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Characterisation of high molecular weight glycoconjugates from conjunctival mucus

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CHARACTERISATION OF HIGH MOLECULAR WEIGHT GLYCOCONJUGATES FROM CONJUNCTIVAL MUCUS

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A thesis submitted in partial fulfilment of the requirements for admission to the degree

of

Doctor of Philosophy

School of Optometry and Vision Science, University of New South Wales, Sydney,

Australia

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Malcolm Ball, February 2002

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ABSTRACT

Due to constant exposure to the environment, the ocular surface requires continuous protection. A multi-component layer known as the tear film provides protection from bacteriological invasion and particulate damage, as well as providing an optically clear surface. The tear film contains not only aqueous proteins but also lipids and a mucus layer in direct contact with the epithelium. When the mucus layer of the tear film is interfered with in some way the stability of the tear film is compromised and it subsequently collapses. Mucus layers on other epithelial surfaces have been shown to be predominantly composed of a high molecular weight, heavily glycosylated class of proteins, known as mucins. However, as the conditions in the eye vary considerably from those of the gut or lung, it is conjectured that the mucus layer of the tear film may contain modified or otherwise different proteins from those found in other tissues. As all mucins are difficult to purify and study, new methods of purification, reducing the amount of losses, and analysis were developed to aid in the elucidation of composition of mucin-like glycoproteins found in the bovine conjunctiva. These methods were used to analyse the high molecular weight glycoconjugate fraction of bovine conjunctival mucus and human tears. Analysis of these fractions by amino acid and monosaccharide composition, ion exchange chromatography, protease digestion, and mass spectrometry have indicated that mucin-like glycoproteins may not be the major glycoconjugates of ocular mucus. Rather the predominant species of ocular mucus may be proteoglycan or proteoglycan-like glycoconjugates.

CHAPTER 1: INTRODUCTION

Due to constant exposure to the environment, the ocular surface, comprising the conjunctiva and cornea, requires continuous protection. A multi-component layer known as the tear film provides this protection. The tear film contains not only aqueous proteins but also a lipid component in contact with the air interface and a mucus layer in direct contact with the epithelium. A stable and structured tear film is required to protect the surface epithelium from bacteriological invasion and particulate damage, as well as providing an optically clear surface.

Mucus layers on other epithelial surfaces have been shown to be predominantly composed of a high molecular weight and a heavily glycosylated class of proteins known as mucins. However, as the conditions in the eye vary considerably from those of the gut or lung, it is conjectured that the mucus layer of the tear film may contain modified or otherwise different high molecular weight proteins from those found in other tissues. When the mucus layer of the tear film is disrupted, for example, by the insertion of a contact lens, the stability of the tear film is compromised and it subsequently collapses. In order to produce contact lenses that can be worn for extended periods of time (up to months), and for general understanding of the function of the epithelial surface of the eye, it is necessary to understand the tear film and its interactions.

This project has focussed on the mucus layer of the tear film and specifically the high molecular weight glycoconjugates. High molecular weight glycoconjugates are difficult to purify and analyse using traditional protein techniques such as high pressure (performance) liquid chromatography (HPLC) and polyacrylamide gel electrophoresis

(PAGE). Those methods that are most commonly used for mucin-like glycoproteins result in large losses. By developing new methods of purification and analysis, or modifying those previously described for mucin glycoproteins, it was possible to determine the composition of the high molecular weight components of the mucus layer of the bovine conjunctival tear film.

Initial studies into the physical properties of the mucus layer indicated that a major component of the tear film might not have been the mucins that have been previously reported. Compositional analysis of the whole high molecular weight glycoconjugate fraction supports this hypothesis. Further analysis of the high molecular weight fraction, by separation into species and analysis of glycopeptides and oligosaccharide fractionation, has indicated that, while some mucin species are present, the major component of the tear film mucus layer is a proteoglycan-like glycoconjugate.

1.1 TEAR FILM STRUCTURE AND FUNCTION

1.1.1 Tear Film Function

The tear film of a healthy eye has many functions. Firstly, it keeps the ocular epithelium moist and allows for gas transfer from the air (Sweeney, Holden 1987). The epithelium and lens of the eye have no vascularisation and, thus, the gas transfer (oxygen in, carbon dioxide out) is by direct transfer with the environment. Secondly, the tear film provides a smooth ocular surface to allow for clear vision. The outer surface of the ocular globe has two regions; the conjunctiva - a layer of tissue that overlies the white tissue outside the

optical pathway, and the cornea - the transparent tissue covering the pupil and iris (Figure 1.1).

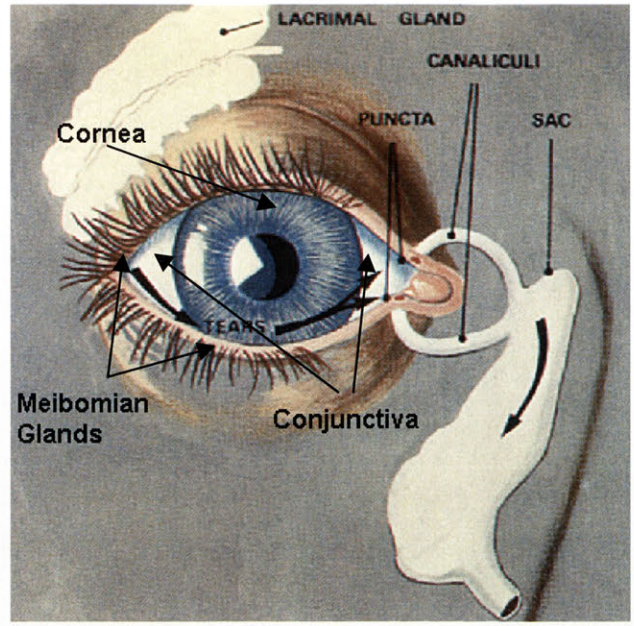

Figure 1.1: *Diagrammatic representation of the eye including the source of tear film components. Goblet cells in the conjunctiva and epithelial cells in both the conjunctiva and cornea secrete the mucus layer. The lacrimal gland produces the aqueous component, and meibomian glands at the edge of the eyelids supply the lipids. The components from these three sources are combined to form the tear film on the surface of the eyeball (Image Courtesy of CRCERT, UNSW Sydney Australia)*

Both of these surfaces have epithelial cells as their outermost layer. The surface epithelium is not smooth due to cell junctions, microvilli and cell surface proteins. This "micro topography" is sufficiently large to diffuse and diffract light, therefore, a liquid layer covers the epithelium effectively making the surface of the eye smooth with a single co-efficient of diffraction which is allowed for by the mechanics of the eye (Boonstra *et al.* 1985). Thirdly, the tear film is the outer barrier of the eye against the environment. It provides protection from microbial invasion and physical debris. The components and structure of the film are designed to neutralise invaders and lubricate or wash away any particulate matter that could damage the cells of the corneal and conjunctival surface (McMaster *et al.* 1999; Hemsley *et al.* 2000).

1.1.2 Tear Film Structure and Components

Historically, the tear film has been said to have a three-layered structure, consisting of a very thin lipid layer exposed to the air on top of an aqueous layer containing most of the dissolved proteins and finally a mucus layer that sits on the epithelial surface (Holly and Lemp 1971). The entire structure was believed to be only quite small (in the order of 10-15 μm) of which the aqueous layer was the largest component (Van Haeringen 1981). However, more recent work has shown that the tear film has a more complex structure and relationship of the layers (Prydal *et al.* 1992). Figure 1.2 depicts the old and current models of the tear film.

