides used so far were considered to be long chain molecules with a random conformation, and no predetermined secondary structure. The collagen however has a well defined triple helical structure (section 1.3.2). It may be considered that the rigid nature of the triple helix may to some extent

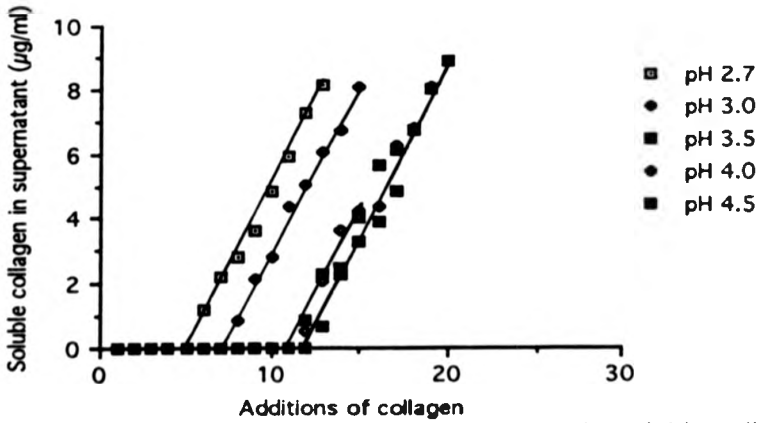


Figure 3.19 The titration of Hyaluronic acid with pepsin soluble collagen at pH 2.5-4.5

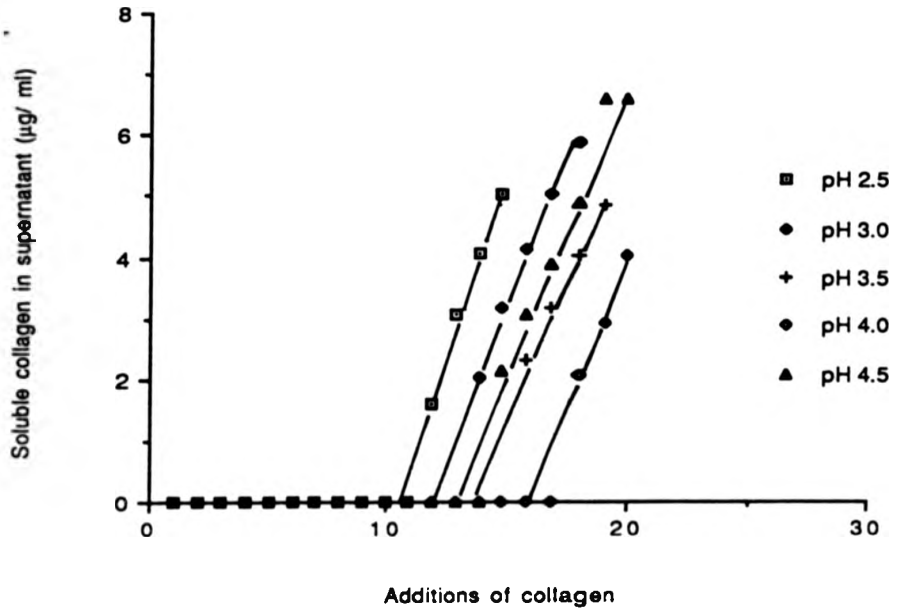


Figure 3.20 Titration of carrageenan with pepsin soluble collagen at pH 2.5-4.5

interfere in preventing the interaction of collagen with the polysaccharide. To test this titrations of alginate (LF1040) were performed using gelatin instead of native PSC. The gelatin was prepared according to Methods 2.1.3 and was used because of its flexible structure. The results obtained are shown in Figureure 3.21.

As in previous experiments, the binding ratio increased with the pH. The denatured collagen however, bound approximately 50% more alginate than native collagen per alpha chain.

3.4.5 Effect of Denaturants on collagen-alginate interactions

Guanidine hydrochloride (Gdn HCl) and urea were included in a set of alginate titrations to determine their effect on the interaction of alginate with collagen since they act by disrupting hydrogen bonding.

Titrations were performed at two different concentrations of urea and Gdn HCl, the results obtained are shown in Figure 3.22. The results showed that neither urea nor Gdn HCl at a concentration of 0.1M had an effect on the precipitation of the collagen/alginate complex since collagen appears in solution after 5 additions whether the denaturants are present or not. At a concentration of 1M, urea still had no effect, however Gdn HCl completely prevented the formation of insoluble complexes.

3.5 The release of alginate from sponges

The initial experiments to measure alginate release from the collagen alginate sponges was performed according to methods 2.2.1.1, where samples of sponge were weighed and shaken in 10 ml of 0.9% saline. After a set time, the sponge was removed and the saline was assayed by the carbazole method to determine the amount of alginate released. It was often found that a sponge removed from saline after 10 minutes, released more alginate than an identical sponge, shaking for 20 minutes. This was considered to be due to

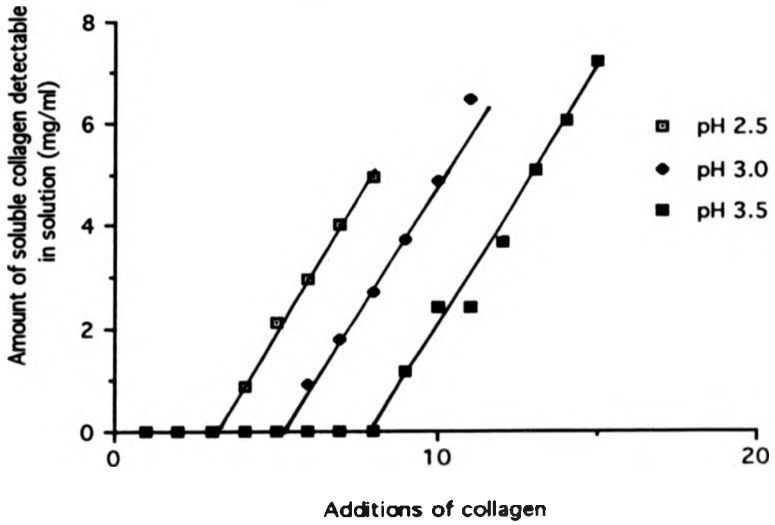


Figure 3.21 Titration of alginate (LF1040) with heat denatured soluble collagen at pH 2.5 -3.5. The collagen additions of 1.2 mg were made in each case to 20ml of a mixing alginate solution (0.1mg/ml)

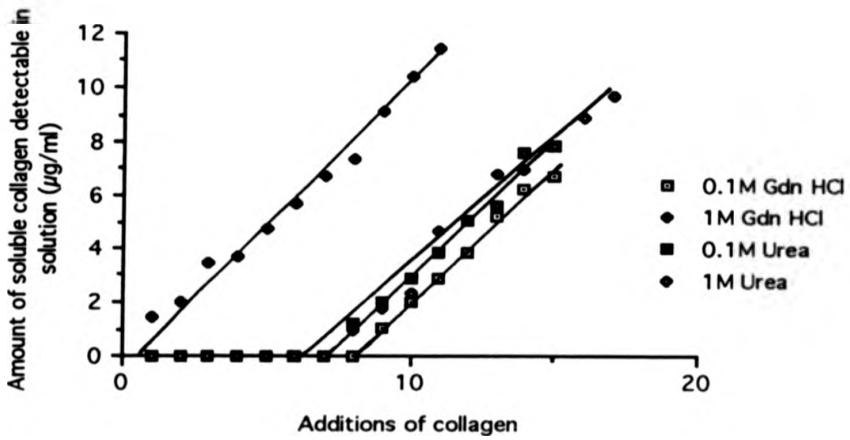


Figure 3.22 The titration of alginate LF1040 with pepsin solubilised collagen at pH 2.5 in the presence of guanidine hydrochloride and urea. The titration was carried out according to method 3.2.9. but adding 0.8 mg of collagen per addition to a mixing solution of 20ml alginate (0.1mg/ml)

heterogeneity of the samples. To test this samples cut from one sponge were assayed for total alginate content (methods 2.2.1.2). The alginate content (n=9) was 7.3% +/- 0.7, i.e. a 9% coefficient of variation. This variability was significantly less than that for alginate extracted into solution from similar samples. A second possibility was that the sample dimensions, in addition to the sample weight, were important. The dimensions were standardised by cutting the samples with a cork borer.

Given a constant size, any difference in the sample weight was due to variations in sponge density. Therefore to determine the effect of sponge density on alginate release, high density and low density sponges were prepared and extracted in the saline solution. It was shown (Fig 3.23) that high density sponges released more alginate than their low density counterparts, when expressed as a percentage of the total weight.

The sponges used in these initial experiments were production Fibracol™ sponges which had been cut from a 25 mm block of freeze dried material. When these blocks of slurry are frozen, the rate of freezing affects ice crystal formation and thus pore size in the resultant sponges, leading to a progressive decrease in density from the bottom to the top of the sponge (P. Watt, personal communication). The remaining studies were therefore done on material prepared in the laboratory, where this relative density difference was eliminated by casting individual sponges of 3 mm thickness.

3.5.1 Relationship between alginate type and release from collagen sponges.

Five alginate preparations were used to prepare a series of 10% alginate/collagen sponges and the release of alginate from 15 mg samples of the sponges into 10 ml volumes of saline was measured. The results (Fig 3.24) showed a rapid release over the first 15 minutes, followed by a rapid decrease in the rate, plateauing in three of the samples from approximately 30 minutes. In two of the preparations (LF1040 and LAM LV) there was a

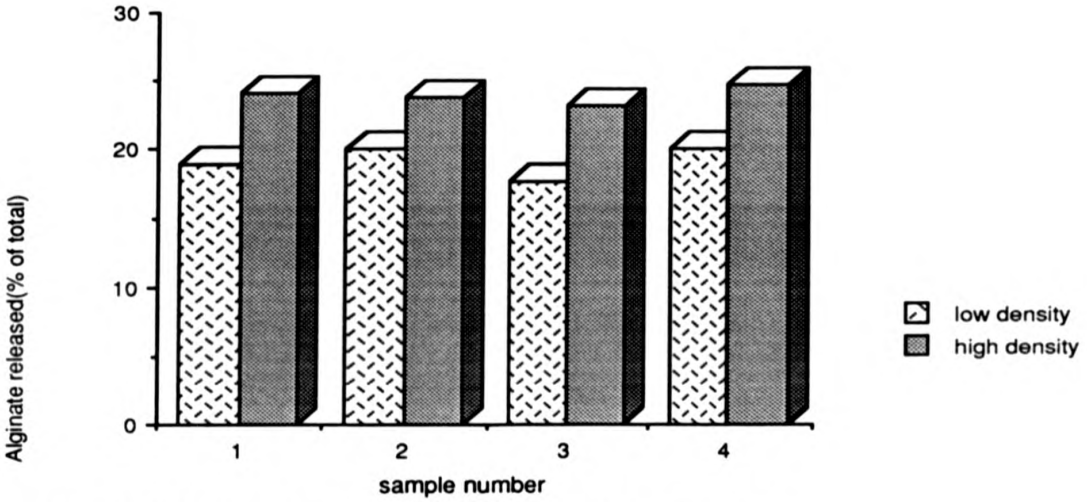


Figure 3.23 The release of alginate from two fibracol™ sponges determined by shaking in saline overnight, and expressed as a % of total alginate

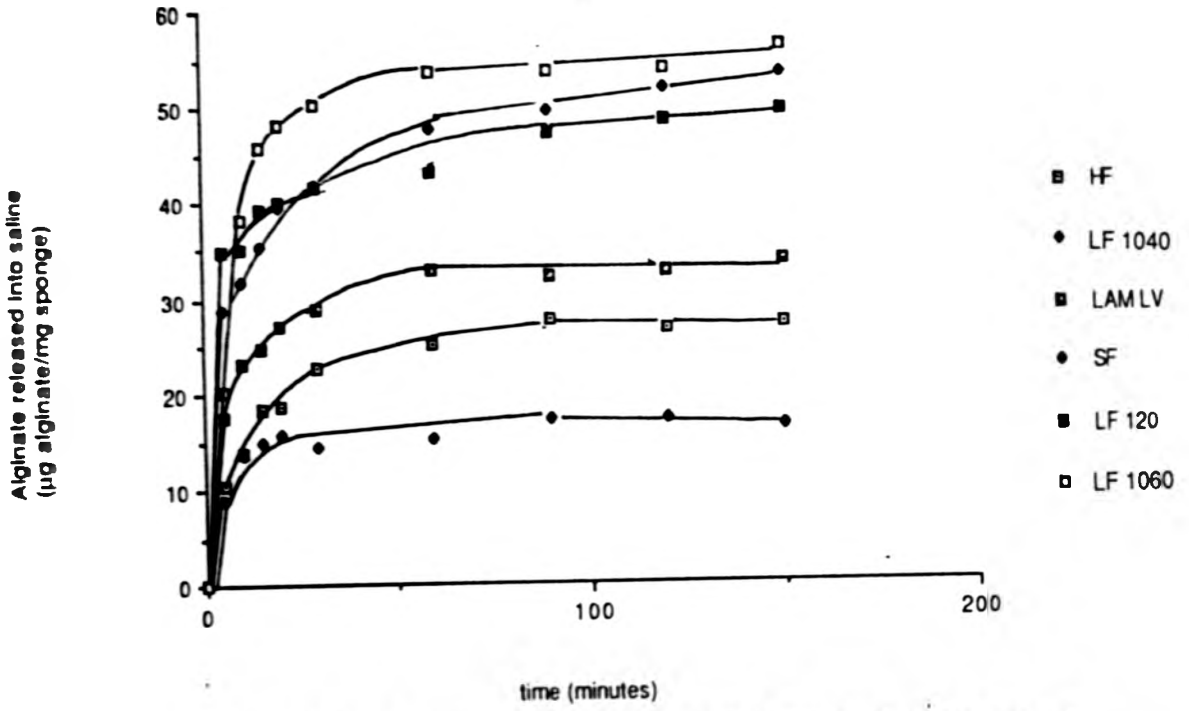


Figure 3.24 The time dependent release of alginate from collagen alginate sponges

continued, albeit slower, release between 30 and 160 minutes. There are obvious difference between the rates of alginate released from the sponges dependent on the type of alginate used to make the sponge.

3.5.2 Wound model

The design of the wound model made it impractical to measure the rate of alginate release from the sponges. Instead, the total amount of alginate released from the sponge was measured after twenty four hours. The results (Fig 3.25) show that the amount of alginate released into the wound model is considerably smaller, than the values measured by shaking in saline solution. Sponges prepared with alginate HF released only 4% of the total alginate compared to 32% in the saline solution. The order of alginate release, was found to be LF1040> LF120>LAM LV> HF> SF. The wound model was difficult to use and very slow, as a result all further samples were tested by shaking in saline solution.