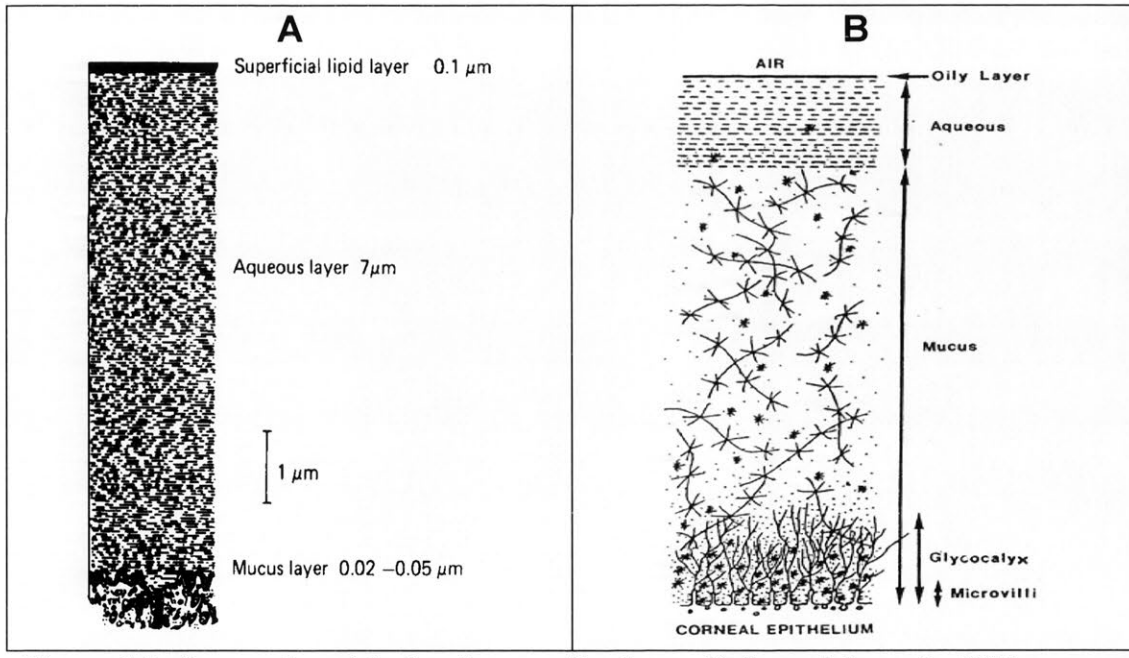

Figure 1.2: *Proposed models for the tear film layer. Holly and Lemp in 1971 proposed a three-layered structure for the tear film: (**A**) This structure involved a thin mucus layer covering the surface epithelium which, in turn, was covered by a relatively thick aqueous component. Finally a lipid layer covered the tear film and presented to the air interface. In total, the tear film was proposed as being only 7-10 μm in length. However, in 1992 Prydal et al. proposed a thicker tear film that was more of a continuum rather than three defined layers. (**B**) The mucus layer is very dense when next to the glycocalyx, becoming less thick as it moves away from the surface epithelium. The space is taken up by the aqueous components of the tear film until the point that there is no mucus gel. A small aqueous layer covers the surface of the gel with a thin lipid layer presented to the air interface. In this model the tear film is between 30 and 50 μm. Interestingly, in both models, the aqueous layer is approximately 7 μm. Accurate measurements of the tear film are difficult as it is hard to detect the epithelial cell/mucus layer boundary*

Slam freezing, a process whereby excised tissue is placed against a super-cooled copper mirror freezing the tear film *in situ*, has allowed a more detailed study of the mucus layer. The research has shown that, in rats, this layer is thicker than originally thought (in the order of 10 μm) (Nichols *et al.* 1985). This technique has also allowed analysis of the tear film structure. Molecules, which could be components of a glycocalyx, have been visualised by electron microscopy (Pfister 1975, Figure 1.3) suggesting a more structured relationship between the tear film and surface epithelium than originally thought.

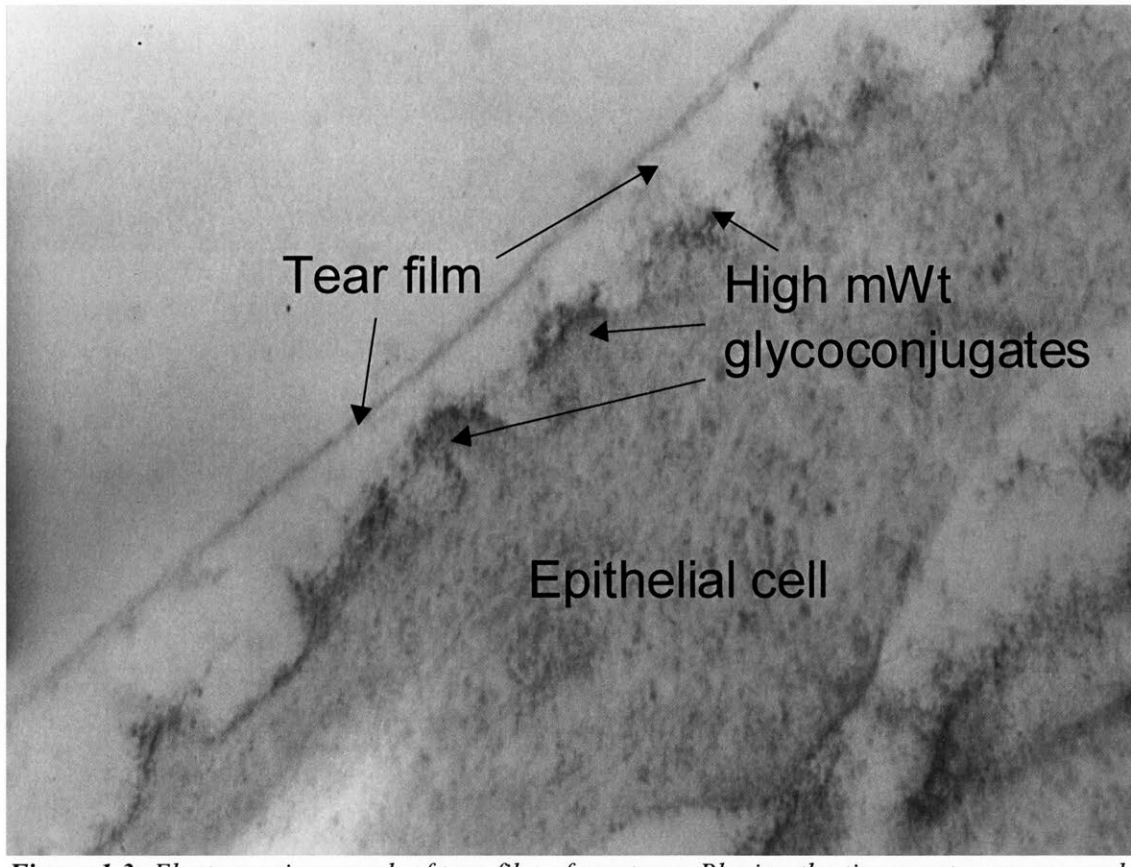

Figure 1.3: *Electron micrograph of tear film of a rat eye. Placing the tissue onto a super-cooled copper mirror (a process known as slam freezing) froze the eye. Clearly visible on the surface of the epithelial cells are large glycoconjugate molecules, suggesting that a glycocalyx may be present (photograph supplied by CRCERT)*

Additionally, Anderton and Tragoulias (1998) have shown, by electrical impedance, that the tear film of a rat is between 20-40 μm thick. However, King-Smith *et al.* (2000) report a tear film layer of only 3 μm. The method of analysis described in this paper involved changes in refractive index. They claim to observe a large refractive index

change from the lipid layer followed by several smaller changes that they attribute to epithelium and the basement membrane. However it is possible that a larger refractive change would occur between the aqueous layer of the tear film and the mucus layer, than the mucus layer and the ocular epithelium. Therefore, it is most likely that these measurements are of the free aqueous component of the tear film. This evidence suggests a continuum rather than three layers. A thin lipid/protein layer exists at the air interface (Mathers *et al.* 1997). This layer most likely acts to inhibit evaporation of the tear layer (Craig and Tomlinson 1997) and consists mainly of cholesterol esters with some phospholipids (about 15%) (Tiffany 1985) and stabilising proteins. A reasonably thin aqueous component which contains the dissolved proteins such as lysozyme, lactoferrin and IgA, leads into an increasingly thick mucus matrix that then binds to the glycocalyx of the epithelial cells (Prydal and Campbell 1992) (Figure 1.2B).

The mucus layer of the eye is secreted primarily from goblet cells in the conjunctiva and the squamous cells of the corneal epithelium (Bjerrum *et al.* 1991). The aqueous component of the tear film is secreted by the lacrimal gland located in the corner of the eye (the tear duct) (Chao *et al.* 1987), and the lipid component is secreted from the meibomian glands found on the leading edge of the eye lids (Tiffany 1985).

1.1.3 Effects of Contact Lens Wear on the Tear Film

When a contact lens is placed into the eye it causes a disruption on many levels. When the lens is placed into the eye it possibly separates the mucus layer from the aqueous and lipid layers with a physical barrier (Sharma and Ruckenstein 1985). Liquid tears are still able to pass behind the lens (as determined by fluorescein visualisation) (McNamara

et al. 1999). However the air interface/continuum structure is disrupted causing the tear film to dry at an accelerated rate (Figure 1.4).

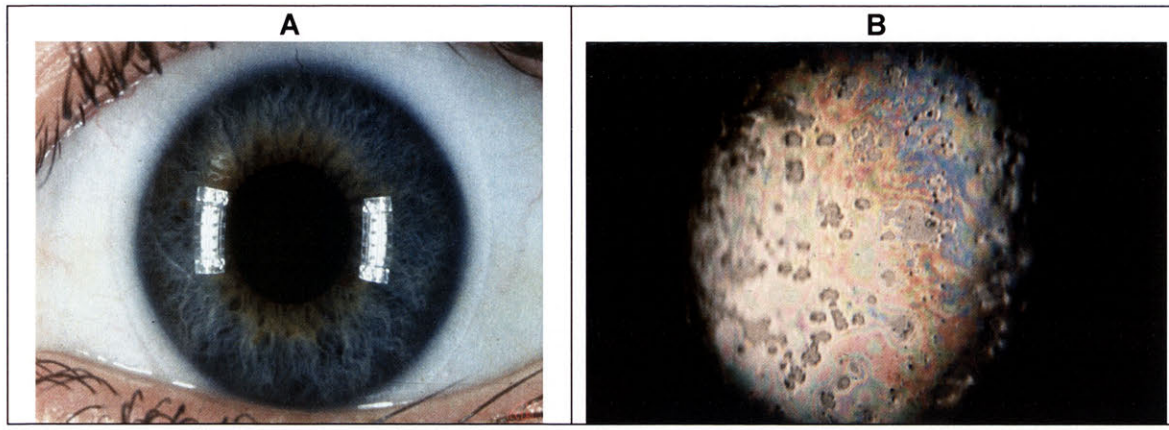

Figure 1.4: *Effect on the tear film of contact lens wear. Upon insertion of a contact lens, a tear film is evident both between the eye and the lens and the lens and the air interface [**A**]. However the tear film destabilises and breaks up after only a few seconds [**B**]. In contrast the cornea can keep a stable tear film for 20-30 seconds. This suggests a structured relationship between the tear film components (photographs courtesy of CRCERT)*