3.5.3 Effect of calcium

Calcium is used in the commercial preparation of Fibracol™ sponges, where it has been shown to stabilise the alginate component and reduce its solubility. To determine the effect of calcium on the release of alginate from the sponges, samples were prepared without calcium or containing 0.8% (standard production concentration) calcium. The total amount of alginate released from the sponges was measured, and the results (Fig 3.26) showed that in the absence of calcium more alginate was released from the sponges. In the case of sponges prepared from alginate SF, the amount of alginate released from the sponge more than doubled in the absence of calcium. However, with sponges containing alginate LF1040 the omission of calcium resulted in only a 5% increase in the level of alginate released, probably because the level of alginate released from sponges containing calcium is

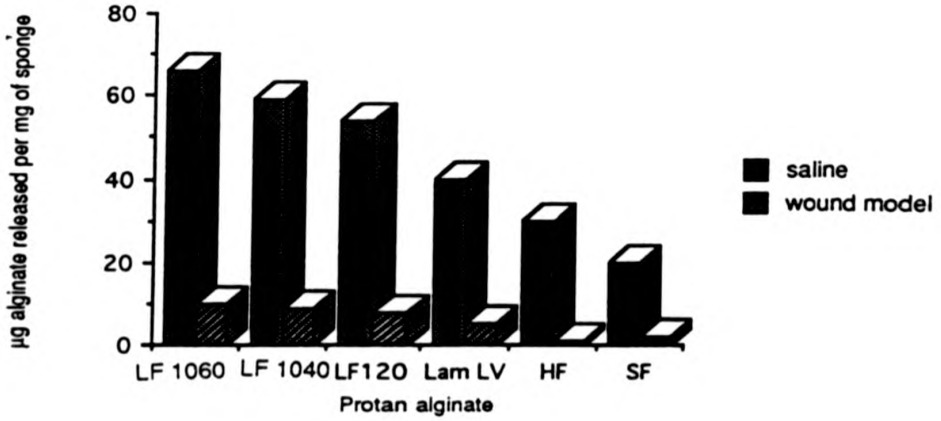


Figure 3.25 Release of alginate from collagen alginate sponges into saline solution in a free system and also the wound model

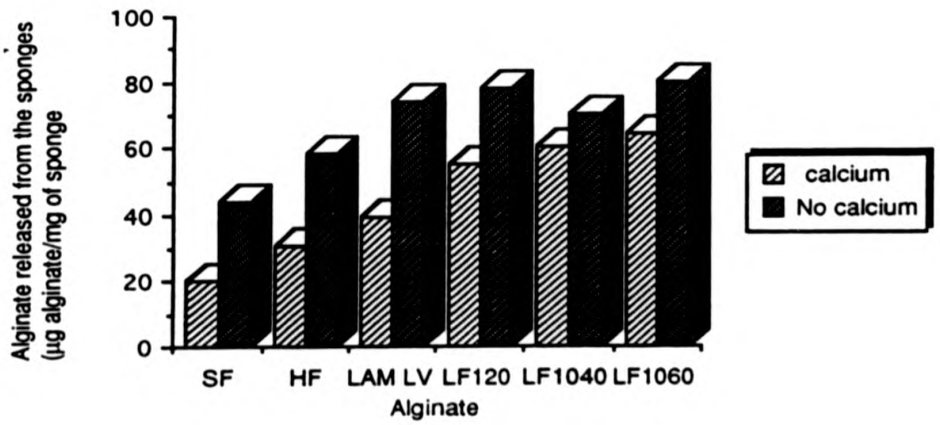


Figure 3.26 Comparison of alginate release from collagen/ alginate sponges with and without calcium

already high, and so removing the calcium has a limited affect. In general sponges prepared without calcium showed proportionally smaller differences in the amount of alginate released, ranging from 45-80 μg/mg of sponge, compared with 20-65 μg/mg of sponge when calcium was present.

3.5.4 The effect of different types of collagen on the rate of alginate release.

To measure the effect that different collagen structures had on the release of alginate from composite sponges, samples were prepared from three collagen preparations made from the same bovine hide source: pepsin solubilised collagen, fibrous insoluble collagen, and heat-denatured (gelatinised) collagen. Sponges were prepared containing 0.8 and 1.6% calcium. Six different commercial alginates were used giving thirty-six possible variations shown in Table 3.4.

The amounts of alginate released from the sponges over a 24 hour extraction period in saline solution (Methods 2.2.1.1) are shown in Figures 3.27, 3.28 and Table 3.5. In Table 3.5 the amount of alginate released is expressed as a percentage of the alginate released from control sponges: which were fibrous collagen sponges containing 0.8% calcium and 10% of the six commercially available alginates (sponges 1-6, Table 3.4)

3.5.5 Fibrous collagen/alginate sponges

The release of alginate from the fibrous collagen sponges with 0.8% calcium (sponges 1-6, Table 3.4), were similar to the values observed in the rate release experiments (section 3.5.1). The order of alginate release was SF<HF<LAM LV< LF120<LF1040<LF1060.

When compared to the control sponges those prepared with fibrous collagen and 1.6% calcium (sponges 9-24), showed the same or lower levels of alginate released. Alginates LF1060 and LAM LV showed similar levels of release, irrespective of the calcium content. LF 120 released 85% of the

Table 3.4 Comparison of collagen alginate sponge samples

SPONGE NUMBER	COLLAGEN USED	ALGINATE USED	CALCIUM (%)
1	fibrous	LF 1060	0.8
2	fibrous	HF	0.8
3	fibrous	LF 120	0.8
4	fibrous	LF 1040	0.8
5	fibrous	LAM LV	0.8
6	fibrous	SF	0.8
7	denatured	LF 1060	0.8
8	denatured	HF	0.8
9	denatured	LF 120	0.8
10	denatured	LF 1040	0.8
11	denatured	LAM LV	0.8
12	denatured	SF	0.8
13	soluble	LF 1060	0.8
14	soluble	HF	0.8
15	soluble	LF 120	0.8
16	soluble	LF 1040	0.8
17	soluble	LAM LV	0.8
18	soluble	SF	0.8
19	fibrous	LF 1060	1.6
20	fibrous	HF	1.6
21	fibrous	LF 120	1.6
22	fibrous	LF 1040	1.6
23	fibrous	LAM LV	1.6
24	fibrous	SF	1.6
25	denatured	LF 1060	1.6
26	denatured	HF	1.6
27	denatured	LF 120	1.6
28	denatured	LF 1040	1.6
29	denatured	LAM LV	1.6
30	denatured	SF	1.6
31	soluble	LF 1060	1.6
32	soluble	HF	1.6
33	soluble	LF 120	1.6
34	soluble	LF 1040	1.6
35	soluble	LAM LV	1.6
36	soluble	SF	1.6

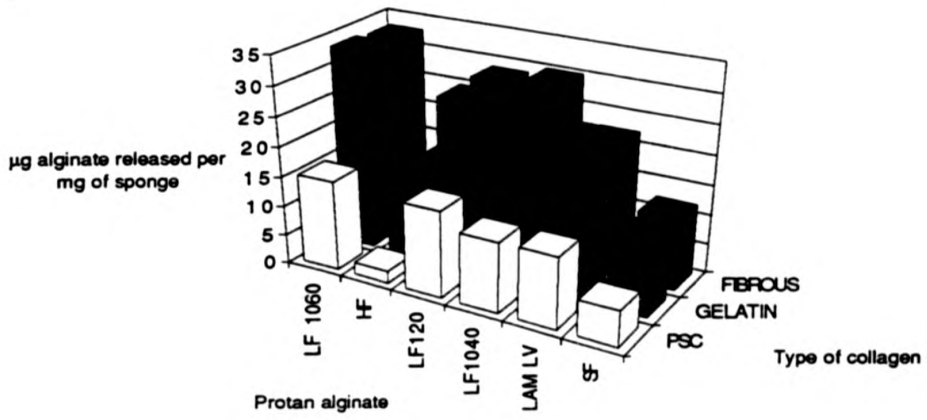


Figure 3.27 The release of alginate from collagen/alginate sponges, prepared from three different forms of collagen, and containing 0.8% calcium.

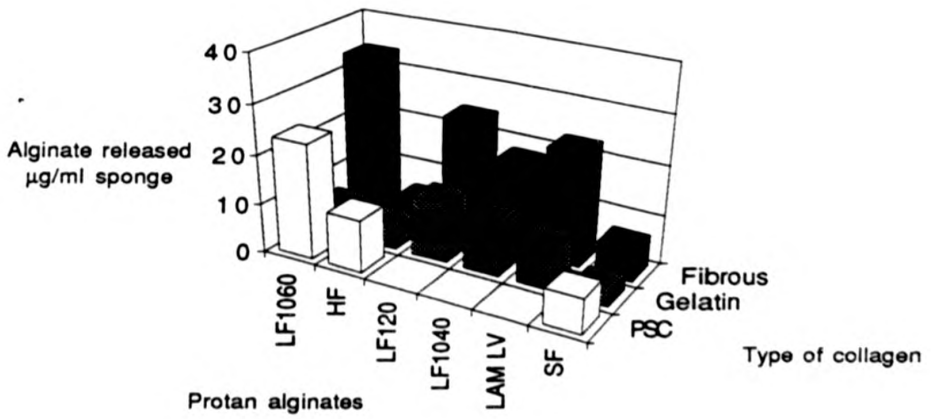


Figure 3.28 The release of alginate from collagen/alginate sponges, prepared from three different forms of collagen, and containing 1.6% calcium

Table 3.5 The alginate released from collagen/alginate sponges prepared from different types of collagen, expressed as a percentage of control sponges

	0.8% Calcium			1.6% Calcium		
Alginate	Fibrous	Denatured	soluble	Fibrous	Denatured	Soluble
LF1060	100	101.7	46.2	101.2	71	18.3
HF	100	101.6	15.3	36	78.9	32.3
LF120	100	99	50.9	84.1	-	41.9
LF1040	100	87.5	39.6	58.5	-	35.1
LamLV	100	111.6	51.8	96.8	-	37.2
SF	100	104.1	48.7	50.2	58.6	24.4

control value, however, both SF and HF sponges released less than half that of the control. The order of release in the sponges with 1.6% calcium was similar to that observed in the control sponges, except for LF1040 and LF1060, whose positions were interchanged: SF and HF <LAM LV, LF1040 and LF120 < LF1060. (Fig 3.27 and 3.28).

3.5.6 Denatured collagen sponges

The amount of alginate released from the sponges prepared from denatured collagen, with 0.8% calcium (sponges 13-18) was not significantly different to the control sponges (Table 3.5), and the order of alginate release was unaltered.

When slurry was prepared from denatured collagen with 1.6% calcium, flocculation of the slurry was observed with alginates LF120, LF1040 and LAM LV. This prevented subsequent processing into sponges, and therefore no alginate release measurements could be made. The three sponges which were prepared with 1.6% calcium (alginates LF1060, HF and SF), showed 20-40% less alginate released than their equivalents with 0.8% calcium. As in the previous sets of sponges, the order of release was SF < HF < LF1060.

3.5.7 Soluble collagen sponges

The amount of alginate released from the sponges prepared with soluble collagen is also shown in Figure 3.27 and 3.28. The sponges with 0.8% calcium (Table 3.4, sponges 13-18) were shown to release less alginate than the control sponges; typically, this was less than 50%, and with the sponge prepared from alginate HF, only 15%.

Despite lower amounts of alginate being released, the order of release was largely unchanged i.e. HF <SF <LAM LV <LF1040 <LF120 <LF1060, although the sponges containing HF and SF, and LF1040 and LF120 reversed order.

The higher calcium concentration (1.6%) reduced the release of alginate from the sponges to 18-42% of that of the control sponges (Table 3.5)

In contrast to the other groups of sponges tested, the order of release of the six alginates changed: the sponge containing alginate LF1060, which was released most readily from the other sponges, showed the second lowest release of alginate; the release of alginate from the other sponges was unaffected.

3.5.8 Methylated collagen sponges

The effect of modifying the primary structure of collagen on the collagen/alginate interactions in the sponges was investigated by methylation. This was chosen since although altering the charge on the collagen, it was thought unlikely to cause steric hindrance to the collagen/alginate interaction. Fibrous collagen was methylated according to the method of Wang (section 2.1.1.2), the modified collagen fibres were used to make collagen alginate sponges. Amino acid analysis was performed on the collagen fibres (methods 2.2.8.2) to determine whether the methylation was successful. The HPLC elution profile of modified and unmodified collagen are shown in Figures 3.29 and 3.30. The modified collagen elution profile had an extra peak (No. 12) which was assumed to be a methylated amino acid derivative.

Since modification of the collagen primary structure may have altered the tertiary structure and fibre organisation, this could have altered the pore structure of the sponges and affected the rate of diffusion. To determine whether this had occurred, blue dextran (2 mg/ml) was incorporated into the sponges and its rate of diffusion out into solution was measured. Sponges were prepared from modified and native collagen fibres with and without alginate, shown below.

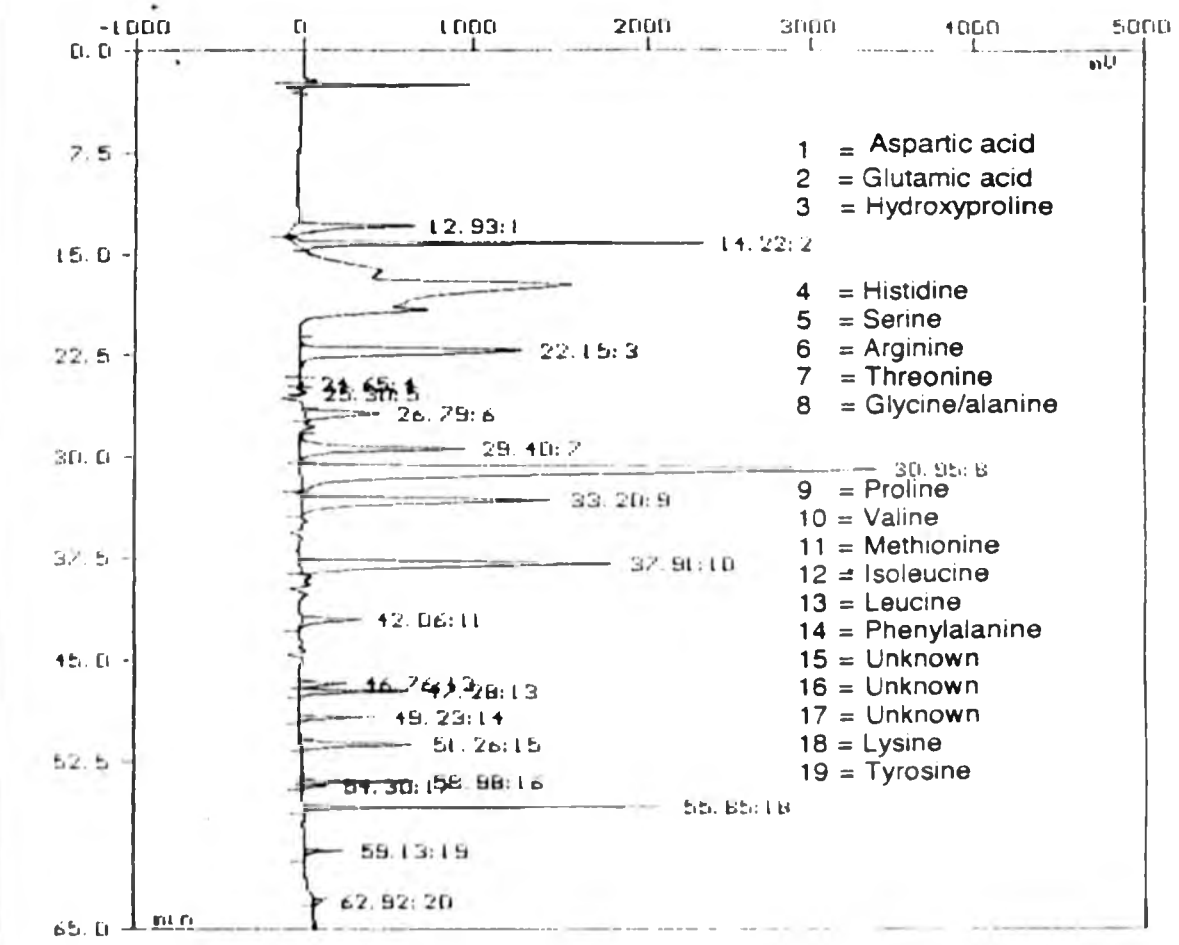


Figure 3.29 HPLC of dansylated amino acids of fibrous collagen on Spherisorb™ ODS (column dimensions 25cm x 4.6mm).