It has also been observed that the total mucus in the tears is reduced when a contact lens is placed in the eye (Bode and Lam 1986). A thinning of both the pre-lens (between the cornea and the lens) and post-lens (between the lens and the air interface) tear films is also observed (Lin *et al.* 1999; Creech *et al.* 1998). The material which makes up the lens is also recognised as a foreign body. Protein deposition on lenses is a serious problem, with the lenses requiring regular cleaning for comfortable use (Pritchard *et al.* 2000). Deposition is caused by the surface energy state of the lens being such that it is energetically favourable for the proteins to denature and layer onto the surface (Kita *et al.* 1994). This is a passive process. However, there may also be an active process in effect. One of the functions of the proteins in the lens (particularly tear-specific pre-albumin or lipocalin, lysozyme and possibly mucin) is to render foreign bodies harmless by binding, to allow them to be washed out by a blink (Fullard and Kissner 1991; Moore and Tiffany 1981; Gachon *et al.* 1986). This may be partially what occurs with the contact lens being coated to prevent damage to the epithelial surface and lubricated for ease of removal from

the eye. Circumstantial evidence to support this is the fact that mucus layer components are some of the first proteins to deposit onto a lens and make up a large proportion of the total protein bound to a lens (Kaluzny and Szatkowski 1998). By adsorbing large amounts of protein in this way, the lens may also disrupt the tear film. A healthy, unaltered tear film will take up to 20-30 seconds to dry to the point of film breakdown (Chao *et al.* 1983A), however, when a contact lens is placed on the eye, the break-up time is more in the order of 6-8 seconds (Morris *et al.* 1998). Contact lenses have also been shown to cause an inflammatory response in the eye (Thakur and Willcox 2000), and have been linked to damage of the eye surface (Itoh *et al.* 1999) and discomfort (Korb 1994) due to loss of tear film integrity.

One possible way to develop a contact lens which does not disrupt the tear film is to coat it with moieties that cause the tear film to treat it as though it were part of the corneal surface. The mucus layer of the tear film is considered by many to be the stabilising factor. It has been shown that disrupting the mucus layer causes the tear film to collapse (Saso *et al.* 1999A), and that placing mucus on a contact lens increases the non-invasive break-up time (NIBUT) *in vitro* (Fatt 1991). It follows then that the mucus layer could be the source of the component required to create a non-fouling, tear film-stabilising contact lens. In order to successfully mimic something as complex as a mucus layer, it is first necessary to understand the components that are present. The major components of secretions that form matrices (such as a mucus gel layer or cartilage and stroma) have been shown to be high molecular weight glycoconjugates, such as mucins.

1.1.4 Species Variation of Tear Film Composition

Little work has been performed on the variation of tear film composition in the animal kingdom. However, the small amount of work that has been reported has provided some interesting information. Marine mammals have a tear film consisting almost exclusively of mucus (Tarpley and Ridgeway 1991). Dolphins have no lipid producing glands and produce only one tenth of the aqueous secretions of humans. In addition, dolphins produce between 1 and 5 ml of tears per minute. Pinnipeds have a tear film which is stable in both water and air. When the seal hunts underwater it has its eyes open. Upon emerging from the water a stable tear film is visible without a blink on the part of the seal. Anderton and Tragoulias (1998) have shown that the rat tear film is thicker than even the most generous estimates for humans (approximately 70 μm as opposed to 30-50 μm for humans). In addition, it is difficult to disrupt the tear film of rats by physical means (Anderton and Tragoulias 1998). When a thread is placed into the tear film layer of a rat's eye it gouges a channel that remains for some seconds before being filled in. Interestingly, what little chemical analysis of tear film components has been performed indicates that the composition of animal and human tear films may be comparable (Tseng *et al.* 1987).

1.2 HIGH MOLECULAR WEIGHT GLYCOCONJUGATES

1.2.1 Mucins

1.2.1.1 Functions of Mucins in the Body

Mucus gels are present on most of the epithelial surfaces that are exposed to the environment (Strous and Dekker 1992). Mucin glycoproteins are the major component of mucus so far investigated (accounting for greater than 40% of the total protein present) (Perez-Vilar and Hill 1999), the other components being serous and aqueous proteins and some lipids. Mucins are the component of the mucus layer that is believed responsible for its gel-like characteristics (Thornton *et al.* 1997).

The mucus layer provides protection for the epithelial layer in many ways. In the stomach and intestines it provides a lubricated "buffer zone" for food to travel down. It also protects the cells from hydrolysis by the strong acid of the stomach and the alkali of the intestines (Turner *et al.* 1999). It provides a barrier against bacterial or amoebic invasion of the intestinal wall and possibly provides a solid support for the many enzymes that function in this area (Loomes *et al.* 1999). Protection against pathogenic invasion is also one of the major functions of tracheobronchial mucins, as well as a buffer zone to allow the removal of dust and smoke from the lungs (Thornton *et al.* 1996). By coughing the mucus layer out of the lungs and down the oesophagus, particulate matter can be cleared at regular intervals (Dwyer and Farley 2000). It is interesting to note that one of the major detrimental effects of smoking is the degradation of the mucus layer (Bhaskar

et al. 1985). The initial line of defence in the airway is the mucus layer that covers the nasal passages. Mucins are also present in the bladder to protect the cells from the high levels of waste chemicals present (Miquel *et al.* 1995; Ruggieri *et al.* 1992). The oviductal tract of humans uses mucus layers for lubrication and protection from pathogenic invasion (Gipson *et al.* 1995). Mucus is also used by other species such as fish as a part of the egg coat to provide protection from the environment (Arranz *et al.* 1997). The function of mucus in the eyes is somewhat more delicate as, not only does it have to provide protection against the environment but, the tear film must be optically transparent for proper function of vision (Glasson *et al.* 1998). Many cases of disease or dysfunction of epithelial tissues (such as in dry eye, carcinoma or ulcers) result in loss or alteration of mucin species. In diseases of other mucus producing tissues such as the gut or respiratory system the mucins can be truncated (Cornberg *et al.* 1999) or glycosylation on the amino acid backbone altered (Devine and McKenzie 1992; Yu *et al.* 1996).

1.2.1.2 Mucin Structural and Physical Characteristics

Much of the analysis of mucins has relied on genomic work in humans and antibody detection. In humans, 10 genes have been definitely characterised (MUC-1 to MUC-9 including MUC-3A and MUC-3B, and MUC-5AC and MUC-5B) (Van Klinken *et al.* 1995; Labat *et al.* 1999; Lagow *et al.* 1999), with three further gene products proposed as mucin molecules (Jumblatt *et al.* 2000). These mucins can be separated into three classes – membrane bound (MUC-1, MUC-3 and MUC-4), gel forming (MUC-2, MUC-5AC and MUC-6) and non-gel forming secreted (MUC-3, MUC-7 and MUC-8) (Gum 1995; Porchet *et al.* 1995).

Figure 1.5 demonstrates the characteristics of these three classes. Some mucin species can have multiple forms. MUC-1, for example, exists as membrane bound and secretory types (Hanisch *et al.* 1996).

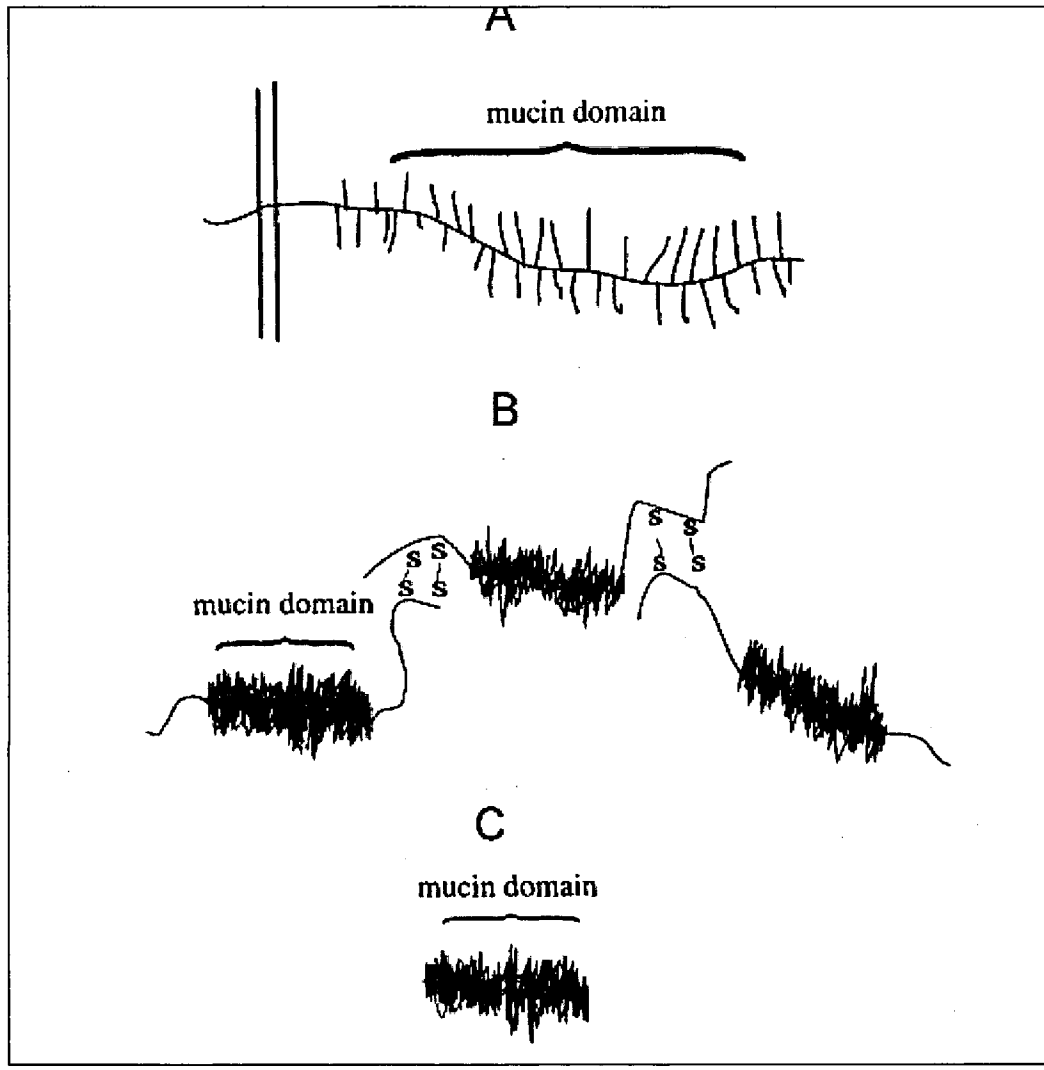

Figure 1.5: *Graphical representations of the three classes of mucins. Membrane bound mucins (such as MUC-1 and MUC-4) [A] have a membrane spanning sequence at the C-terminus and a small "naked" region at the N-terminus with a large "spacer" mucin domain. The gel forming mucins (such as MUC-2,MUC-5B, MUC-5AC and MUC-6) [**B**] have one or more large glycosylated "mucin" regions, which are flanked by "naked" regions. These regions contain cysteine rich D domains similar to Von-Willenbrand factor, which enable the sub-units to form large polymers by S-S bonding. Finally the non-gel forming mucins (such as MUC-7 and MUC-3) [**C**] are merely a large glycosylated "mucin-like" region (taken from Karlsson 1997)*

MUC-1 is believed to be almost ubiquitous on epithelial surfaces, whereas MUC-4, the other membrane bound mucin, has so far only been found in the lung, eye and gastro-intestinal tract (Gum *et al.* 1992). The larger gel forming mucins

occur in the colon (MUC-2, MUC-6, MUC-5B) (Toribara *et al.* 1993), the intestinal tract (MUC-2, MUC-5AC), airways (MUC-2, MUC-5AC, MUC-5B) and gall bladder (MUC-5B, MUC-6) (Jany *et al.* 1991). The smaller non-gel forming mucins are found in the small intestine and gall bladder (MUC-3) (Fox *et al.* 1992), saliva (MUC-7) (Mack and Hollingsworth 1994) and lungs (MUC-8) (Carlstedt *et al.* 1995; O'Hara *et al.* 1994). Many of the mucin genes so far characterised have been shown to be expressed in multiple tissues (Gum *et al.* 1992), meaning that the amino acid backbones of these mucins are similar. However there is evidence to suggest that the glycosylation that is attached to the amino acid backbones is tissue specific (Brockhausen 1999). As it is widely believed that the sugar component of the mucin is largely responsible for it's characteristics, it would be fair to treat each of these mucins as a separate glycoprotein with different functions.

The mucin glycoproteins which have so far been characterised have a defined general structure that can be applied to all species, with some variation due to their role. All mucins have large glycosylated regions, many of which contain "tandem repeat" sequences (several amino acid residues repeated next to each other) (Seregni *et al.* 1997). The number of repeats may vary within mucin species. MUC-1, for example, has a different number of repeats in its secreted and membrane bound forms (Baeckstrom *et al.* 1994). Table 1.1 lists the known tandem repeat sequences so far characterised.

Table 1.1: *Some known mucin species and their tandem repeat sequences (taken from Cooper 1999)*

Mucin Glycoprotein	Species	Tandem Repeat Sequence
MUC-1	Human	PDTRPAPGSTAPPAHGVTSA
MUC-2	Human	PTTTPITTTTVTPTPTPTGTQT
MUC-3	Human	HSTPSFTSSITTTETTS
MUC-4	Human	TSSASTGHATPLPVTD
MUC-5AC	Human	TTSTTSAP
MUC-5B	Human	SSTPGTAHTLTVLTTTATTPTATGSTATP
MUC-6	Human	SPFSSTGP..(153aa)...THSPPTGS
MUC-7	Human	TTAAPPTPSATTPAPPSSSAPPG
MUC-8	Human	TSCPRPLQEGTRV and TSCPRLPQEGTPGSRAAHALSRRGHRVHELPTSSPGGDT
MUC-9	Human	GX(K/M)(T/A)(M/L)T(S/P)VG(H/Y)QS(V/M)TP
RSMG	Rat	PTTDSTTPAPTTK
Muc-3	Rat	TTPPDV
PSM	Pig	GAGPGTTAS..(66aa)..QAAGTS
BSM	Cow	GTTVAPGSSNTGTTVAPGSSNTGTTVAPGSSNT
MUA-1	Frog	TTTVPTIPE
MUB-1	Frog	GESTPAPSETT
Muc-1	Gibbon	PVTRPAPGSTTSPAQDVTS
MUC-1	Frog	APTTAAATT
MLP	Rat	PSTPSTPPPST

If the mucin is membrane bound then the molecule will have a small "naked" region at the N-terminus and a transmembrane domain at the C-terminus (Figure 1.5A). The gel forming mucins may have more than one highly glycosylated domain joined by lightly glycosylated or "naked" regions. The naked regions on each end of a gel forming mucin contain cysteine rich "N" and "C" domains (Figure 1.5B) that are responsible for the formation of disulfide bridges with other sub-units to form polymeric gel structures (Thornton *et al.* 1991; Perez-Viler *et al.* 1996; Carlstedt *et al.* 1993). There may also be a region of the mucin that is relatively highly glycosylated but does not contain a tandem repeat sequence (Herrmann *et al.* 1999). The non-gel forming mucins that are secreted have no cysteine rich domains in their naked regions and are effectively a "mucin-like" domain only (Karlsson 1997) (Figure 1.5C).

The oligosaccharides attached to mucins are mainly di- or tri-saccharides attached through O-linkages to serine (Ser) and threonine (Thr). However, several larger structures have been characterised (van Halbeek *et al.* 1994; Ropp *et al.* 1991). Some structures which have been characterised are shown in Figure 1.6.

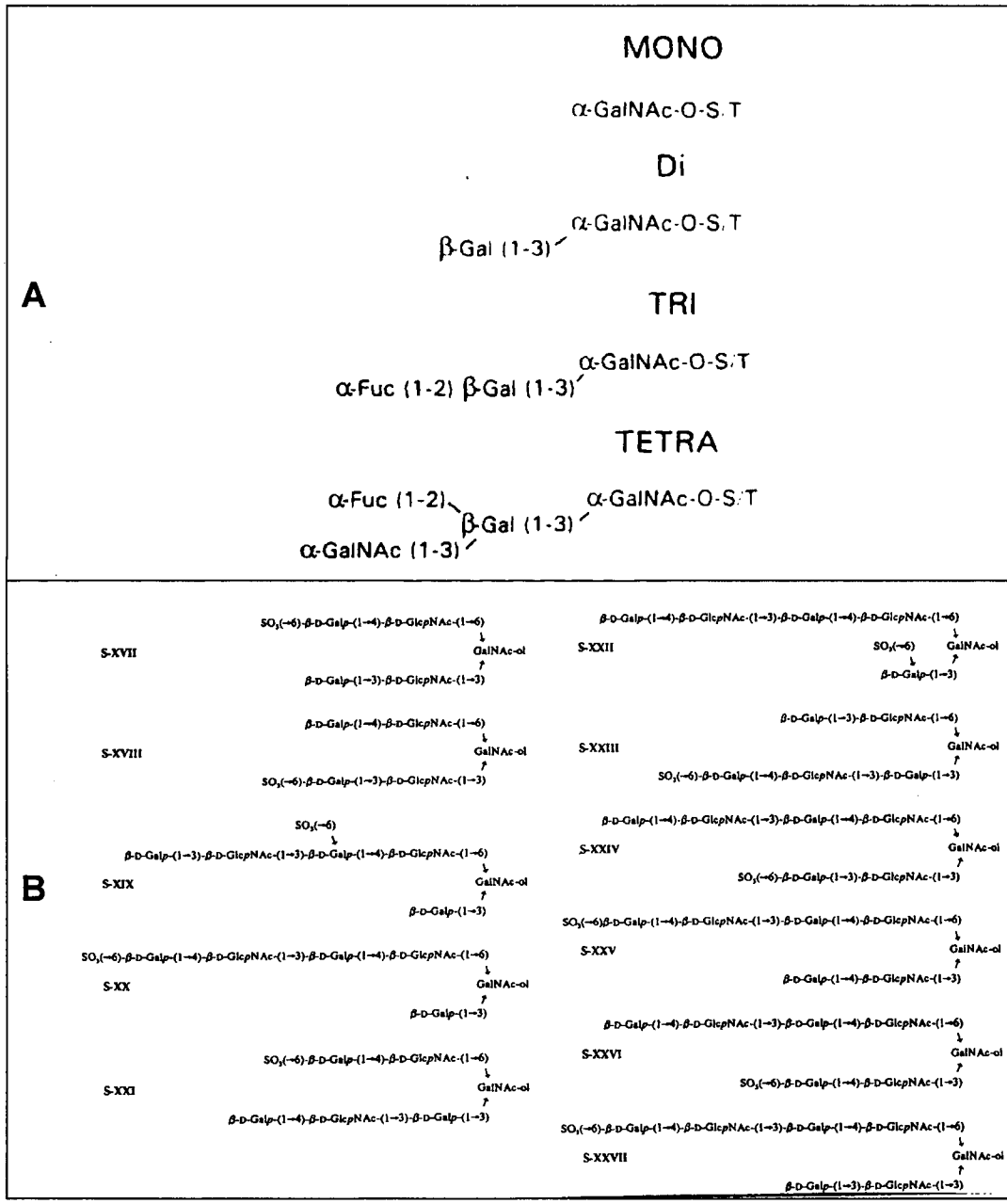

Figure 1.6: *Examples of the glycosylation present on mucin glycoproteins. Listed are the small acidic oligosaccharides described by Gerken et al. (1998) for porcine sub-maxillary mucins [A] and some of the large sulfated mucins found on human tracheobronchial mucins by Mawhinney et al. in 1992 [B]*