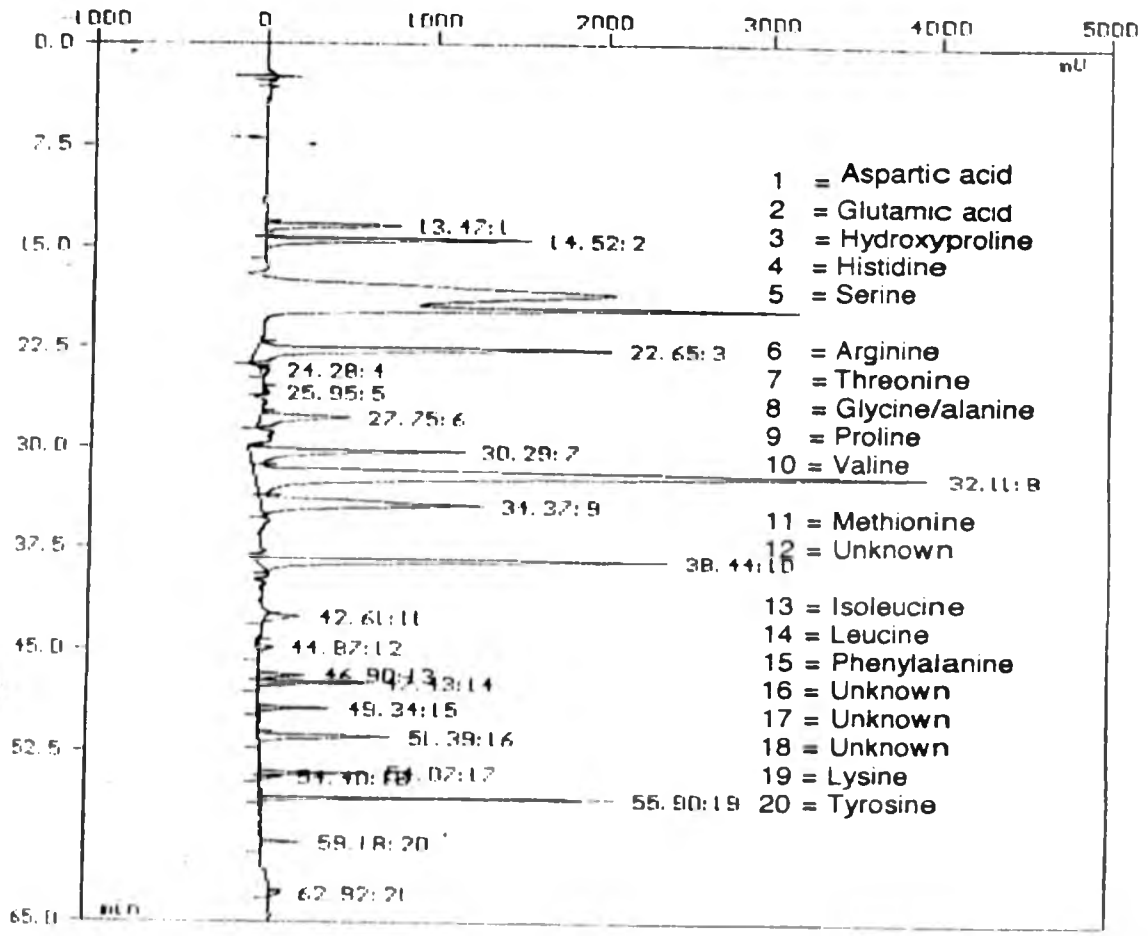


Figure 3.30 HPLC of dansylated amino acids of methylated fibrous collagen. (Column details on Fig 3.29)

Sponge number	collagen	alginate	blue dextran
1	modified	+	+
2	modified	-	+
3	fibrous	+	+
4	fibrous	-	+

Approximately 15 mg of the sponges were soaked in 10 ml of saline, samples of the saline were taken at times up to 24 hours and the absorbance measured at 660 nm. The results (Table 3.6) show there were no significant differences between the sponges prepared from methylated collagen and those of native collagen, indicating that macromolecular diffusion, and presumably the physical structure, had not been affected.

The amount of alginate released from the methylated collagen/alginate sponges was measured by shaking samples of the sponges in saline solution (Methods 2.2.1.1).

The results are shown in Figure 3.31 and Table 3.7, where the values are expressed as a percentage of the total alginate released from control sponges (Table 3.4 sponges 1-6).

It was shown that the total amount of alginate released was significantly lower than that of the unmodified sponges. The higher molecular weight alginates (HF and SF) showed only 24 and 52% of the control level released, whilst those prepared from the other alginates released between 70 and 80 %.

As with sponges prepared from other collagen sources, the order of release of the alginates was unchanged.

3.6 Tensile strength of collagen alginate sponges

The tensile strength of collagen alginate sponges prepared from the Protan alginates was measured according to the previously described method

Table 3.6 The release of blue dextran from control sponges

		A630	
sponge no.	2 hours	4 hours	24 hours
1	0.068	0.1	0.113
2	0.039	0.067	0.087
3	0.088	0.083	0.095
4	0.042	0.063	0.079

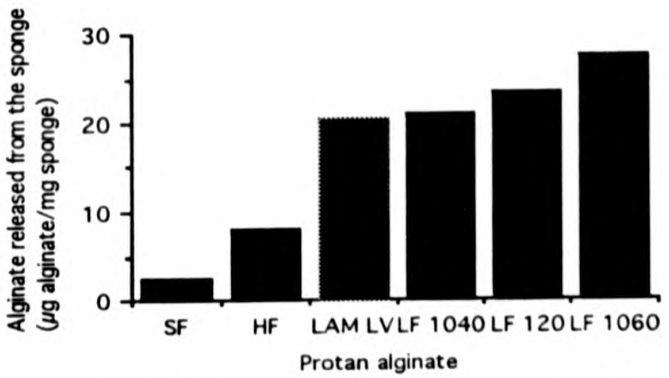


Figure 3.31 The release of alginate from sponges prepared from methylated collagen

Table 3.7 The amount of alginate released from the methylated collagen sponges. (expressed as a percentage of the fibrous control sponges)

Alginate	% of control
LF 1060	79
HF	52
LF 120	80
LF 1040	70
LAM LV	87
SF	25

(Section 2.2.3).The mean values ranged from 4.213 to 6.939 N (Table 3.8). It was thought that the tensile strength of the sponges would increase with the molecular weight of the alginate added. Although the sponge with alginate SF had the highest tensile strength (6.93N) it does not have the highest Mr. As Figure 3.32 shows, there was no correlation with the other sponges.

3.6.1 Absorption of collagen alginate sponges

The ability of collagen alginate sponges to absorb saline solution was measured (methods, section 2.2.4). The sponges were soaked for 8 hours and weighed to determine the amount of saline absorbed. The results (Table 3.8) are expressed as µl of saline absorbed per mg of sponge and show no significant difference between sponges illustrating that the alginate used in the sponge does not affect the ability of the sponge to absorb saline.

3.7 Galactomannan sponges

To obtain a comparison between anionic polysaccharides and neutral gums, sponges were prepared containing locust bean gum or guar gum which are structurally similar galactomannans. Since no electrostatic interactions occur between the galactomannan molecules and the collagen fibres, it was hypothesised that all the polysaccharide in the sponges would be capable of being released from the sponge.

Borate ions were added to some sponges, since borate ions cross-link galactomannans it was thought that they might have a similar effect as calcium ions cross-linking alginate within the collagen matrix. The anions cross link the polysaccharide chains which leads to a thickening of galactomannan/borate solutions. The cross-links are via *cis*-diol groups in the sugar residues (Pittet 1965). It was thought that the addition of borate ions would affect the rate of galactomannan release from the sponges. A set of sponges containing calcium ions were prepared as a control.

Table 3.8 Effects of different alginate preparations on tensile strength and saline absorbance in collagen alginate sponges

Alginate	Tensile strength (Newtons)	S.D	Absorbance (µl per mg)	S.D.
SF	6.94	1.63	36.1	5.36
HF	5.97	0.99	32.88	2.5
LF 120	4.93	1.17	32.12	5.4
LAM LV	4.21	1.227	30.73	3.2
LF1060	5.87	1.14	33	2.03
LF1040	6.09	0.479	39.81	1.6

In each case a minimum of 20 samples were tested

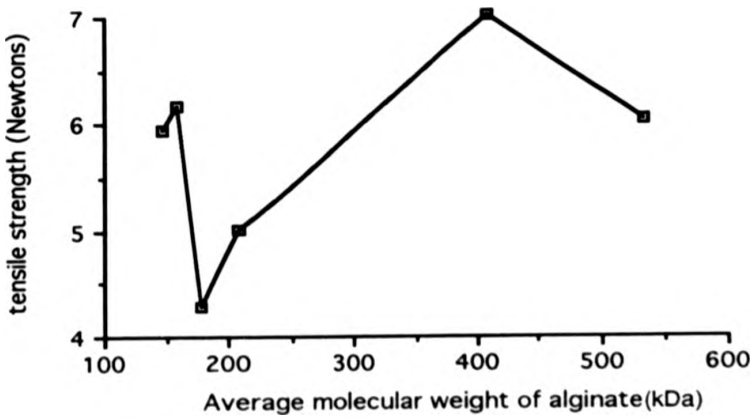


Figure 3.32 The effect of different alginate molecular weights on the tensile of collagen/alginate sponges

The amount of galactomannan released from the sponges was measured by shaking samples of the sponges in saline (Methods 2.2.1.1), and the amount of eluted polysaccharide determined by the phenol sulphuric acid assay (section 2.2.11.2). The results showed a time dependent elution of the polysaccharide up to 24 hours (Fig 3.33 and Table 3.9).

3.7.1 Guar sponges

The sponge containing neither calcium or borate was taken as the control. At 1 hour the sponge containing calcium had released 10% less guar than the control, whilst that containing borate ions released 20% more. At 24 hours the sponges containing calcium and borate had released less guar than the control sponge (23 and 17% respectively).

3.7.2 Locust bean gum

As above, the sponge containing no ions was taken as a control. The results (Fig 3.33) show that at the three time points the sponges containing ions had released less LBG into solution than the control sponge. The exception to this was at 4 hours, when the sponge with calcium had released more guar than the control. After 24 hours the sponges containing calcium and borate ions had released 6 and 30% less LBG than the control.

3.8 Collagen alginate films.

3.8.1 Tensile strength

Samples of film were cut using the dumb-bell press (Methods 3.2.3) and clamped using the same method as for the sponges. The tensile strength was taken to be the load at the point of fracture, measured in Newtons. Samples which fractured at the clamps were considered unrepresentative and rejected. A minimum of seven measurements were made.

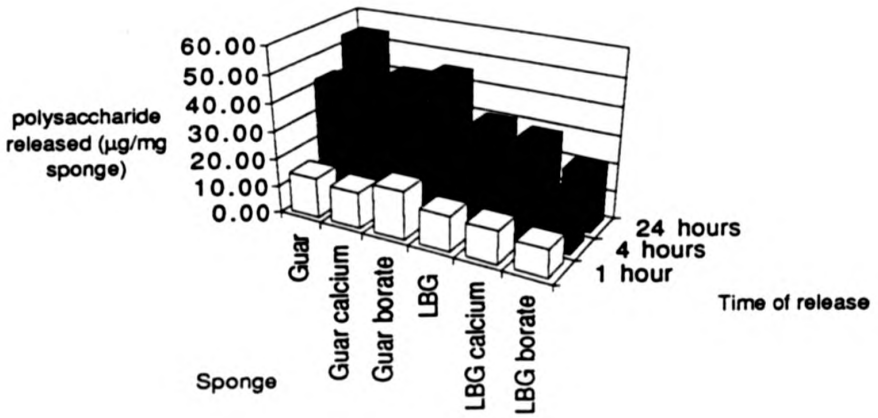


Figure 3.33 The release of galactomannan into saline from collagen/guar and collagen/LBG sponges

Table 3.9 The effect of calcium and borate ions on the release of galactomannan from collagen/guar and collagen/ Locust bean gum sponges

values are expressed as µg of polysaccharide released per mg of sponge

Sponge	1 hour	4 hour	24 hour
Guar	14.6	42.7	54.0
Guar calcium	13.2	34.8	41.8
Guar borate	17.6	38.7	45.2
LBG	12.8	19.9	30.3
LBG/ calcium	12.1	27.4	28.6
LBG/ borate	10.1	18.0	21.0

The results showed that there were considerable differences between the tensile strengths, with the values ranging from 24.5 to 61.7 N.

The tensile strength of the collagen/alginate films was not related to the molecular weight of the alginate; as illustrated in Figure 3.34. In some cases such as the film prepared from alginate SF, which has the highest molecular weight, the length of the alginate chain corresponds to the highest tensile strength, however there is no obvious correlation with the other five films.

3.8.2 Film Permeability

To determine if there was any difference in the pore structure of the films, their relative permeability to saline was measured.

A model was developed (methods 2.2.2) which allowed accurate, repeatable measurements of the film permeability. The film was clamped in place, the tube inverted, and 10 ml of saline solution was added through the base of the tube to fill the container. The initial weight of the container and solution were noted, and the tubes were then placed on a mesh support in an incubator at 37°C. At 1,4 and 24 hours the tubes were removed from the incubator and weighed to determine the mass of solution lost. The permeability values for the films containing 10% alginate are shown in Table 3.11

There were small differences in the amount of solution lost through the films at 1 and 4 hours, but it was difficult to compare the samples since the volume lost was so small. At 24 hours a larger volume had permeated the film, however, the differences between films were not significant.

3.8.3 Release of polysaccharide from collagen alginate films.

The release of alginate from collagen/alginate films, was measured by shaking samples of film in saline solution (methods 2.2.1.1). The solution was sampled at time intervals and alginate assayed by the carbazole method. After 24 hours no detectable alginate was released from the films.

Table 3.10 The effect of different molecular weights of alginates on the tensile strength of collagen/alginate films.

Alginate used	average strength (N)	standard deviation
LF 1040	25.42	5.8
LF10 60	53.6	13.8
SF	61.7	8
HF	42.8	7.3
LF120	35.9	14.2
LAM LV	55.3	9.4

Table 3.11 The effect of different alginates on the permeability of collagen alginate films.

Results are expressed as volume of saline (ml) lost by permeation through the collagen/alginate membranes

Alginate used	1 hour	4 hour	24 hour
LF120	0.097	0.27	5.137
LF1040	0.086	0.25	5.269
SF	0.107	0.269	5.118
HF	0.059	0.223	5.228
LF1060	0.088	0.225	4.904
LAM LV	0.044	0.207	5.839

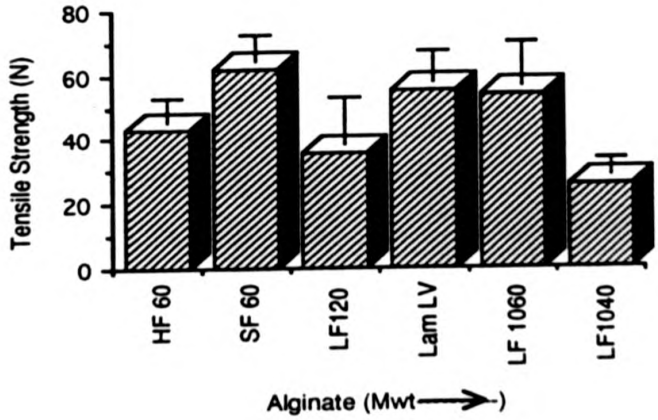


Figure 3.34 The effect of different alginate molecular weights on the tensile strength of collagen/alginate films

3.9 Investigation of alginate /Sepharose™ 4B interaction.

Initial results from the Sepharose™ 4B gel permeation chromatography suggested that the Protan alginates had similar molecular weight distributions. It was thought that an interaction between the alginate and the Sepharose™ 4B was occurring, which was preventing the resolution of alginate on the column. To verify this, the fractions eluted from the column after addition of alginate LF1060 were pooled into apparent high and low molecular weight sample, and re-chromatographed (Fig 3.35). The alginate sample eluted between fractions 11 and 40, the high Mr fraction between 11 and 32, and the low between 16 and 40. The area of overlap of the high and low curves was 30% indicating reasonable separation of this poly-disperse preparation.

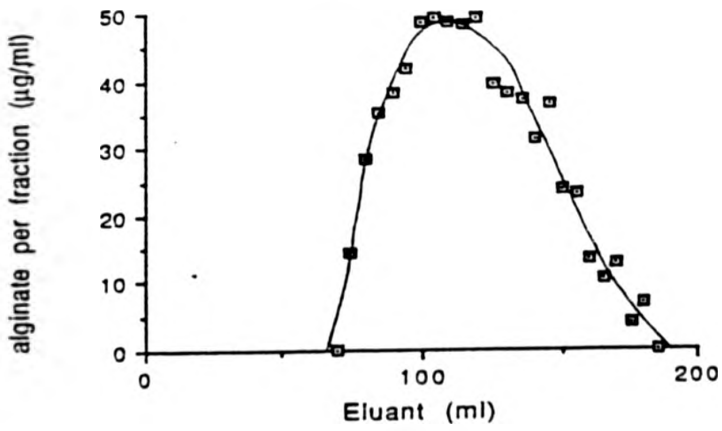
3.9.1 The molecular distribution of the alginates, determined by gel exclusion chromatography

The six commercially available alginates were chromatographed on a Sepharose™ 4B column. The elution curves (Fig 3.36 and 3.37) show there was little difference in the range of molecular weights. Elution of all six alginates commenced immediately after the void volume (120 ml), and the end of the peak was typically after 450 ml was collected. The exception to this was with Protan SF which was retained by the column until 500 ml had been collected.