Many mucins have a high ratio of sialic acid to neutral sugar (being as high as 0.9:1 sialic:GalNAc) making them highly hydrophilic and having a large net negative charge (Padhye *et al.* 1991). However, some mucins, such as rat gastric mucin, have very little sialic acid and appear to remain charged due to sulfate moieties (van Beurdan-Lamers *et al.* 1989). There are only a few sites in most of the mucins that may have N-linked oligosaccharide attachments (see Section 1.3.1) and not all of these are utilised. In one mucin at least (MUC-2) the N-linked oligosaccharides have been shown to be important in the formation of the gel matrix (Van Klinken *et al.* 1998). In some cases there are no N-linked sugars attached to the mucin molecule (Bertolini and Pigman 1969).

1.2.2 Proteoglycans

1.2.2.1 Location and Functions of Proteoglycans

A proteoglycan is defined as a glycoprotein, which carries a post-translational modification of glycosaminoglycan (GaG) oligosaccharides attached to the amino acid backbone (McArthur *et al.* 2000). Some of the proteoglycans well characterised are Fibril Associated Collagens with Interrupted Triple helices (FACIT), collagens (Types IX, XII and XIV), decorins, fibromodulins and aggrecans (Schwartz 2000). Proteoglycans are present in the extracellular matrixes of the body, such as cartilage and stroma (Carney *et al.* 1986) and on the surface of cells as part of the glycocalyx (Yamagata and Kamada 1999). They have also been shown to be present in the cell walls of plants (Braam 1999). Some groups have suggested that they may play a role in the formation of mucus layers (Sheehan *et al.* 1995). The functions of proteoglycans so far characterised

either relate to the formation or stabilisation of extracellular matrices (Barry *et al.* 1992) or to signalling pathways (Dobra *et al.* 2000). Aggrecans bind with hyaluronic acid and a link protein in cartilage to form a stable matrix. This matrix is then hydrated to give cartilage its shock absorbing characteristics (i.e. cartilage has the ability to resist compressive stress) (Iozzo 1998). The class of proteoglycans, known as the small leucine-rich proteoglycans, bind to collagen Type I and II fibrils, thus stabilising them into a matrix (Hobby *et al.* 2000). The GaGs attached to proteoglycans have also been shown to inhibit proteases *in vitro*. Proteoglycans are also involved in the transfer of signal molecules, such as non-peptide hormones, and their subsequent degradation (Prydz and Dalen 2000).

1.2.2.2 Proteoglycan Structural and Physical Characteristics

The definition of a proteoglycan is quite general and, as such, the proteins that fall into this class have varied structures. Over 40 full-length gene sequences have been so far characterised with many other partial sequences (Schwartz 2000). The proteoglycans described fall into four major families (however there are several proteoglycans which do not fall into these groups) (Iozzo 1998). The aggrecan gene family consists of four known proteoglycans - aggrecan, versican, neurocan and brevican (Yamaguchi 2000). These proteoglycans are typical of the larger matrix-forming group. They consist of areas of heavy carbohydrate attachment flanked by "naked" regions that serve as areas of binding to form polymers or attachment to other components in the ECM (Iozzo 1998). These proteoglycans resemble the gel forming mucins in many ways, having a large variable length repeat region that is heavily glycosylated. There are two groups of cell

membrane-associated proteoglycans, the heavily glycosylated of which syndecan and serglycin are members (Ethell *et al.* 2000), and the lightly glycosylated such as perlecan (Friedrich *et al.* 2000). The heavily glycosylated cell membrane-associated proteoglycans have a short C-terminal cytoplasmic domain followed by a membrane spanning region and a large highly glycosylated extracellular domain (Schwartz 2000). These proteoglycans show similarities to the membrane bound mucins such as MUC-1 and MUC-4. These glycoconjugates also have a repeat region which is heavily glycosylated, though the repeat is usually smaller than those of mucins (the repeat for serglycan is, for example, only Ser/Gly). The lightly glycosylated cellular-associated proteoglycans contain large modular structures that have multiple receptor and binding functions. In this case these regions are not glycosylated. A small amount of glycosylation is present at the N-terminus. In the case of perlecan, only a single heparan sulfate chain is attached (Friedrich *et al.* 2000). The other major group of proteoglycans so far characterised is the small leucine-rich proteoglycans of which biglycan, decorin and luminican are examples. These molecules have a similar structure to the aggrecan family, consisting of a central, heavily glycosylated, repeat region (in this case containing leucine) flanked by globular "naked" domains responsible for binding (Matsushima *et al.* 2000; Waddington and Langley 1998). Figure 1.7 graphically represents some known proteoglycan structures.

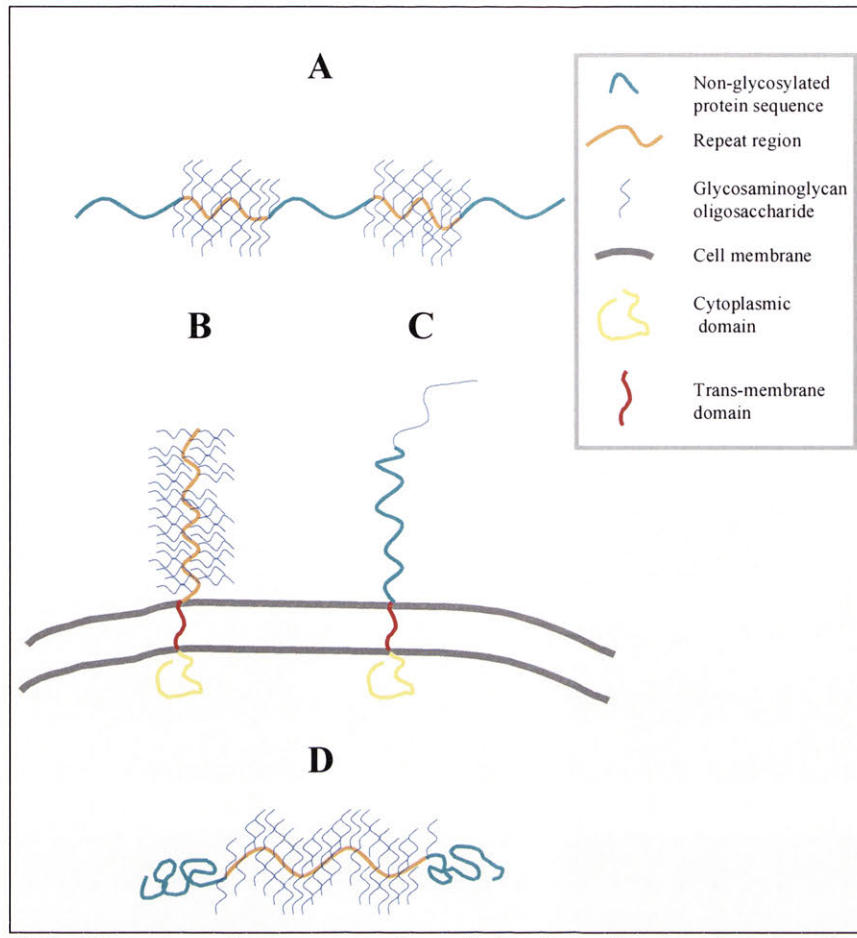

Figure 1.7: *Diagrammatic representation of the four classes of proteoglycans. The aggrecan family [**A**] contains proteoglycans that have heavily glycosylated repeat regions flanked by "naked" protein. The unglycosylated protein contains binding domains for other ECM components. The heavily glycosylated cell membrane bound proteoglycans [**B**], including syndecan and serglycin, contain a small C-terminal cytoplasmic tail and a transmembrane domain, topped at the N-terminal end with a large, heavily glycosylated, repeat region. The lightly glycosylated cell bound proteoglycans [**C**] such as perlecan have their transmembrane domain capped with a large non-glycosylated region containing various binding domains. Glycosylation is only found at the N-terminus. The small leucine-rich proteoglycans [**D**] include luminican and biglycan. These proteoglycans have a central leucine rich tandem repeat region which is heavily glycosylated. This region is flanked by globular, non-glycosylated domains that contain binding sites for collagen and other ECM components (Iozzo 1998; Iozzo 1999; Carey 1997; Schwartz 2000)*

Proteoglycans differ from mucins in some important areas. Generally proteoglycans contain less sugar by weight than mucins (carbohydrate making up only 10-50% of the mass of the molecule as compared to as much as 90% for mucins) (Bleckmann and Kresse 1980). Even the heavily glycosylated proteoglycans such as aggrecan are easily digested by proteases (Hassell *et al.*

1981). In addition proteoglycans have markedly different amino acid and monosaccharide compositions to that of mucins (Schwartz 2000; Iozzo 1999). Proteoglycans do not have the high levels of Ser, Thr, Pro, Val, Ala that mucins typically have. Also, they contain monosaccharides which are not associated with mucins, such as O-linked mannose and xylose (Iozzo 1998; Schwartz 2000). Finally proteoglycans contain hexuronic acids, such as glucuronic acid (Iozzo 1999). Proteoglycans and mucins may be considered counterparts, mucins forming gel layers on epithelial surfaces and proteoglycans forming extracellular matrices and connective tissue.