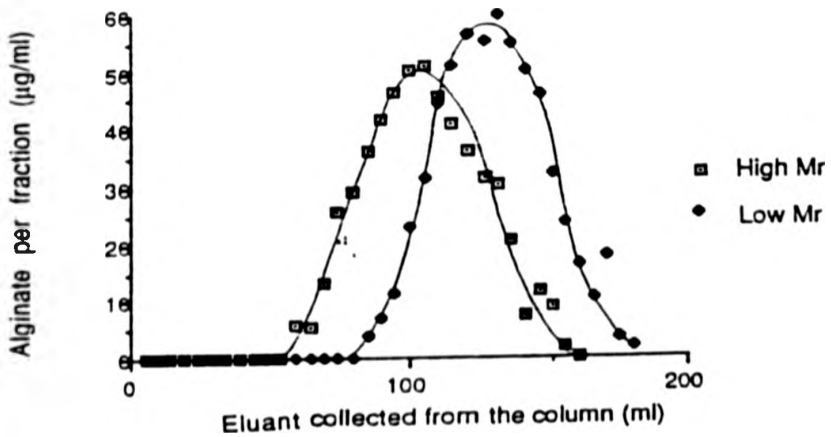
The peaks of the elution curves (Table 3.12) showed that alginates SF and HF had the highest apparent molecular weight. This corresponded with the molecular weights given by Protan Ltd. The remaining alginates, which were reported to have similar molecular weights, showed peak absorbance between 255 and 300 ml. Table 3.12 shows that for the other alginates

Table 3.12 The elution peaks of different alginates from a Sepharose ™ 4B exclusion chromatography column.

Alginate	Peak (ml)	Mr (kDa)
SF	220	400-600
HF	205	450-600
LF1040	300	130-170
LAM LV	300	150-180
LF 1060	255	120-160
LF120	260	150-250



A



B

Figure 3.35 Chromatography of alginate LF 1060 on Sepharose 4B (Fig 3.35A) Fractions 14 to 22 , and 23-35 were pooled to give high and low molecular weightpools, respectively. The pooled fractions were concentrated and rechromatographed on the same column (B).

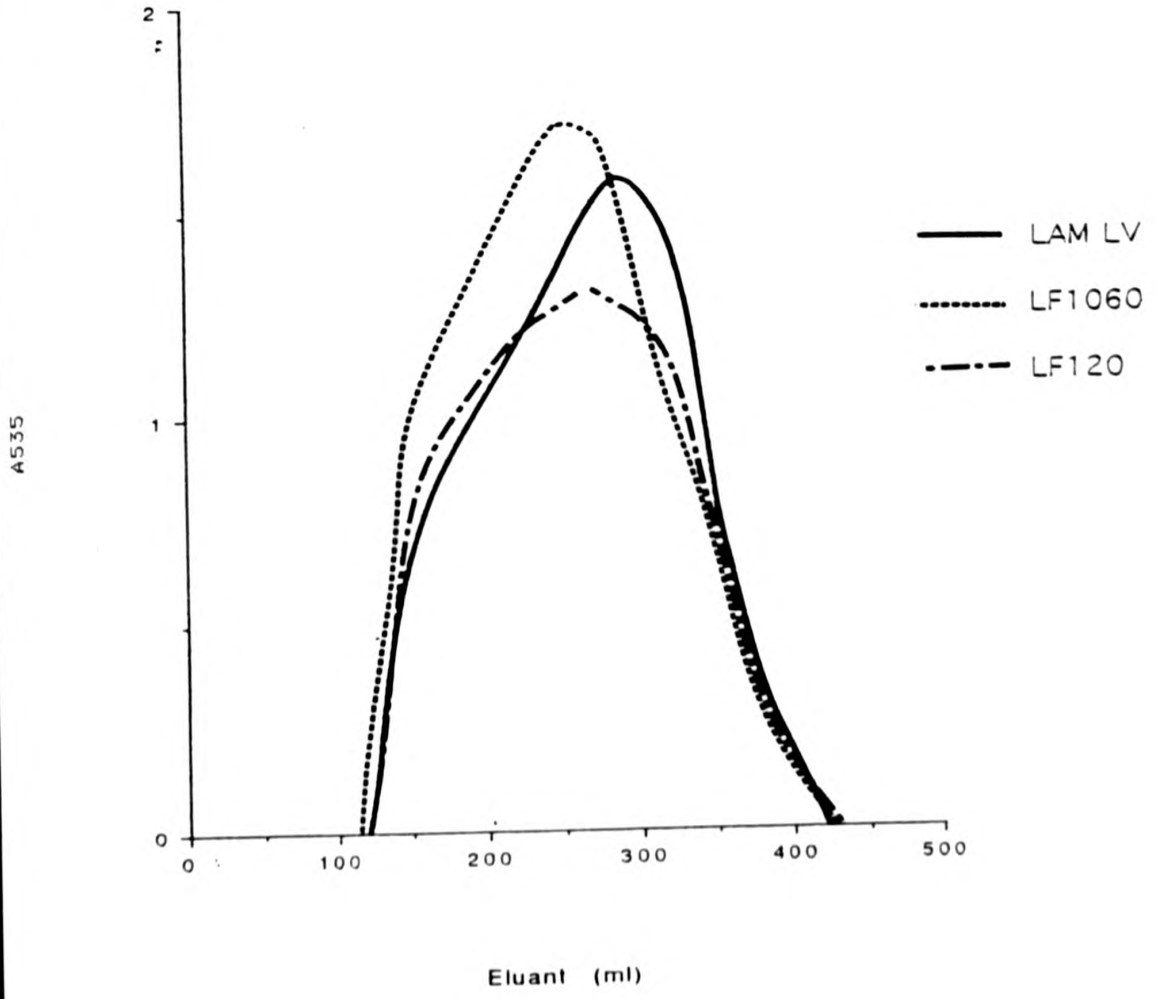


Figure 3.36 Preparative chromatography of Protan alginates on Sepharose™ 4B. 100mg of each alginate was applied to the column in 20ml of eluant (column dimensions 450x42mm)

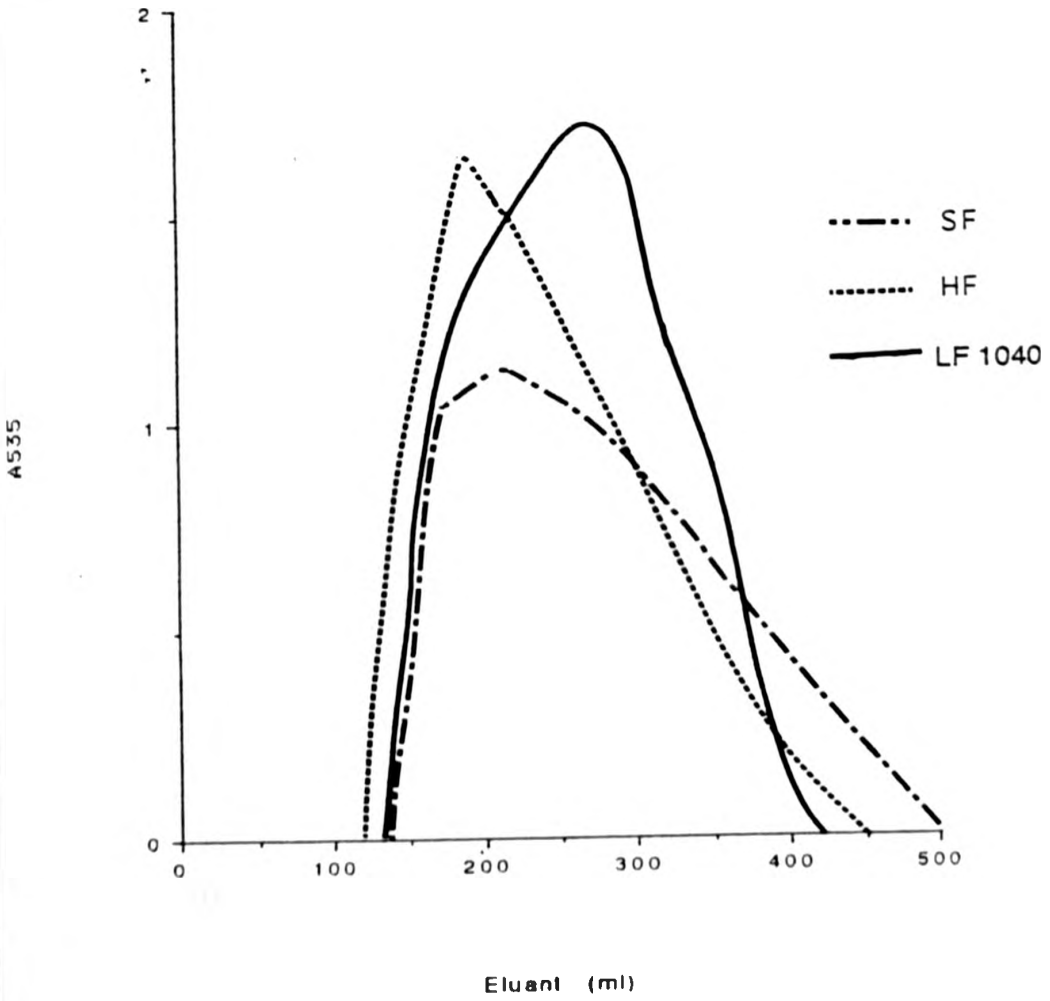


Figure 3.37 Preparative chromatography of Protan alginates on Sepharose™ 4B. (column details on Fig 3.36)

(LF1040, LAM LV, LF1060, and LF120) there was no relationship between the retention volume and the molecular weights described by Protan Ltd.
Each of the six alginates eluted from the column as a single peak with a distribution approximating to normal. The exception was again with alginate SF which 'tailed off' more slowly than the other alginates, forming a skewed curve.

3.9.2 Molecular distribution of alginate released from collagen alginate sponges.

The alginate that was eluted from a collagen/alginate sponge, prepared from alginate LF1040, was collected after 4, 24 and 72 hours and chromatographed on a Sepharose™ 4B column. Before application to the column the alginate was concentrated according to methods 2.2.1.1. The elution profiles obtained are shown in Figure 3.38. Protan alginate LF1060 eluted from 45-180 ml with a peak at 110 ml. The alginate released from the sponge during the first 4 hours was eluted between 145 and 200 ml with a peak at 160 ml. The samples taken at 24 and 48 hours eluted between 50 and 190 ml with peaks at 130 and 140 ml respectively.
The elution profiles show that after 4 hours only alginate molecules from the lower end of the molecular distribution have been released into solution. After 24 and 72 hours molecules from the complete molecular weight range, i.e. the largest and the smallest molecules are released, however, the only a small proportion of the large molecules have been released.

3.9.3 Analysis of the molecular distribution of the alginate retained in collagen sponges.

After extraction into saline of soluble alginate from a sponge approximately 40% of the total alginate was retained in the sponge. The sponges were treated by EDTA extraction (methods 2.2.1.2) or pepsin digestion (Methods

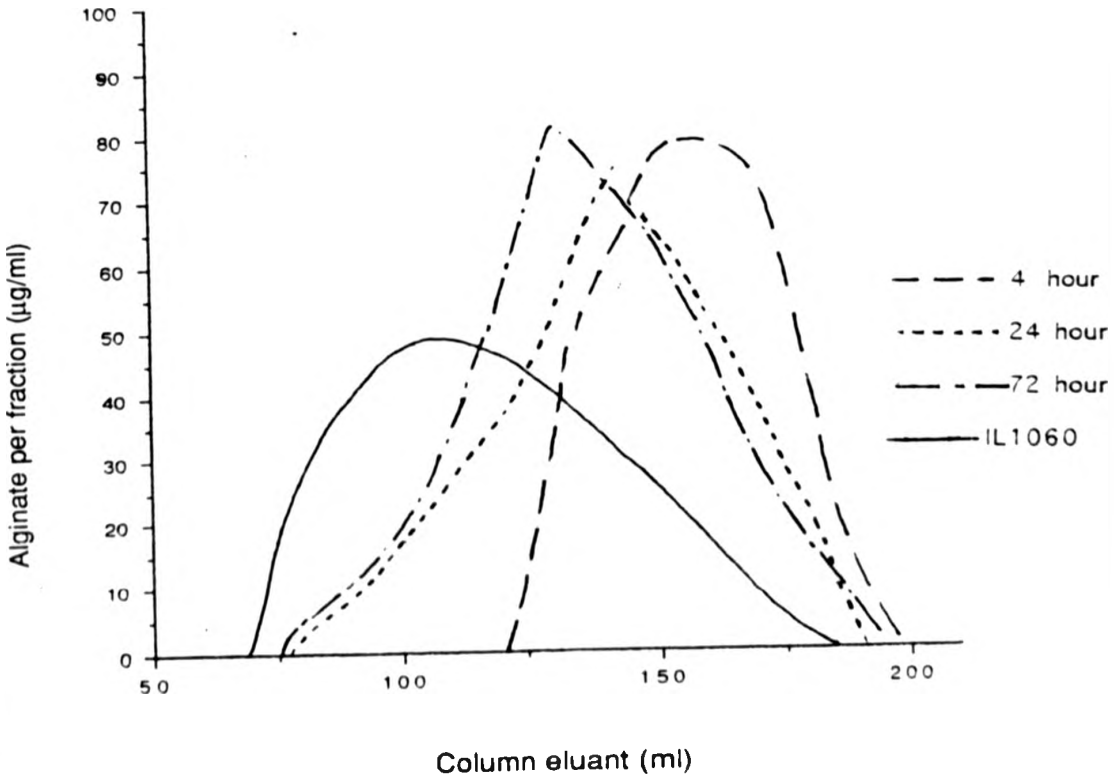


Figure 3.38 The elution of alginate washed fromaFibracol™ sponge. Alginate extractions were made at 4, 24, and 72 hours by extracting the sponges in saline. A profile of the alginate which was used for the sponge preparation is included as a comparison

2.2.1.1) to release the alginate remaining. As shown in Figure 3.39, the alginate from the EDTA extracted sponge eluted from the column between 65 and 175 ml with a peak at 110 ml. The alginate from the pepsin treated sponge eluted between 80 to 180 ml with a peak at 140 ml, indicating that alginate isolated by enzyme treatment had a lower molecular weight than that extracted by EDTA.

3.9.4 The effect of irradiation on the molecular distribution of the alginate released from collagen /alginate sponges.

Industrially prepared biopolymer sponges are routinely sterilised by gamma irradiation, therefore, the affect of irradiation on the molecular distribution of alginate was investigated

Soluble alginate from gamma irradiated and non-irradiated collagen alginate sponges was eluted, concentrated and chromatographed on a Sepharose™ 4B column as described in section 2.2.1.1. The elution profile (Fig 3.40) showed a normal distribution. The alginate extracted from the irradiated sponges eluted with a peak at 130 ml, indicating that it had a lower average molecular weight than the non-irradiated alginate (peak at 120 ml), however the reduction in molecular weight could not be determined since the Sepharose™ 4B column was not calibrated with suitable standards.

3.10 HPLC Chromatography of the Protan alginates

The six alginates were chromatographed on HPLC gel permeation chromatography columns (Fig 3.41). The alginate was eluted from the column between 9 minutes and 17 minutes, regardless of the alginate applied. The void volume of the column, detected by exclusion of 600 kDa dextran was 9 minutes, indicating that the alginate was released at the void volume. The ascending part of the peak can therefore be ignored, since the larger polysaccharide chains were not resolved by the column. The descending side

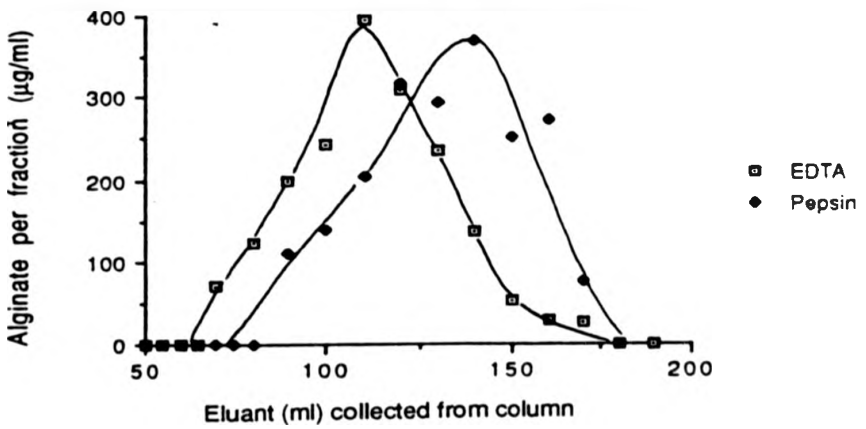


Figure 3.39 The alginate retained in collagen alginate sponges after 72 hours washing in saline. Two methods of isolating the alginate are compared; pepsin digestion of the collagen and washing the sponge in 10mM EDTA.

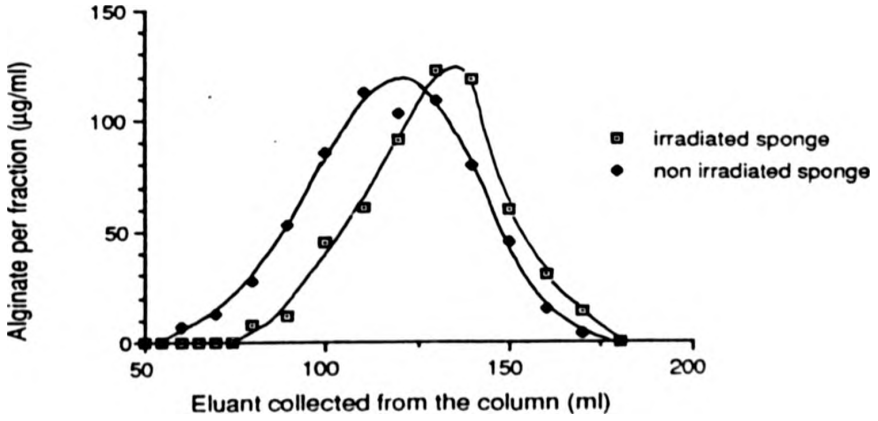
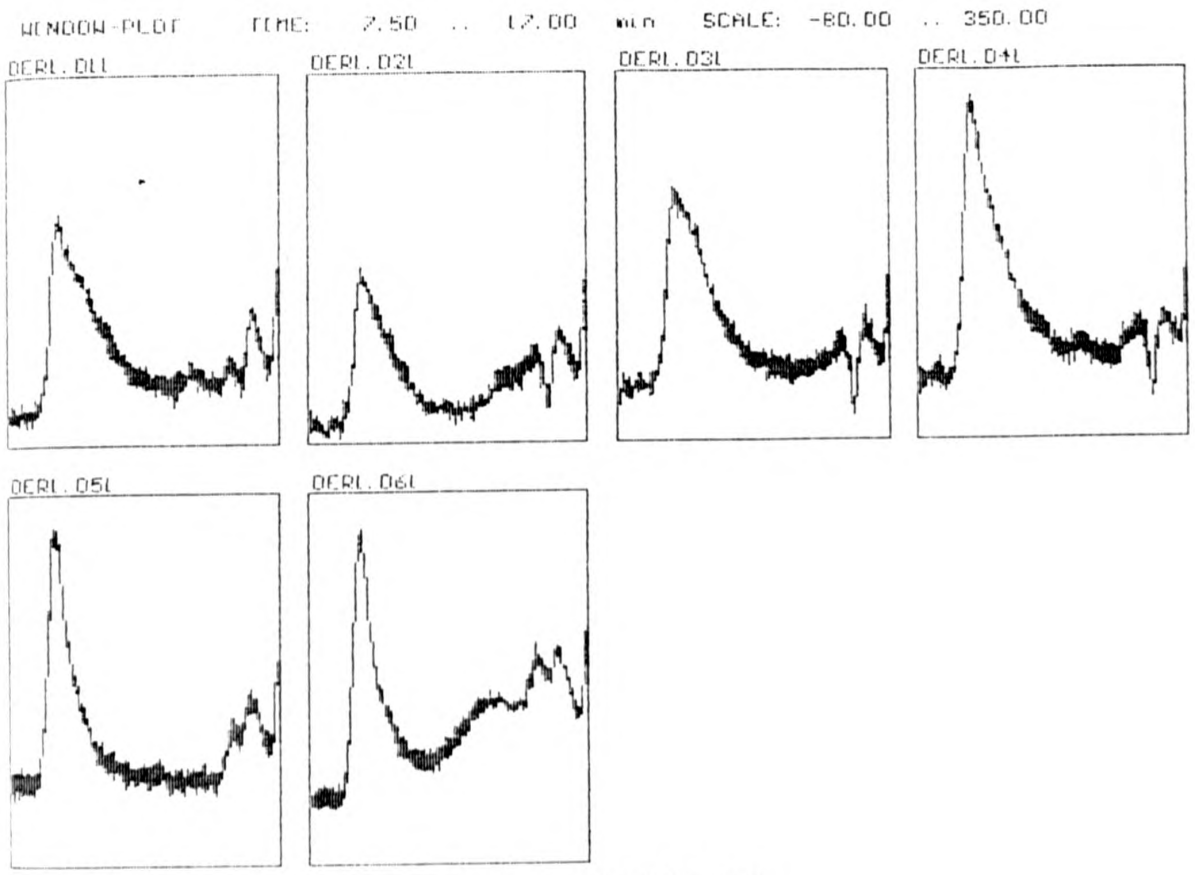


Figure 3.40 The comparison in the molecular weight distribution of alginate released from irradiated and non irradiated collagen alginate sponges. 5 ml fractions were collected and assayed for alginate content by the carbazole method.



X axis time scale ranges from 7.5 to 17.0 minutes (left to right)

Figure 3.41 HPLC size exclusion chromatography of six commercial alginates

Der D1l = Alginate LF1040
Der D2l = Alginate LF1060
Der D3l = Alginate Lam LV
Der D4l = Alginate LF120
Der D5l = Alginate HF
Der D6l = Alginate SF

of the peaks, however, can be used to compare the molecular distribution of the alginates. In the case of alginates HF and SF the peak was steep and very narrow, indicating, that the polysaccharide chains are high molecular weight and have a narrower molecular weight distribution than the other alginates. Alginate SF had a second, broader, peak, eluting between 12 and 17 minutes corresponding to a molecular weight of 2500 Da. Similar peaks were found with the other alginates; however, they were less intense. Alginate LF120 was shown to have a steep, narrow peak, indicating a relatively small size distribution. The remaining alginates were more polydisperse.

3.11 Agarose/polyacrylamide gel electrophoresis

The alginates were electrophoresed on a 1% agarose/ 1% PAGE gel (Plate 2). Lane 1 was hyaluronic acid of 375 kDa and was included as reference. The alginates (lanes 2-6) migrated further and showed a high degree of polydispersity. The R_f value for the point of maximum absorbance determined by scanning densitometry is shown in Table 3.13

The alginates were streaked over a large area of the gel. Alginates HF and SF had only a low intensity staining on the lower part of the gel, suggesting that there only small amounts of low molecular weight polysaccharide in the samples. The remaining alginates had a wide spread area of intense staining, indicating a wider molecular weight distribution than alginates SF and HF.

3.12 Determination of the average molecular weights of the Protan alginates by viscometry

The average molecular size of the alginates (shown below) determined by the Ubbelhode viscometer are within 30000 Da of the values supplied by Protan Ltd; the exception was alginate SF which was 80000 Da lower than the manufacture's value.

Plate 2 Agarose polyacrylamide gel of commercial alginates

Lane	Polysaccharide
1	hyaluronic acid
2	Alginate SF
3	Alginate HF
4	Alginate LF1060
5	Alginate LAM LV
6	Alginate LF120

Table 3.13 The range and peak intensity of different alginates electrophoresed on a polyacrylamide/ agarose gel

polysaccharide	range (RF)	intensity peak
Hyaluronic acid	0-0.238	0.143
SF	0-0.666	0.33
HF	0-0.666	0.238
LF1060	0-0.761	0.428
LAM LV	0-0.809	0.476
LF120	0-0.859	0.428

The values were calculated according to the formula given in section 2.2.5.1

Alginate	n_{sp}	n_{rel}	n	c (mg/ml)	Mwt (Da)	Protan (Da)
SF	.686	.522	7.53	.0761	312000	400000
HF	.823	.600	10.60	.0630	493000	525000
LF1060	.754	.562	4.648	.133	164000	150000
LF120	.837	.609	5.459	.1237	230000	200000
LAM LV	.737	.553	4.161	.1456	141000	165000

n_{sp} :- specific viscosity, n_{rel} :- relative viscosity, n :- intrinsic viscosity

c :- alginate concentration, Mwt :- average molecular weight. Protan= commercial molecular weights

3.13 The effect of pH on the secondary structure of collagen

3.13.2 Circular dichroism

The spectra of PSC was measured at pH 2.5, 3.0, 3.5, 4.0 and 4.5. The results (Fig 3.42) show that the curves obtained from the PSC are identical, with the exception of pH 4 where a deeper trough is observed at 198 nm. This was considered unusual, particularly since a similar effect did not occur at pH 4.5. The trough was thus considered to be an anomaly, rather than an actual change in the collagen structure.

3.13.2 The effect of guar and locust bean gum on the structure of PSC

The spectra from the galactomannan/collagen mixture (Fig 3.43) showed that the collagen had an identical spectrum, irrespective of the presence of galactomannan. Guar and LBG showed no significant dichroism over the full range of wavelengths.

Figure 3.42 The change in the circular dichroism spectra of pepsin solubilised collagen at pH 2.5-4.5

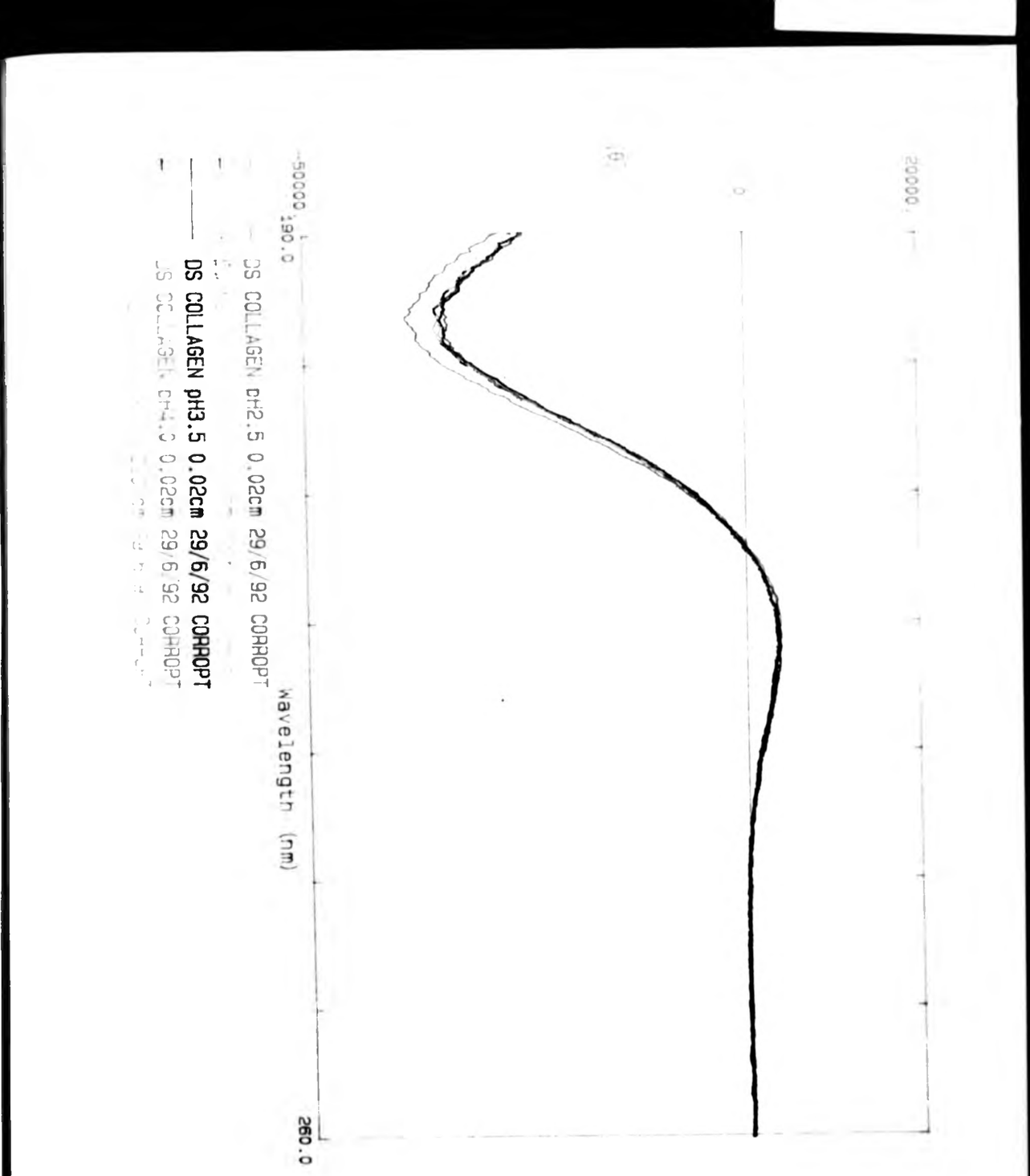
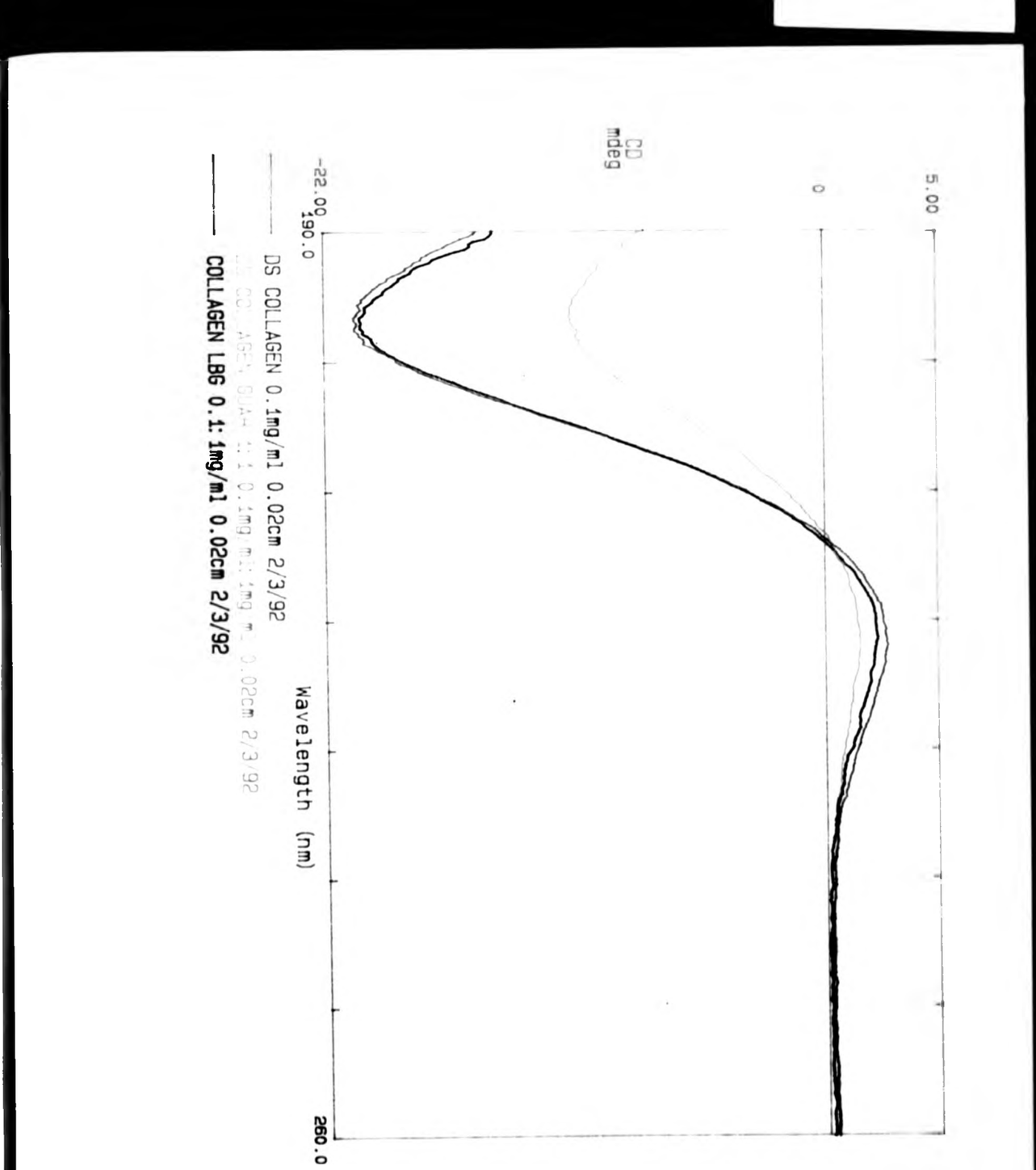


Figure 3.43 The effect of guar and LBG on the structure of pepsin solubilised collagen determined by circular dichroism



3.13.3 The effect of galactomannans on the stability of the collagen triple helix.

The melting curve of the PSC (Fig 3.44) showed a rapid loss of ellipticity between 32 and 42°C. The addition of LBG to the PSC did not alter the melting curve. However, the addition of guar gum to the PSC caused the collagen helix to melt at a lower temperature. If the melting point is assumed to be midway on the linear section of the graph, then the values are: PSC 34.5°C, PSC and LBG 34.5°C, and PSC and guar 33°C. Therefore, there was no significant change in the melting point when LBG was added, however, guar appeared to de-stabilise the triple helix.