1.3 GLYCOSYLATION OF GLYCOCONJUGATES

When an eukaryotic cell produces a protein, it undergoes several processes. Firstly the DNA is transcribed to messenger RNA in the nucleus with any introns being excised. The messenger RNA is attached to ribosomes and the peptide core is constructed according to the code. Once the peptide core is completed, many proteins are folded and then are ready in a functional form. However, a number of proteins undergo post-translational modification. Modifications include the removal of a signal sequence, attachment of sulfate or phosphate, or the attachment of oligosaccharides to specific amino acids. These attachments are typically described as N-linked (as they are linked to the amine group of asparagine) or O-linked (linked to the hydroxyl group of Ser or Thr) (Gooley *et al.* 1991).

1.3.1 N-linked Glycosylation

N-linked glycosylation is the most understood process of glycosylation and is a "simpler" process of attachment than that of O-glycosylation. An N-linked attachment occurs when the reducing end of an oligosaccharide is linked to the amino group of an asparagine residue (Lehrman 1991). Not all asparagine residues are glycosylated, however, the Asn must be in the amino acid sequence Asn-Xaa-Ser/Thr, where Xaa is any amino acid except proline (Wang *et al.* 1993). The attachment is made by a pre-synthesised, high mannose containing polysaccharide that is attached to the Asn residue in the endoplasmic reticulum. Exomannosidases and exoglucosidases in the endoplasmic reticulum and Golgi apparatus remove the glucose and some of the mannose residues. Finally various glycosyltransferases attach other monosaccharide residues to the "trimmed" structure as it passes through the trans Golgi (Varki 1991) (Figure 1.8).

A core structure always remains present in N-linked oligosaccharides consisting of two N-acetyl glucosamine residues in line followed by three mannose residues forming a "branch" structure. To this core there may be several "antennae" of GlcNAc and galactose residues (complex), the mannose residues may remain (having not been trimmed - high mannose), or there may be some mannose present with antennae springing from it (hybrid). Figure 1.9 depicts an example of each of the three types of N-linked oligosaccharides.

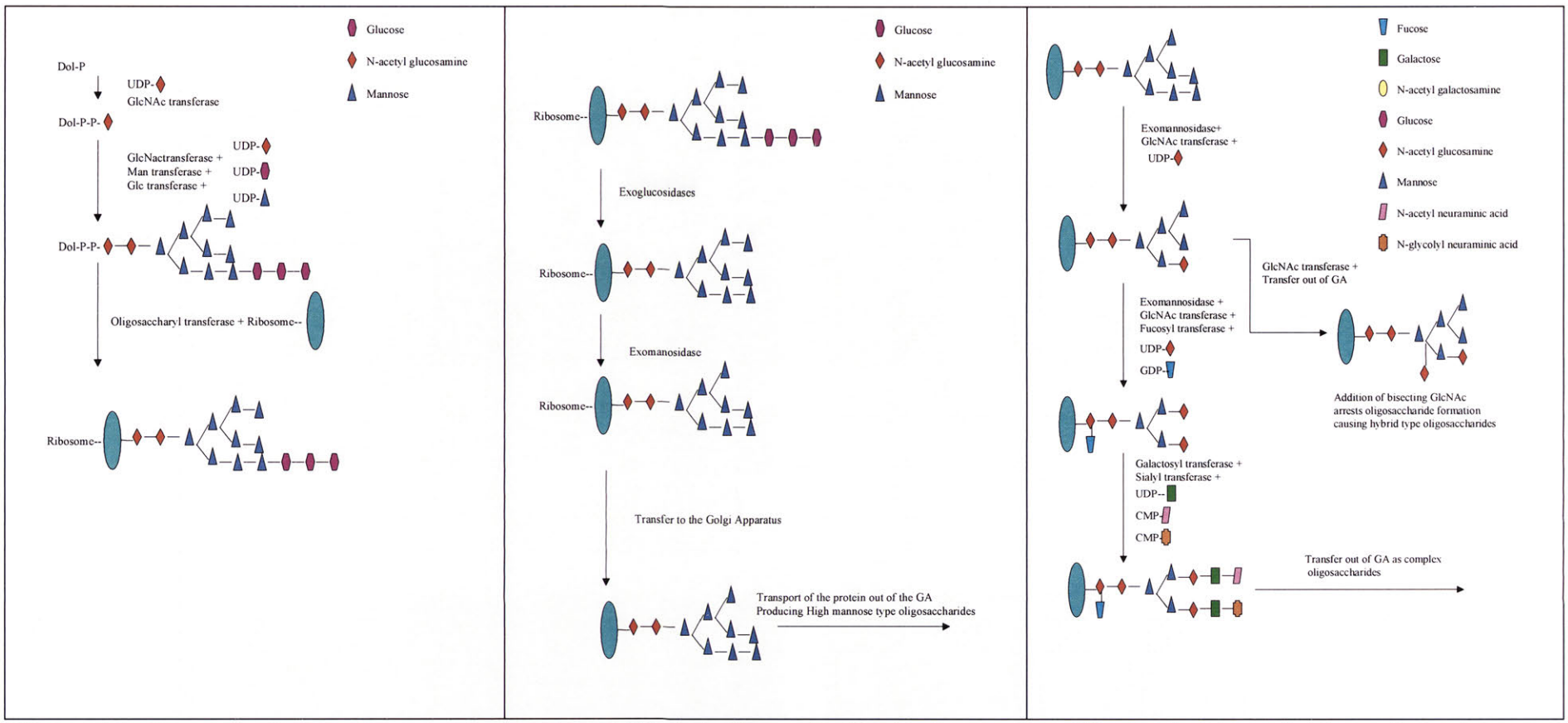

Figure 1.8: *The production, attachment and processing of N-linked oligosaccharides in glycoproteins. Oligosaccharide production and attachment occurs in the endoplasmic reticulum with processing moving to the Golgi apparatus. Production may be stopped at the high mannose or hybrid stages depending on what protein is being glycosylated (Lehrman 1991)*

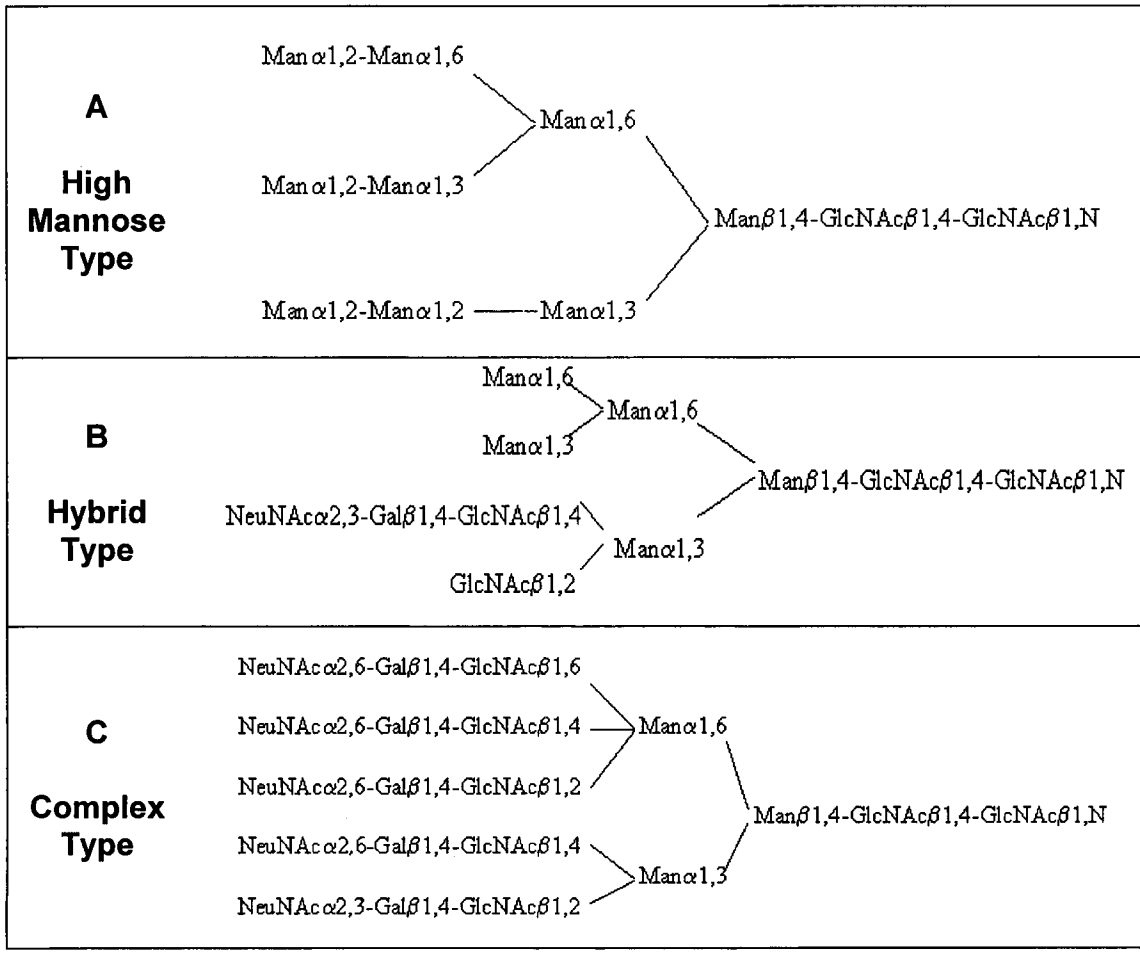

Figure 1.9: *Types of N-linked glycans attached to protein cores. The glycans attached to asparagine may be of the high mannose [A], hybrid [B] or complex [C] type depending on what stage the process was arrested in the Golgi apparatus (Lehrman 1991)*