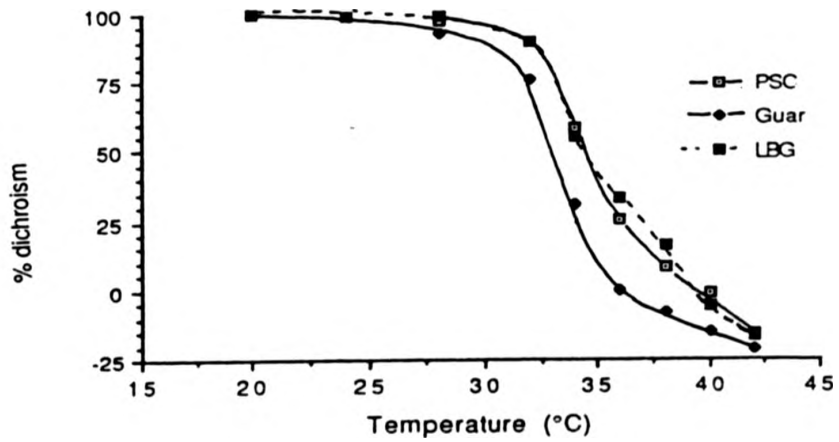


Figure 3.44 The effect of guar and LBG on the stability of the soluble collagen triple helix.

The integrity of the structure was expresed as a percentage of the native structure, taking the value at 20°C as 100%. The collagen concentration was 0.1mg/ml, galactomannans were added to the relevant samples at 0.1% w/v.

CHAPTER 4 DISCUSSION

4.1 Interaction of soluble collagen with polysaccharides in solution

Upon mixing protein polysaccharide solutions it has been shown that one of three things may happen (Tolstoguzov 1986). In the first instance a coprecipitation may occur, often caused by an electrostatic interaction between the two polymer systems. In the second instance the two polymers may co-exist in solution with no interaction between them. The third case is when the polymers interact so weakly that it does not result in precipitation. Examples of this last situation include polymer entanglement or complex formation through hydrogen bonding.

In the third case it is often difficult to detect or quantify any interaction which may be occurring, and Gidley and Robinson (1990) have shown that rheological measurements can help demonstrate such weak interactions.

Formation of insoluble complexes between collagen and charged polysaccharides precludes the use of rheology to investigate their formation and nature. However, the phenomenon of complex precipitation can be used as the basis for titration experiments. These two approaches were the main tools used in the investigation of interactions between soluble polysaccharide and soluble collagen.

4.1.1 Interaction of soluble collagen with charged polysaccharides.

With the six alginates investigated, the pH of the mixing solution was shown to be important in determining the concentration of each biopolymer in the collagen/polysaccharide precipitate. This was expected since the pH of the solution affects the net charge on the collagen molecules. As the pH approaches 4.8 (the IEP of the protein) the net charge on the collagen becomes less positive. If an alginate has x negative charges available to attract collagen molecules, then at pH 2.5, where the collagen has more

positive charges, fewer collagen molecules will be needed to cancel out the charge on the alginate. It follows that at pH 4.0 there will be a smaller net positive charge on the collagen and so a larger number of collagen molecules will be needed to interact with the alginate.

The alteration of the net charge on the collagen helices appears to be the most likely reason that the binding ratio of the two macromolecules is pH dependent. The fact that the different alginates exhibit similar binding ratios at the same pH values is more unexpected. This may be explained by the fact that the charge density of each alginate is very similar and that on such a large scale, steric effects such as chain size have no influence on the electrostatic interaction. In effect this would mean that two small alginate chains could interact as well as one large chain.

That chain length does not appear to affect the interaction, could be explained by the fact that alginate is a flexible molecule and regardless of chain length, the polysaccharide would be capable of orienting to interact with the collagen.

Hyaluronic acid appears to interact in a similar manner to the alginate, this is despite the fact that it has a greater chain length and a lower charge density. This supports the previous suggestion that for a simple interaction in solution the size of the polysaccharide chain has little or no effect on the collagen/polysaccharide binding ratio. It was expected that HA, which has a lower charge density, would have a smaller binding ratio than that of alginate. The fact that this was not the case, suggests that the charges on the polysaccharide are in excess and do not interact fully with the positive charges on the collagen molecules, this would lead to the precipitate having a net negative charge. Although the charge density may affect the strength of binding in the coprecipitate it does not appear to affect the stoichiometry.

This is supported by the fact that the charges on the collagen molecules are not evenly distributed (Asghar and Henrickson 1982) and so there will be

localised areas of charge where there is the potential for interaction. It is unlikely that such a large molecule as HA could orientate in a manner that would permit each of its negative charges to interact with a corresponding positive charge on a collagen molecule.

With the sulphated polysaccharide carrageenan the collagen/carrageenan binding ratios were similar irrespective of the pH. The largest difference in the collagen/polysaccharide binding ratios of the uronic acid containing polysaccharides and carrageenan were observed at pH 2.7 and 3.0. The most likely explanation of this difference, is the fact that the charges on both the collagen and the alginate (pKa 2-4) are ionising over this pH range. This would give rise to a larger range of end points than with carrageenan where only the collagen is capable of ionisation.

The titration of alginate LF1040 with denatured collagen showed that the denatured collagen was capable of binding a higher number of alginate molecules than native collagen. It was found that a denatured collagen molecule could bind three alginate repeating unit (ru) per denatured ru, whilst the native triple helix bound only two alginate ru per collagen ru. Therefore, in total the denatured collagen bound 50% more alginate than the native. This result was expected since denaturing soluble collagen produces a less constrained molecule, allowing more flexibility in the interaction of the two biopolymers. Since the charged amino acids on the collagen molecule will be on the outside of the helix, then unfolding will not significantly increase the number of positive charges available for interaction. Denaturation will however lead to a more flexible collagen molecule where the charged residues are not locked in position, but can move more freely, leading to a more complete interaction with the polysaccharide.

Guanidine hydrochloride (Gdn HCl) is a denaturant used in protein unfolding studies. It is generally accepted that it acts by interrupting the water structure around a molecule through competition between Gdn HCl and water for

protein binding. In globular proteins Gdn HCl concentrations of 1-3M are used to cause subunit dissociation. For complete unfolding 6M is required. The finding that Gdn HCl disrupted the binding between PSC and alginate was surprising since guanidine hydrochloride at this concentration had no detectable effect on the structure of the collagen as shown in the CD spectra of PSC in the presence of 1M Gdn HCl. More unusual was the fact that urea, which is thought to act in a similar way to the Gdn HCl has no effect on the interaction. The most likely reason for this is that Gdn HCl is a charged molecule and would be expected to interrupt electrostatic bonding between the biopolymer chains. The fact that urea has no effect on the precipitation of the collagen/polysaccharide complexes, is evidence that the interaction is mainly electrostatic in nature. This cannot be taken as evidence that hydrogen bonding does not occur, simply that any hydrogen bonds which are formed, are not critical in maintaining the collagen/ polysaccharide complex. The finding that Gdn HCl disrupts the complexes, shows that the electrostatic bonds are important in preserving the structure, and that in the absence of the electrostatic interaction other intermolecular bonds are ineffective.

4.1.2 Interaction of soluble collagen with neutral polysaccharides

Rheological measurements on mixtures of collagen with modified celluloses, or starch, showed no appreciable enhancement of viscosity indicating a lack of interaction

The modified celluloses (MC, HPMC, and HPC) caused a small decrease in the viscosity of PSC at low torque, but this effect disappeared at high torque. The initial decrease in viscosity is unlikely to be caused by entanglement, because there are very few cellulose molecules in the mixture; in addition an entanglement would be expected to lead to an enhanced viscosity. Similarly, electrostatic interaction can be discounted since the modified celluloses are neutral gums. A possible explanation is that the modified celluloses interact

hydrophobically with the collagen, which would increase order in the mixture, thereby reducing the viscosity. Henderson (1987) has shown that methylated cellulose can aggregate hydrophobically, to produce a gel. However, the temperature has to be raised to 50°C to achieve this phase transition. That HPcellulose, which does not hydrophobically aggregate, also resulted in a decreased viscosity, casts doubt on the role of hydrophobic interaction. It is also possible that the reduced viscosity was a result of differences in the ionic strength of the solutions, which could have been caused by ion contamination in the modified cellulose powders. There are no known instances of modified cellulose interacting with proteins, therefore the presence of ions seems the most likely cause of the reduced viscosity. Since the reduction in viscosity was relatively small, no further investigation of this system was performed.

The addition of potato starch to PSC solution resulted in no change in viscosity.

Since these uncharged polysaccharides did not precipitate PSC from solution, it was not surprising to find that they caused no, or minimal, viscosity changes. However, mixtures of the galactomannans with PSC surprisingly produced significant increases in viscosity, indicating that interactions between them had occurred. The dilute guar solutions may have shown less viscosity enhancement since there were fewer polymer chains to interact. This suggests that any complex which may have be formed would be weaker and more difficult to detect. Another reason for the smaller peaks with the dilute sample could be due to the shear rate of the measurements. Applying a particular force to a sample causes the plate of the rheometer to rotate at a particular speed (the shear rate). The viscosity is a measure of the resistance to this flow at a particular shear stress. Therefore although the two samples were measured at similar shear stresses, the shear rate in each case was very different. In the more dilute sample the plates were rotating more quickly, as a result, any complex formed would be more likely to be broken down.

The fact that the other galactomannans tested also showed an enhancement of the viscosity suggests that some form of interaction was occurring.

Since both gums have similar structures but interact to different extents, it is possible that the degree of branching is important. A similar effect to this has been reported for galactomannans interacting with the bacterial polysaccharide xanthan gum (Brownsley *et al* 1987). The exact method of interaction is poorly understood, but it has been shown that guar gum interacts with xanthan to a lesser extent than the LBG. This was considered to be due to the less branched structure allowing larger areas of the mannose backbone to be exposed, and therefore be available for interaction. The results recorded on the Deer rheometer suggest that the order of interaction is also LBG>guar. This corresponds with the degree of branching, where LBG 1:3.5> Guar 1:1.1. Therefore, there is a likelihood that the interaction is occurring between the PSC and the mannose units on the backbone of the polymer.

Xanthan gum is considered to be a stiff rod-like molecule which consists of a single stranded helix. It has been proposed that the interaction of xanthan with galactomannans is via intermolecular bonding between the mannose backbone of the galactomannans and ordered elements of the xanthan (Dea *et al* 1977). More recently an interaction has been suggested via the tri-saccharide branches of the xanthan. In both cases the interaction is proposed to be stabilised by intermolecular bonding, owing to the stereochemically compatible backbones of both molecules. Although these models of interaction are different with respect to the role of xanthan, it is accepted that the galactomannan molecules interact through their mannose units. The extent of interaction is dependent on the degree of substitution of the galactomannans (Dea *et al* 1977).

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As the interaction of PSC with galactomannans was also found to be dependent on the degree of substitution of the mannose chain, this suggests

that although the precise method of interaction between PSC and galactomannans is unknown it is likely to be via intermolecular bonding between the mannose units and the triple helix.

Rheometry used in this manner can do little more than suggest an interaction. In order to define the method of interaction, more detailed molecular studies are necessary.

4.3 Circular dichroism measurements

Circular dichroism was used to detect any changes in secondary structure of the collagen triple helix that occurred when PSC was mixed with galactomannans. The stability of the collagen triple helix in the presence of guar or locust bean gum was also determined. In addition the structure of the PSC was investigated over the pH range 2.5-4.5 to ensure that the differences observed in collagen/alginate binding at different pH values (section 3.4.1) were not caused by conformational changes in the collagen triple helix.

It was found that the pH of the solution had no detectable effect on the secondary structure of the collagen. This was expected since raising the pH would only effect the acidic amino acids in the collagen, namely glutamic acid (pKa 3.65) and aspartic acid (pKa 4.25) causing them to ionise. This would increase the number of charged groups in the molecule, thus altering the electrostatic bonding within the helix. However, the acidic amino acids only constitute 9.1% of the total collagen. Therefore, the overall destabilisation of the structure would be minimal, since the majority of the intramolecular forces will be unchanged. The collagen triple helix is stabilised by a mixture of non-ionic bonding. Although the energy required to break each of the bonds is small, the total energy required to disrupt the structure is considerably higher. It was proposed that the galactomannans may interact with the collagen molecule through hydrogen bonding. The number of sites available for

interaction, and the resultant energy of interaction, are likely to be too small to cause a significant change in the collagen conformation. Therefore, the galactomannans were not expected to alter the secondary structure of the collagen. This was shown to be the case in mixtures of collagen and LBG, however, the addition of guar gum significantly reduced the intensity of the collagen trace; this may represent a change in the structure of the collagen triple helix in the presence of guar gum.

The melting point of the collagen helix can easily be detected by CD, and is characterised by the loss of secondary structure.

Gelman and Blackman (1974) investigated the effect of GAGs on the melting temperature (T_m) of the collagen triple helix. They found that the addition of anionic GAGs to the collagen stabilised the helix, resulting in a higher T_m, and postulated that the increase in thermal stability was caused by aggregation of the collagen molecules, which was facilitated by the GAGs.

The finding that guar lowered the melting point of the collagen, suggests that it does so by reducing the aggregation of the molecules. This is supported by the fact that PSC precipitates at pH 6, but could by kept in solution if guar was present.

The stabilisation imposed by the GAGs, was greater than the observed destabilisation caused by guar gum. LBG failed to produce a similar destabilisation. There is no obvious explanation for the lack of destabilisation caused by the LBG. However, the ratio of polysaccharide to collagen was shown to be important in the stabilisation, therefore it is possible that there was not enough LBG in the collagen/polysaccharide mixture. This would have resulted in a biphasic melting curve. It was shown that the melting curve of PSC with LBG may be slightly, but not significantly biphasic. A second reason that LBG may have had no effect on the collagen, could be the molecular weight and distribution of the polysaccharide. If guar destabilises the collagen helix by preventing aggregation, then the size of the polysaccharide is likely to

be important. It could be that the LBG used in this study was too small to act in a similar manner to the guar gum.

4.4 Interaction of alginate with insoluble collagen

The extent of interaction of the polyanionic alginates with insoluble collagen was studied indirectly by measuring the rates of release of free polymer from three dimensional sponges made from mixtures of the two.

The rate of alginate release from sponges prepared with a range of alginates differing in structure and Molecular weight was shown to vary, depending on the alginate used. The curves obtained from a plot of alginate release with time, could be divided into three main parts (Fig 3.24). The first was associated with an initial rapid release of alginate; a second phase where the rate gradually decreased, and a third 'plateau' showing little change in the release rate.

With all of the sponges tested, phase 1 lasted for approximately 15 minutes, during which time the rate of alginate release was constant, though different for each sponge. The initial release of alginate was likely to be caused by soluble sodium alginate, located near the surface of the sponge pore walls and hence with a small diffusion path, being released into the saline most rapidly.

The second phase, may be due to either sodium alginate diffusing out of the sponge, and/or an ion exchange effect between calcium and sodium alginate resulting in an increasing concentration of the more soluble sodium alginate form. The length of this phase varied considerably between sponges, in the case of SF sponges phase 2 was almost undetectable, but with sponges prepared from LF1040, it lasted almost 30 minutes.

In the third phase of alginate release, the concentration of alginate in solution was almost constant, indicating that the release of alginate had effectively stopped.

Interestingly, the initial rate of release, and the duration of phase 2, appeared to be related to the total amount of alginate released. For example, sponges prepared from alginate SF had the slowest initial rate of release, the shortest phase 2 time, and released the lowest amount of alginate. By contrast the sponge prepared from alginate LF1040 sponges showed the opposite: the initial rate, the length of phase 2, and the total amount of alginate released were higher than the other sponges.

When sponges prepared from the different alginates were measured to determine the total amount of alginate that was released, it was found that the amount varied between sponges. The order of release was: LF1060> LF1040 > LF120 > LAM LV > HF > SF. Initially, it was considered that the factors responsible for the retention of alginate within the sponges, would be a combination of electrostatic interaction and biopolymer entanglement.

If electrostatic interaction was occurring, then since the charge density was the same on each alginate, the effect should have been constant. Alternatively if simple entanglement of the biopolymers was occurring, then by diffusion, the highest molecular weight alginate would take longer to be released from the sponge.

The results from the titration studies in section 3.4.1 showed that the ratio of interaction between the collagen and the different alginates were similar, irrespective of the type of alginate. In addition to this, the alginate release experiments were performed at pH 6, where the insoluble collagen and alginate have the same net charge. Although this would not have prevented localised charge interactions, however, it is unlikely that any electrostatic interactions would be sufficient to cause the large differences observed in the amounts of polysaccharides released from the sponges.

If entanglement of the fibres was occurring, then, the larger molecular weight chains would be expected to be more interwoven within the sponge and, as a result, take longer to be released. When the total alginate released was

plotted against the average molecular weight of the alginates, it was shown that although the high molecular weight alginates (SF and HF) were released from the sponge in smaller amounts, the other alginates did not follow this trend. It must therefore be assumed that the average molecular weight of the alginate chains was not the only factor controlling the release of alginate from the sponges.

The alginates were known to have different percentages of guluronic (G) and mannuronic acid (M). The G and M content was considered important, because only the G residues are capable of binding calcium (section 1.2.1). The binding of calcium to sodium alginate results in the formation of insoluble calcium alginate fibres, therefore the proportion of soluble sodium alginate in the sponges would be expected to influence the amount of alginate released. A plot of alginate released against the percentage of guluronic acid in the alginate (results not shown) showed no correlation however, suggesting that the % G in the alginate, had no influence on the amount of alginate that was released.

The amount of soluble sodium alginate in the sponges was estimated by the ability of calcium ions to precipitate a known amount of alginate. The results showed that the binding and subsequent dissociation of calcium and alginate were closely related to the extent to which sponges retained the alginate. There was a linear relationship between calcium binding, and alginate released from the sponges, the only exception being LF 1060. As the binding affinity of alginate for calcium increased, more insoluble calcium alginate fibres form, resulting in less soluble alginate being released from the sponges. It was assumed therefore that the calcium binding affinity of the alginates is the critical factor in determining alginate release from the sponge.

Although the presence of calcium was shown to be important in the release of alginate from the sponges, the reason for this is not understood. One explanation is that if the calcium alginate fibres were entangled in the collagen

matrix, fibre length (determined by extent of calcium binding) would dictate the rate of diffusion. Alternatively the calcium could have formed alginate-collagen bridges. Methylated collagen sponges were prepared to investigate whether collagen chains were being cross linked by calcium ions. If collagen-calcium-alginate bridging was occurring, then the methylation of the collagen would reduce the number of charged groups available for calcium bridging. This in turn would lead to an increase in the amount of alginate released from the sponges. Surprisingly, however, the total alginate released was lower in methylated collagen sponges than in unmodified sponges. This indicated that calcium bridging between the alginate and the collagen chains is not likely to be responsible for retaining the alginate within the sponges. A reduction in the number of charges on the collagen would also reduce the electrostatic interaction with alginate, and it would be expected, therefore, that the amount of alginate released from the sponges would increase. The fact that no increase was found supports previous evidence that electrostatic interactions do not influence the release of alginate from the sponges.

The results showed that the interaction between calcium and alginate is most important in determining the amount of alginate released from the sponge. The manner in which the alginate release is controlled is not understood, but could be due to entrapment, entanglement, or a mixture of both. It was hypothesised that in sponges, collagen would form a matrix with alginate fibres within it. The collagen would act like a sieve, allowing alginate molecules of a certain size to diffuse from the sponges. Therefore, in each batch of sponge there would be a population of calcium alginate fibres which were retained in the sponge. There is however little evidence to prove this hypotheses as the correlation of molecular weight and release rates were poor. Investigations at the molecular level are necessary to provide information on the manner in which the biopolymers are interacting.

Sponges made from the different structural forms of collagen, containing 0.8 or 1.6% of calcium, were prepared in an attempt to provide details on the effect of calcium in the sponges.

The results of the alginate release studies on the fibrous collagen sponges, containing 1.6% calcium, showed the amount of alginate released from the sponges was reduced compared with the 0.8% sponges, the effect was not uniform, ranging from a 4% (LAM LV) to a 64% (HF) reduction

The sponges containing soluble, and denatured collagen, also showed that an increase in the concentration of calcium, reduced the levels of alginate that were released from the sponges, proving that additional calcium can reduce the amount of alginate released. However, it is difficult to predict the extent of the reduction.

When preparing sponges made with denatured collagen, three of the slurry mixtures flocculated. This effect did not occur with the native soluble and fibrous collagen preparations. It has been shown that at a pH below its isoelectric point, collagen will interact with alginate to form a coprecipitate. Therefore, it would be expected that the collagen and alginate would interact in the low pH (pH 3) conditions used during sponge preparation. The addition of calcium to the collagen/ alginate slurry is designed to reduce or prevent flocculation by blocking ionic sites on the alginate chains. The slurries that precipitated were prepared from alginates LF120, LF1040 and LAM LV, which contained the lowest percentage of guluronic acid (Table 2.1), and as a result, could not bind as high a level of calcium. It is, therefore, likely that the higher calcium levels resulted in the saturation of the calcium binding sites. A flocculation effect was not observed in the other slurries made from these alginates which also contained 1.6% calcium, thus the flocculation was not caused by the formation of calcium alginate alone. It must therefore be assumed that a denatured collagen/calcium alginate network had formed, which led to the precipitation. The reason a similar effect was not observed

with the other forms of collagen is unclear; however, the flexible structure of the denatured collagen may allow a higher potential for interaction with the alginate. This is supported by earlier results (section 3.4.4), where denatured collagen was found to interact more strongly with alginate than native collagen.

The effect of different collagen structures on the rate of alginate release showed that there was little difference between sponges prepared from fibrous and denatured fibrous collagen. This was unexpected since denaturation of the collagen increases the protein flexibility, and hence, the potential for interaction with alginate. The fact that no additional interaction occurred in the denatured collagen sponges appears to prove electrostatic interactions are not important in controlling the release of alginate from the sponges. The increased flexibility of the denatured collagen polypeptides was expected to result in a closely packed matrix which would retain more alginate molecules within the sponge. The amount of alginate released from the two types of sponges was comparable, therefore it can be assumed that the collagen sponge matrices are similar.

The release of alginate from the sponges prepared from soluble collagen, was less than 50% of that from fibrous collagen sponges. This can be explained by considering the structure of the collagen molecules. In sponges prepared from fibrous collagen, the fibrous structure, i.e. fibril orientation and the gaps between fibrils within each fibre, will be dictated as it is laid down in the animal. However, once solubilised each of the helices can interact to form a more dense network on drying, without having a predetermined fibre structure imposed on it. Furthermore, alginate will be able to interact with only a minority of collagen molecules on collagen fibres, since the majority will be internal to the helical and super-helical arrangement of fibrils and fibres. In contrast, each individual collagen molecule in a solution of PSC will be

theoretically available for interaction. In this way soluble collagen sponges would retain more alginate within the collagen matrix.

4.4.1 Physical characteristics of collagen-alginate sponges

The effect of different alginates on the physical characteristics of collagen-alginate composite sponges was investigated by measuring the tensile strength and the absorption of a saline solution.

The absorption of the samples varied with the type of alginate incorporated into the sponge: however, the standard deviation of the samples was high, and the differences relatively small. This indicates that any effects due to alginates were less than the variability between sponges of the same type.

The tensile test results showed differences between the sponges prepared from different alginates, but the S.D values were very high (coefficient of variation up to 25%). Sponges prepared from alginate SF appeared to be stronger than the other sponges, but not significantly so.

The measurements of the tensile strength, and the ability to absorb saline, showed no correlation with the release of alginate from the sponges.

The fact that there were no significant physical difference between the sponges is perhaps not surprising, since the different alginates are unlikely to alter the dispersion of the fibrous bundles of collagen molecules which will inevitably dictate the strength of the matrix in conjunction with the pore size determined by ice crystal formation (P Watt unpublished results).

4.5 Release of Galactomannan from collagen/galactomannan sponges

The release of polysaccharide from sponges containing galactomannans showed that the sponges with guar and locust bean gum released more polysaccharide into solution when the sponges contained no cations. This proves that addition of a cation such as calcium, which has no direct effect on the galactomannans, interacts with the collagen fibres in a way that entraps

more polysaccharide within the sponge. It may be that the calcium ions act as cross links between adjacent collagen molecules, since at the pH the release experiments were performed, collagen is largely negatively charged. This contradicts the earlier observation that methylated collagen sponges behave in a similar manner to native collagen sponges, suggesting that calcium was not crosslinking the collagen fibres. This discrepancy may be explained by the fact that the methylation of the collagen would not be 100% complete, thus, enough unmodified sites may have remained for crosslinking to occur.

Borate ions, which are known to interact with the galactomannans (Pittet 1965), showed a mixed effect on polysaccharide release from the sponges. In both guar and LBG sponges, the presence of borate ions resulted in lower amounts of polysaccharide being released, than in the control sponges. The guar sponges containing borate ions released less polysaccharide than those with calcium, however, with LBG sponges the opposite was true. None of the sponges showed a 100% release of the galactomannans from the sponge, even after 24 hours It appears that entanglement of the polysaccharide chains occurred, preventing their released from the collagen matrix. This supports earlier evidence that charge interactions are of little importance in controlling the release of alginate from the sponges, since in this case entrapment is sufficient to retain the polysaccharides within the sponge.

Attempts to measure the rate and total alginate released from collagen/alginate films were unsuccessful, since no detectable level of alginate was released. It appears that the dense structure of the films does not allow the alginate chains to diffuse from the matrix. This was unexpected since it had previously been shown (personal communication E Lorimer) that the polysaccharide hyaluronic acid (Mwt >1000 kDa) was eluted from similar films, thus, it was expected that alginate chains of less than half the size would be released. The difference between the HA-collagen films and the alginate case was the presence of calcium, therefore, it is likely that the

alginate is in the form of insoluble calcium alginate, which is entangled in the collagen matrix and protected from release or exchange with sodium ions.

The permeability of the films to saline solution was not affected by the type of alginate used in the film preparation. This was expected, since it would be unlikely that the alginate molecules could alter the alignment and surface activity of the collagen fibres, and hence, change the pore size of the films. It has been shown (Gilbert and Lyman 1990) that diffusion through collagen membranes is dependent on the degree of cross-linking, quaternary structure, and fabrication technique; the method of permeation was "pore" type. In this study, no cross-linking was performed, and the type of collagen and the method of preparation were the same in each case. Therefore, it is not surprising that the inclusion of different alginates did not significantly alter the pore size, and hence, permeability of the films.

The tensile strength of the films varied considerably, depending on the alginate used. The values recorded were far higher than those of the sponges. The difference in the strength of the films is most likely due the alignment of the collagen fibres, as opposed to a change in fibril structure. Although the strongest film was prepared with alginate SF, the alginate with the highest molecular weight, there was no direct correlation between molecular weight and film strength.

4.6 Molecular weight analysis of alginates released from collagen-alginate sponges

The discovery that different alginates were leached from collagen/alginate sponges at different rates suggested that the differences may be due to the molecular weight distribution of the alginates. Several methods were used to determine the molecular weight distributions.

The elution curves of the alginate types from the Sepharose 4B column indicated that they had an almost identical molecular weight range. It was shown that the alginates did not interact with the Sepharose™, therefore it was assumed that the broad peaks eluted from the column indicated that Sepharose 4B was incapable of separating the alginate molecules. It was reported (manufacturers information) that Sepharose 4B would fractionate polysaccharides between 3 and 500 kDa; unfortunately this proved not to be the case, under the conditions used in this work. The elution profiles from HPLC supported the gel exclusion results, suggesting that the molecular size distributions of the six alginates were very similar. The elution profiles from the HPLC columns showed that the alginates were eluted as two peaks. However, the second smaller peak was common to many samples prepared in this laboratory, and was assumed to be an anomaly of the solvent system.

Unfortunately the supply of alginate LF1040, was exhausted at this stage, and an identical batch could not be obtained. As a result, the viscometry and gel electrophoresis experiments were performed with the five remaining alginates. Polyacrylamide/ agarose gel electrophoresis showed the molecular weight distribution of the alginates to vary considerably. Although the apparent molecular weight could not be calculated, the migration of the alginates through the gel verified the commercial data that alginates HF and SF had a higher molecular weight that the other samples.

The molecular size distribution values calculated by viscometry, used the 'molecular shape' constant for Hyaluronic acid. This was considered acceptable since both alginate and HA are unbranched polysaccharide molecules.

The average molecular weight of the alginates determined by viscosity, were, in the majority of cases, within 30 kDa of the values supplied by the manufacturer. This was considered reasonable, when the use of the HA

constant, and the inaccuracy and batch variability of the alginates was taken into consideration (personal communication R. Stead).

The molecular weight calculated for alginate SF was considerably less than the value supplied by Protan Ltd. However, the gel permeation and the HPLC elution curves offer an explanation for this irregularity. The elution profiles showed a high level of low molecular weight material (10-50 kDa), which would reduce the average molecular weight. The origin of this material is unlikely to be depolymerisation of large alginate molecules, since it has been reported (McDowell 1977), that sodium alginate (approx Mwt 100 kDa) can be stored at room temperature for 3 years without a detectable change in the size of the alginate molecules. Similarly degradation of the alginate molecules by shear stress during preparation can be eliminated, since the alginate solutions were prepared in an identical manner and other preparations did not show this low Mr fraction. Therefore, it is likely that the low molecular weight material was present in the alginate when obtained from the supplier.

It was found that alginate molecules with a smaller degree of polymerisation were released from the collagen alginate sponges more rapidly than larger molecules. This was expected since smaller molecules can diffuse through the sponge matrix more rapidly. After 4 hours in saline, only low molecular weight alginates were released from the sponges; by 24 and 72 hours, larger molecules were released, but only a small proportion of the total. Analysis of the alginate molecules retained in the sponge showed two elution curves depending on the method used to released the alginate from the sponge. The alginate obtained by pepsin digestion had a lower average molecular weight than that obtained by EDTA extraction. The pepsin extracted alginate was degraded by impurities in the enzyme preparation, leading to the lower apparent molecular weight. Hence, the EDTA extracted alginate was accepted as the most representative.

These results indicate the importance of the size distribution of the alginate molecules in determining their release from sponges. This explains why the amount of alginate released from collagen alginate sponges is not directly proportional to the average molecular weight, but is dependent on the molecular distribution.

Interestingly, sponges prepared from alginate SF (which was shown to contain substantial low molecular weight material), released only small amounts of alginate into solution. The reason that high levels of alginate SF were not released, may been due to other factors, e.g Guluronic acid content, pore size of the sponge and high calcium affinity, increasing the retention of alginate SF within the sponge.

Irradiation of the Fibracol™ sponges reduced the apparent molecular weight of the alginate that was released. In particular, the higher molecular weight molecules from non-irradiated sponges were not detected in the alginate extracted from irradiated samples. There are two possible reasons for this: (1) that the alginate molecules are degraded by the radioactivity, (2) that a change in collagen structure has occurred, enabling more alginate to be eluted from the sponge. It was reported (Bowes and Moss 1962; Bailey and Tromans 1964), that irradiation of collagen fibres in a 'dry' state, resulted in fragmentation, and an increase in intermolecular crosslinking of the collagen fibres. However, the degree of crosslinking was reported as almost undetectable in dry collagen products. Therefore the density of the sponge matrix would decrease due to depolymerisation of the collagen, allowing more alginate molecules to be released from the sponge.

Alternatively, the alginate in the sponges may have been degraded by irradiation, this would result in a lower degree of polymerisation, enabling more alginate to be released from the sponge in the form of smaller molecules. There have been no publications on the effects of irradiation on alginates. However, it has been shown (Kennedy 1991) that HA, which has a

similar structure to alginate, is depolymerised by small doses of irradiation through desaturation of the uronic acid monomers.

CHAPTER 5 GENERAL CONCLUSIONS

The interaction of the anionic polysaccharides with collagen in solution, was dependent on ionic bonding. This is in agreement with published results of the interaction of GAGs with collagen (Obrink 1973a, Obrink *et al* 1975). The composition of the complex formed between the collagen and the polysaccharides was dependent on the pH that the two were mixed, the type of polysaccharide was also shown to effect the ratio collagen:polysaccharide in the coprecipitate. The fact that denatured collagen interacts more effectively with alginate, illustrates the importance of the flexibility of the molecules, in reducing steric inhibition and increasing the interactions.

Several authors (including Obrink *et al*1975, Tolstoguzov 1986), have shown that an electrostatic interaction between a protein and anionic polysaccharide can occur above the IEP of the protein, but does not result in precipitation of the biopolymers. An investigation of a similar effect between collagen and the polysaccharides studied in this thesis, was not performed owing to the insolubility of the PSC above pH 5.