N-acetyl, N-glycolyl neuraminic acid (sialic acid) or sulfate may be attached to the end of the antennae, a GlcNAc residue may be attached to the core oligosaccharide between mannose 2 and 3 (bisecting GlcNAc) and fucose may be attached to the core or the antennae (Sasak *et al.* 1991; Roux *et al.* 1988) (Figure 1.10).

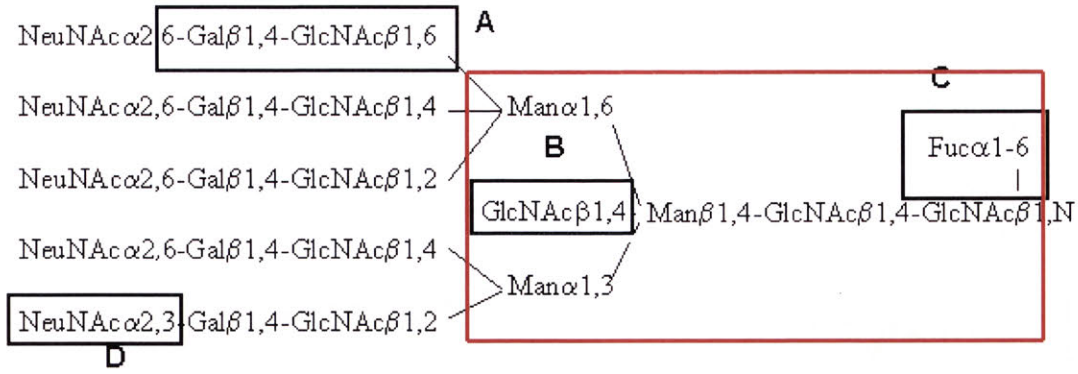

Figure 1.10: *Theoretical oligosaccharide demonstrating possible modifications to the oligosaccharide core (red box). The core may have the mannose structures (present in the high mannose pre-cursor) replaced by from 1-5 GlcNAc antennae [**A**] attached or a bisecting GlcNAc [**B**] may be used to arrest the oligosaccharide in the hybrid form. Fucosylation [**C**] may occur on the core or the antennae. Finally the antennae may be capped with N-acetyl or N-glycolyl neuraminic acid or sulfate [**D**] (Lehrman 1991)*

1.3.2 O-linked Glycosylation

O-linked glycosylation of mucin glycoproteins takes the form of linkages between the reducing end of N-acetyl galactosamine and the hydroxyl group of either Ser or Thr (Meynial-Salles and Combes 1996). However O-linkages of oligosaccharides to hydroxyproline and hydroxylysine do occur in some plants and in proteoglycan attachments (Schwartz 2000). In mucin glycoproteins there is no universal sequential restriction to the addition of oligosaccharides to Ser or Thr. However, the sequence Xaa-Pro-Xaa-Xaa where one Xaa = Thr and the others are not Cys, Met, Phe, His, Tyr or Trp has been suggested as a likely key sequence for one type of O-linked attachment in *Dictyeostelium discoideum* (Gooley *et al.* 1991). A review of O-linked glycosylation by Thanka Christlet and Veluraja (2001) has suggested that proline around the attachment site (particularly at +3 or -1) favours O-glycosylation. Furthermore the surrounding amino acids must have small side chains (large side chain amino acids such as cyteine, and particularly the aromatic amino acids, inhibit O-glycosylation). It has become

apparent, however, that there are several enzymes for attachment of monosaccharides to Ser/Thr residues and each of these glycosyltransferases has a different target amino acid sequence for glycosylation (Kasinathan *et al.* 1991). O-linked oligosaccharides are attached to the protein, one monosaccharide residue at a time, in the Golgi apparatus rather than the attachment of a pre-synthesised polysaccharide built up in the endoplasmic reticulum (Deschuyteneer *et al.* 1988). This process, coupled with the number of glycosyltransferases involved, means there is not a single core structure for O-linked oligosaccharides attached to mucins, rather there are a number that have been suggested (Hanisch and Peter-Katalinic 1992, Table 1.2).

Table 1.2: *Some known core structures of O-linked glycans attached to mucin glycoproteins. The core structures of mucin oligosaccharides to which polylactosamine is attached for larger structures (Hanisch and Peter-Katalinic 1992)*

Core	Structure
Core 1	Galß(1-3)GalNAcα1-O-Ser/Thr
Core 2	Galß(1-3)(GlcNAcß (1-6))GalNAcα1-O-Ser/Thr
Core 3	GlcNAcß(1-3)GalNAcα1-O-Ser/Thr
Core 4	GlcNAcß(1-3)(GlcNAcß (1-6))GalNAcα1-O-Ser/Thr
Core 5	GalNAcα(1-3)GalNAcα1-O-Ser/Thr
Core 6	GlcNAcß(1-6)GalNAcα1-O-Ser/Thr
Core 7	GalNAcα(1-6)GalNAcα1-O-Ser/Thr

In mucins, O-linked sugars are often only attachments of di- or tri-saccharides (Chai *et al.* 1992), however very large and complex structures of 25 residues or more have also been found (Mawhinney *et al.* 1992). In many glycoproteins, particularly mucins and other protective structures (such as PsA in *Dictyostelium discoideum* or cellulases with spacer regions), it is not uncommon for O-linked oligosaccharides to be densely packed together (Jung *et al.* 1997). It is also not uncommon for complex structures to be surrounded by the simple di- and tri-saccharides (Eckhardt *et al.* 1987). Similarly to N-linked oligosaccharides, O-linked structures may have sialic acid, sulfate or fucose attachments (Lo-Guidice *et al.* 1994).

1.3.3 Glycosaminoglycan Oligosaccharides

GaG oligosaccharides are a specific class of structures that can be either attached through the amine group of Asn or the hydroxyl group of Ser/Thr. They may also be attached to hydroxyproline and hydroxylysine. GaGs are linked to the protein core in many ways including O-linkages via a mannose residue (normally in mammals mannose would not be associated with O-linked oligosaccharides) (Endo 1999). They may also have xylose as the reducing terminus. In mammalian glycoproteins and mucins, xylose is only rarely found in O-linked oligosaccharides (in EGF-like domains of blood factors) (Nishimura *et al.* 1992) and not at all in N-linked oligosaccharides (Kornfeld and Kornfeld 1976).

There are several basic types of glycosaminoglycan structures of which examples are shown in Figure 1.11.