Viscosity measurements suggested that there was an interaction between Guar gum and collagen. Interactions between neutral gums and protein, have been reported, e.g. dextran and gelatin (Tolstoguzov *et al* , 1974). In these examples the aggregation of the protein is either prevented or promoted by the addition of the neutral gum. Circular dichroism studies showed that guar gum destabilised the collagen triple helix, resulting in a lower temperature of melting. The guar was thought to act by preventing the aggregation of the collagen molecules, thereby lowering the energy required to unfold the helix.

The type of alginate used in collagen/alginate sponges, influenced the amount of alginate released into saline solution. The size distribution of the

polysaccharide used in the sponges was important, since smaller molecules were released from the sponges most readily.

The addition of calcium to the sponges was shown to reduce the amount of alginate that was eluted, whilst increasing the concentration of calcium above the normal level, further reduced the amount released. However, it was shown that sponges containing no calcium retained up to 40% of the total alginate, proving that other factors are also important in controlling the release.

The structure of the collagen used in the sponges effected the amount of alginate released, probably due to differences in the collagen matrix of the sponge, allowing different sizes of alginate molecules to be released. The release of polysaccharide from collagen/ galactomannan sponges varied depending on the galactomannan used. Guar was more readily released than LBG. The addition of borate and calcium cations, reduced the amount of polysaccharide released. The fact that calcium was as effective as the borate ion, suggests that the cations are crosslinking the collagen, rather than the galactomannans.

No detectable amount of alginate was released from the collagen/ alginate films, due to the less porous structure. However, other larger polysaccharides, in particular Hyaluronic acid, are released from films prepared in an identical manner (unpublished results in this laboratory). Therefore, calcium ions, which were not present in the collagen/HA films, are likely to be responsible for the retention of the alginate within the film. The calcium was thought to have crosslinked the alginate within the sponge matrix. Whether this was by intermolecular bonding of alginate, collagen, or both, was unclear and would require further investigation.

The structure and physical properties of the sponges and films were not significantly changed by the addition of different polysaccharides. This was expected, since the fibrous bundles of collagen can only interact with the polysaccharides on the outside of the collagen fibre. Therefore, any binding

which occurs will be unlikely to change the structure of the collagen fibre, thus the sponges and films will have similar collagen matrices, and similar physical properties.

The results of this thesis could have a direct effect on the production of Fibracol™ sponges, manufactured by Johnson and Johnson Medical Biopolymer Group. If a wound healing device is designed for external appliance to a wound, then it is undesirable to have migration of material from the device to the wound bed. It has been shown within this work that polysaccharides are released from collagen/ polysaccharide sponges. If it were necessary to prevent the release of all material from the sponges, the manipulation of the calcium concentration, would be the most effective method of achieving this. In addition, the alginate used in the Fibracol™ sponges (LF1060) could be changed to a higher molecular weight alginate (SF or HF), which has a higher retention within the sponge.

Small alginate molecules, are more likely to raise an immunological response, than large molecular weight molecules. It has been shown that the smaller molecules are most readily released from the sponges. Therefore, it could be argued that a source of alginate with minimal amounts of low molecular weight material, would be advantageous.

Although the percentage of guluronic acid in the alginate did not directly affect the amount of alginate released from the sponges, it is important for another reason. Skjakbraek *et al* (1991) have shown that the mannuronic acid residues of alginate are ten times more likely to stimulate cytokine release from human monocytes. These compounds have important roles in the acute inflammatory response in humans. Therefore, it is desirable to use an alginate with a high percentage of guluronic acid residues, in wound healing products. Alginate LF1060, which is used in production, has a high G content (65%), but low molecular weight. It would be better to use alginates SF, or HF, which have the same percentage G residues, but a considerably higher Mwt.

Therefore, although all three alginates would raise a similar inflammatory response, smaller amounts of alginates SF and HF would be released from the sponges, to cause the response.

There are also implications for the use of collagen sponges as a slow release drug carrier. If a drug could be associated with the alginate, then the rate of release from the sponge could be controlled by the choice of alginate, and the calcium concentration. This would be particularly useful when a rapid initial release was required, followed by a prolonged slow release.

The apparent increased viscosity observed between PSC and the galactomannans has no direct bearing on any of the present JJMBG products. However. if any future products use soluble collagen in a thick solution, then it would be possible to achieve a high viscosity with lower solids content by adding guar gum. This could be of particular relevance if an absorbent pad was required, since guar gum can swell and absorb a large volume of water.

BIBLIOGRAPHY

Ahmed, Z.F. and Whistler, R.L. (1950) J.Amer. Chem. Soc. 72, 2524 - 2525.

Asghar, A. and Henrickson. R.L. (1982) Advances in Food Research 28, 231-371.

Atkins, E.D.T., Mackie, W., Parker, W.D. and Smolko, E.E. (1971) J.Polymer Sci. 9, 311 - 316.

Ayad, S., Boot-Handford, R.P., Humphries, M.J., Kadler, K.E. and Shuttleworth, C.A. (1994) Extracellular Matrix Facts Book, Academic Press, New York.

Bailey, A.J. and Tromans, W.J. (1964) Rad. Res. 23, 145 - 155.

Barker, S.A., Basarab, O. and Cruickshank, C.N.D. (1967) Carbohydrate Res. 3, 325 - 632.

Barker, S.A., Cruickshank, C.N.D., Morris, J.H. and Wood, S.R. (1962) Immunology 5, 627-632.

Bekturov, E.A. and Bimendina, L.A. (1977) Interpolymeric complexes, Alma-Ata, Nauka Press.

Binder, R.J., Coit, J.E, Williams, K.T. and Brekke, J.E. (1959) Food Technol. 13, 213 - 216.

Bishop, C.T., Perry, M.B., Blank, F. and Cooper, F.P. (1965) Can. J. Chem. 43, p30-39.

Bitter, T. and Muir, H.M. (1962) Anal. Biochem. 4, 330-334.

Bourquelot, E. and Herrisey, H. (1899) Compt.Rend. 129, 391 - 395.

Bowes, J.H. and Moss., J.A. (1962) Rad. Res. 16, 211 - 223.

Brandt, K.D. (1981) Glycosaminoglycans, Textbook of Rheumatology, ed Kelly, W.N, Harris, E.D., Ruddy, S. and Sledge, C.B. Saunders, Philadelphia. 239 - 254.

Brownsley, G.J., Cairns, P., Miles, M.J. and Morris, V.J. (1987) in Gums and Stabilisers for the Food Industry 4, ed. Phillips, G.O., Wedloch, D.J., and Williams, P.A., IRL press, 157 - 163.

Bungeberg de Jong, H.G. (1936) La coacervates, les coacervates et le importance en biology, Paris. Hermann et cie.

Chvapil, M. (1973) U.S. patent No. 3823212.

Clarke, A.E. and Wilson, I.A., (1988) eds. Carbohydrate protein interaction Heidelberg-Springer Verlag.

Conchie, L.B., Scott, J.E. and Faulk, W.P. (1975) J. Immunol. Meth. 7, 393 - 398.

Dea, I.C.M, Morris, E.R., Rees, D.A., Welsh, E.J., Barnes, H.A. and Price, J. (1977) Carbohydrate Res. 57, 249 - 272.

DeGennes, P.G. (1979) Nature 282, 367 - 375.

Effront, J. (1897) Compt. Rend. 125, 38-40.

Fietzek, P.P., and Kuhn, K. (1976) Int. Rev. Connect. Tiss. Res. 7, 1 - 60.

Fischer, F.G. and Dorfel, H.Z. (1955) Physiol. Chem. 302, 186 - 203.

Frannson, L.A. (1970) Chemistry and Molecular biology of the Intercellular Matrix, vol. 2, ed Balazs, E.D. Academic press, New York. 823-842.

Fraser, R.D., MacRae, T.P. and Miller, A. (1987) J. Mol. Biol. 193, 115 - 121.

Gelman, R.A. and Blackwell, J. (1974) Biochim. Biophys. Acta. 342, 254 - 261.

Gidley, M.J. and Robinson, G. (1990) Methods in Plant Biochem. 2, 607 - 642.

Gilbert, G. and Lyman, D.J. (1990) J. Bio. Mat. Res. 21, 642 - 655.

Grant, M.E. and Jackson, D.S. (1976) Essays in Biochemistry 12, 77 - 113.

Greenwald, R.A., Schwartz, C.E. and Cantor, J.O. (1975) Biochem. J. 145, 601 - 605.

Gross, J. and Kirk, D. (1958) J.Biol. Chem. 233, 355 - 360.

Gurov, A.N. and Tolstoguzov, V.B. (1988) Hydrocolloids 2, 285 - 296.

Gurov, A.N., Larichev, N.A., Krylov, V.I. and Tolstoguzov, V.B. (1978) Studia Biophysica 72, 7 - 9.

Gurov, A.N., Lozinskaya, N.V., Poteshnikh, A.V. and Tolstoguzov, V.B. (1981) J. Dairy Sci. 64, 380 - 383.

Gurov, A.N., Wajermann, E.S. and Tolstoguzov, V.B. (1974) Die Starke 26, 172 - 176.

Gurov, A.N., Wajermann, E.S. and Tolstoguzov, V.B. (1977) Die Starke 29, 186 - 188.

Haug, A., Larsen, B. and Smidsrod, O. (1967) Acta. Chem. Scand. 21, 691 - 704.

Henderson, A. (1987) in Gums and Stabilisers for the Food Industry 4,ed. Phillips, G.O., Wedloch, D.J., and Williams, P.A., IRL press, 265 - 275.

Hermanns, P.H. (1949) J. Polymer. Sci. 4, 135-143.

Hirst, E.L., Jones, J.K.N. and Jones W.O. (1939) J.Chem.Soc. 1880 - 1883.

Hulmes, D.J. (1973) J.Mol. Biol. 79, 137 - 148.

Iverius, P.H. (1972) J. Biol. Chem. 247, 2607 - 2613.

Kennedy, J. F. (1991) Product literature, Fermentech Ltd.

Kuhn, K. and Glanville, R.W. (1980) Biology of Collagen, ed. Viidik, A., and Vuust, J., Academic Press, London 1-14.

Laemmli, U.K. (1970) Nature 227, 680 - 685.

Lemieux, R.U. (1988) Carbohydr. Res. 178, 293 - 297.

Lepivert, D., Huc, A. and Gimero, R. (1985) European Patent 153896.

Lowther, D.A. and Natarajan, M. (1972) Biochem. J. 127, 607- 608.

Lowther, D.A., Toole, B.P. and Herrington, P. (1970) Chemistry and Molecular Biology of the Intercellular Matrix. Vol., 2, ed Balazs, E.D. Academic press, New York. 1135 - 1153.

Mathews, M.B. and Decker, L. (1968) Biochem. J. 109, 517 - 526.

Mathews, M.B. (1965) Biochem. J. 96, 710 - 716.

McCormick, D., van der Rest, M., Goodship, J., Lozano, G., Nimomiya, Y. and Olsen, B.R. (1987) Proc. Natl. Acad. Sci. USA. 84, 4044 - 4048.

McDowell, R.H (1977)Properties of Alginates, Alginate Industries Limited.

Miller, E.J. (1971) Biochemistry 10, 1652 - 1659.

Miller, E.J. (1976) Molec. Cell. Biol. 13, 165 - 192.

Morris, E.D. and Welsh, E.J. (1982)Carbohydrates-A Unilever Educational booklet.

Morris, E.R., Rees, D.A. and Thom, D. (1973) J.Chem.Soc. Chem. Comm. 245 - 249.

Na, G.C. (1988) Coll. Rel. Res. 81. 315 - 318.

Nelson, W.L. and Gretcher, L.H. (1930) J.Amer.Chem.Soc. 52, 2130 - 2136.

Noguchi, H. (1960) J. Phys. Chem. 64, 185 - 191.

Obrink, B. (1973a) Eur. J. Biochem. 33, 387 - 137.

Obrink, B. (1973b). Eur. J. Biochem. 34, 129 - 137.

Obrink, B. and Sundelof, L.C. (1973) Eur. J. Biochem. 37, 226 - 232.

Obrink, B. and Wasteson, A. (1971) Biochem. J. 121, 227 - 233.

Obrink, B., Laurent, T.C. and Carlsson, B. (1975) FEBS Letters 56, 166 - 169.

Oegema, T.R., Laidlaw, J., Hascall, V.C. and Dziewiatkowski, D.D., (1975) Arch. Biochem. Biophys. 170, 698 - 709.

Okada, K. (1989) U.S. patent 4880654.

Penman, A. and Sanderson, G.R. (1972) Carbohydr. Res. 25, 273 - 282.

Piez, K.A. (1976) Biochemistry of collagens, Eds Ramachandran, G.N., and Reddi, A.H., Plenum Press New York. 1 - 44.

Piez, K.A., Eigner, E.A. and Lewis, M.S. (1963) Biochemistry 2, 58 - 66.

Pittet, A.O. (1965) Methods Carbohyd. Chem., 5, 3-9.

Poole, S, West, S.I. and Walters, C.L. (1984) J. Sci. Fd. Agric., 35, 701 - 711.

Poole, S. (1988) Leatherhead Fd. R.A. Tech notes No 74.

Quinocho, F.A. (1986) Ann. Rev. Biochem. 55, 287 - 315.

Raghunath, K., Biswas, G., Panduranga Rao, K. and Joseph, K.T. (1983) J. Biomed. Mat. Res. 17, 613 - 621.

Rees, D.A. (1969) Adv. Carbohydrate Chem, Biochem. 24, 267-274.

Rees, D.A. (1972) Biochem J. 126, 257 - 273.

Richardson, I. (1988) Meat manufacturing, October 14 - 16

Rubin, A.L. and Stenzel, K.H. (1969) in Biomaterials, ed. L.Stark and G. Agerwal. Plenum, New York. 157- 184.

Sajdera, S.W. and Hascalls, V.C. (1969) J. Biol. Chem. 244, 77 - 87.

Sakaguchi, O., Yokota, K. and Suzuki., M. (1969) Jap. J. Microbiol. 13, p1

Scott, J.E. (1980) Biochem. J. 187, 887-891.

Scott, J.E., Orford, C.R. and Hughes, E.W., 1981, Biochem. J. 195, 573 - 581.

Sharon, N, and Lis, H. (1990) Chem in Britain 679 - 682.

Skjakbraek, G., Smidsrod, O., Otterleim, M., Soons Hiong, P. and Espevik, T. (1991) Patent No. WO 9111205.

Snowden, J.M. and Swann, D.A. (1980) Biopolymers 19, 767 - 780.

Stanford E.C.C. (1883)J. Soc. Chem. Ind. 44, 943-946.

Thomson, T.E. and McKernan, W.M. (1961) Biochem J. 81, 12 - 16.

Tiebacks, F.W. (1910) Kolloid-Z 8, 198- 201.

Tokaev, E.S., Gurov, A.N., Rogov, I.A. and Tolstoguzov, V.B. (1987) Die Nahrung 31, 825 -834.

Tolstoguzov, V.B. (1986) Funct. Prop. Food 12, 387-412.

Tolstoguzov, V.B. and Wajnermann, E.S. (1975) Die Nahrung 19, 45 - 60.

Tolstoguzov, V.B., Belkina, V.P., Gulov, V.Ja., Gringberg, V., Titova, E.F. and Belavzeva, E.M. (1974) Die Starke 26, 130 - 138.

Toole, B.P. (1976) J. Biol. Chem. 251, 895 - 897.

Toole, B.P. (1982) Connect. Tiss. Res. 10, 93 - 100.

Toole, B.P. and Lowther, D.A. (1968) Arch. Biochem. Biophys. 128, 567 - 578.

Van der Rest, M. and Garrone, R. (1992) FASEB. J. 5, 2814 - 2823.

Vandevelde, M.C. and Fenyo, J.C. (1985) Carbohydrate polymers 5, 251 - 273.

Wajermann, E.S., Grinberg, V., and Tolstoguzov, V.B. (1972) Kolloid-Z polymer 250. 945 - 951.

Wang, C.I, Miyata, T., Weksler, B., Rubin, A.L., and Stenzel, K.H. (1978) Biochim. Biophys. Acta. 544, 568 - 577.

Wen-Sherng, C., Henry, G.A., Gaud, S.M., Miller, M.S., Kaiser, J.M. and Balmadeca, E. (1989) European Patent No. 0340035.

Whistler, R.L. and Stein, J.Z. (1951) J. Amer. Chem. Soc, 73, 4187 - 4188.

Wiedmann, H., Paulson, M., Timpl, R., and Engel, J. (1984) Biochem J. 224, p331 - 333.

Wood, G.C. and Keech. M.K. (1960)Biochem. J. 75, 588 - 598.

Zenzin, A.B., and Rogatsheva, V.B. (1973) Adv. Chem. and Phys. Polymer. ed Z.A. Rogavin, Moscow. 114- 153.