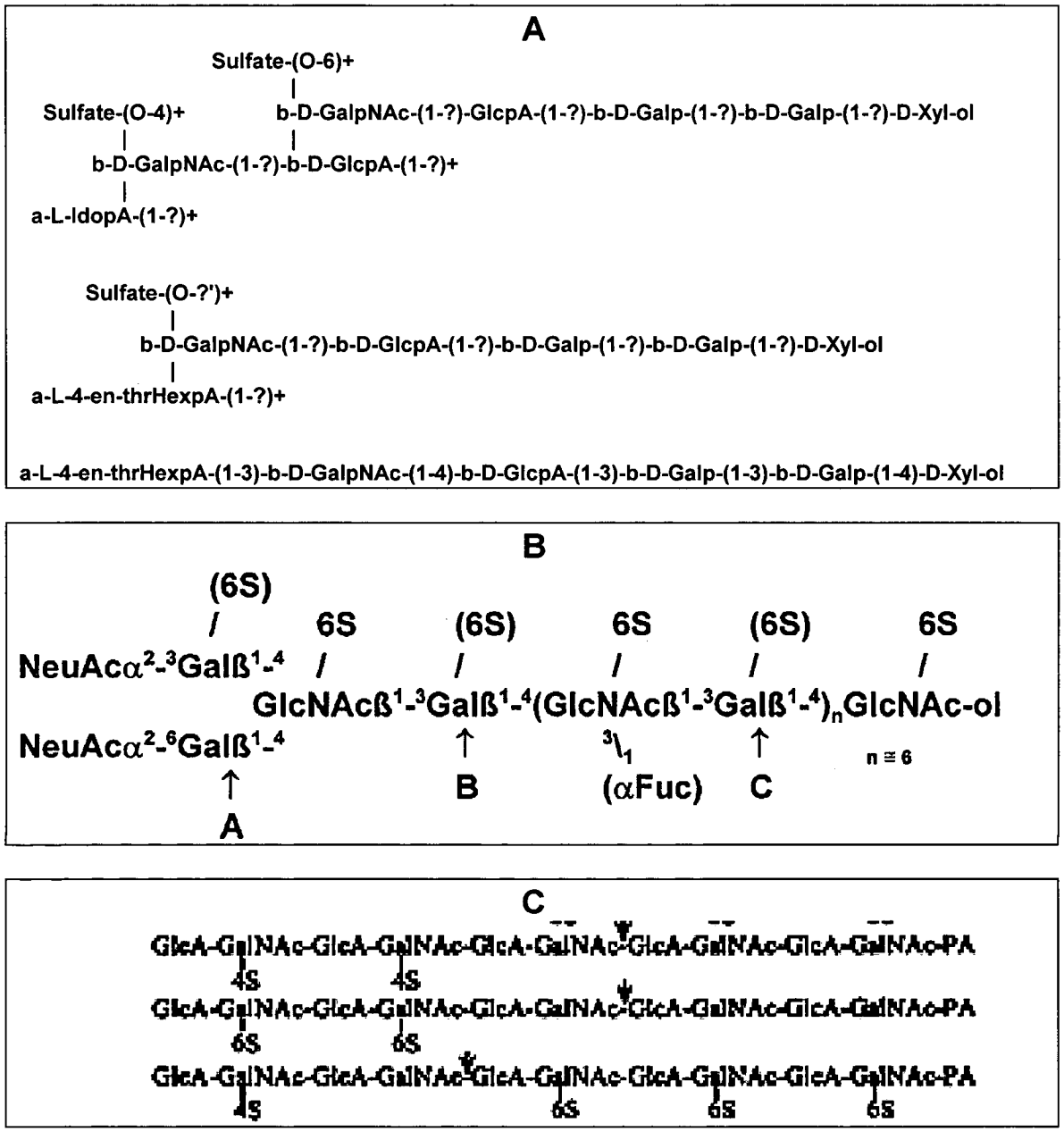

Figure 1.11: *Some examples of glycosaminoglycan oligosaccharides. [A] some reported chondroitin sulfate structures (CarbBank database, http://128.192.9.29/carbbank). [B] a standard keratan sulfate structure (taken from Lauder et al. 1998). [C] some reported dermatan sulfate structures (taken from Takagaki et al. 2000)*

Keratan sulfate: Keratan sulphate is a polymer based upon a repeating N-acetyl lactosamine sequence. The two repeating sugars are N-acetyl glucosamine and galactose. This core structure may have sialic acid or fucose attached to it and is usually sulfated on C6 of GlcNAc. It may also be sulfated on C6 of the Gal residue. Keratan sulfate may

have N-linked or O-linked attachment of the oligosaccharide to the proteoglycan core (Iozzo 1998).

Chondroitin Sulfate / Dermatan Sulfate: Chondroitin sulphate and dermatan sulphate are both derived from the same polymer - β(1-3)D-glucuronic acid β(1-4)D-N-acetyl galactosamine. They can be sulphated at positions 4 or 6 of N-acetyl galactosamine and position 2 of the uronic acid. They are not N-sulphated (i.e. sulfate is not attached to the nitrogen of the amino sugar). The difference between chondroitin sulphate and dermatan sulphate is the epimerisation of glucuronic acid to iduronic acid. In chondroitin sulfate there is more glucuronic acid than iduronic acid present in the oligosaccharide, and *vice versa* for dermatan sulfate. The discrimination of these GaGs is, however, difficult. These GaGs can also contain fucose and sialic acid end caps (Iozzo 1998).

Heparin and Heparan Sulfate: Heparin and heparan sulfates are repeats of N-acetyl glucosamine and glucuronic acid. In the case of heparan sulfate, this glucuronic acid has epimerised to form iduronic acid. Both can be N-sulfated and O-sulfated at C6 or C3 of the GlcNAc and C2 of the uronic acid. Heparin contains a higher percentage of both N- and O-sulfation than heparan (Schwartz 2000). This group of GaGs is associated with growth factors and adhesion molecules (Tumova *et al.* 2000).

There is also one GaG that acts independently, i.e. the polysaccharide is not attached to a proteoglycan backbone. This GaG is a polymer composed of repeating disaccharides of β-1-4-glucuronate-β-1-3-N-acetylglucosamine, known as hyaluronic acid. It is involved in the formation of cartilage with aggrecan, a ligand for cell surface receptors, and as a link for protein-protein interactions (Saso *et al.* 1999B).

1.3.4 Functions of Glycosylation

The functions of post-translational modifications, including glycosylation, have only recently been explored in detail. This was due mainly to the fact that many of the methods of observation and analysis, such as NMR or X-ray crystallography, traditionally used for structure and function studies, are rendered ineffective when glycosylation is present on a molecule in significant quantities (Fontenot 1993). In addition, proteins are often collected in large amounts by transferring the gene responsible for coding the protein to a bacterial expression system (such as *Escherichia coli*). The glycosylation of proteins by bacteria is primitive in comparison to eukaryotes, resulting in the recombinant product having no glycosylation attached. With the advance of techniques and protocols of analysis, however, it is becoming increasingly apparent that glycosylation is important for the correct function of many proteins.

Some of the functions which have been so far determined for glycosylation include:

- Protection from enzymatic or physical degradation by blocking vulnerable sites in the peptide core: the glycosylation of porcine gastric mucin (PGM) makes it possible for the formation of mucus tolerant to the extreme acid conditions of the stomach (Cao *et al.* 1999).

- Creation of areas of linearity in the protein by not allowing it to fold: this is important in molecules such as PsA in *Dictyostelium discoideum.* The glycosylated repeat region creates a linear "spacer" which keeps the active globular region of the protein away from the cell wall (Haynes *et al.* 1993).

- The formation of a matrix between it and other molecules: such as in a cell wall, cartilage or a mucus layer (Rose *et al.* 1989).

- Cell-cell adhesion: Syndecan-1 (CD138) is involved in the adhesion of myeloma cells in cell-cell and matrix-cell interactions (Borset *et al.* 2000).

- Involvement in the active sites of receptors and enzymes: CD44 is a mucin-like protein found as part of the glycocalyx of mammalian cells. The glycosylation of this protein has been shown to be important in the receptor functions of the molecule (Borset *et al.* 2000).

- Antigenic sites for the immune system: extrinsic allergic alveolitis is caused by breathing glycoproteins into the lungs which are secreted from birds (Todd *et al.* 1991).

- Activation of proteins and enzymes: immunoglobins require glycosylation to form an active antigen-binding site (Prat *et al.* 1989).

- As digestion markers, i.e. O-linked glycosylation, controls the digestion of bovine pro-opiomelanocortin (Siciliano *et al.* 1994).

- Receptor sites: FGF-2 uses O-linked oligosaccharides to signal the proliferation of embryonic cells (Jirmanova *et al.* 1999).

- Points of attachment for bacteria, and Protozoan parasites: *Trypanosoma cruzi* use mucin oligosaccharides as their first binding point when invading a host (Di Noia *et al.* 1996).

It is also important to note that the oligosaccharide structure of proteins, such as mucins, is often altered in disease states such as malignancy (Lan *et al.* 1990; Capon *et al.* 1992). This suggests functions which are yet to be determined.

1.3.5 Ocular Mucus

Ocular mucus differs from mucus layers found on other epithelial surfaces in many ways, the most obvious being the physical differences. It is a relatively thin layer of mucus, with a direct relationship to lipid and aqueous components to form a stable tear film. It must also be transparent. There are also differences at a molecular level in both the peptide core and sugar structures of the mucins. Firstly, the high molecular weight components of ocular mucus do not appear to be as large as those of other gel forming mucins (Chao *et al.* 1983A). This may be artefactual due to the nature of the tandem repeat sequences present in the ocular glycoconjugates. The reported amounts of Ser and Thr present is low compared to other mucins (only 20-25% in ocular mucins compared to as much as 75-80% in the larger gel forming mucins) (Chao *et al.* 1983A). The ocular mucus components are also less glycosylated (by weight) than the larger gel forming mucins of the intestines and airways (ocular glycoconjugates are only 25-50% carbohydrate by weight, whereas mucins from other sources can be as much as 80% carbohydrate by weight) (Chao *et al.* 1983A; Thornton *et al.* 1994). Work on ocular mucins so far has been conflicting. Tei, Moccia and Gipson (1999) have identified MUC genes coding for

MUC-1, MUC-4 and MUC-5AC. Furthermore, antibody staining techniques performed by Garcher *et al.* (1994) have identified MUC-2 epitopes in the tear film. Corfield and his co-workers (Carrington *et al.*1998) have performed a great deal of work on both human and canine ocular mucins using both antibody and some chemical characterisation. To date, chemical characterisation of ocular mucins from rabbit and bovine sources has not produced amino acid compositions which are consistent with mucin glycoproteins (Tseng *et al.* 1987). There was no enrichment of amino acids associated with the tandem repeat sequences found in mucins from other tissues. Ocular mucin fractions that have been analysed so far are higher in Ser than Thr, which is a specific difference to the other mucins (the closest being MUC-5AC that has approximately the same amount of Ser as Thr (SWISS_PROT Database, http://expasy.proteome.org.au/sprot/).

One thing worth noting is that, in many cases, the analysis of mucins has actually been performed on the "mucin fraction", i.e. the whole of the high molecular weight glycoconjugates, as separating mucins is very difficult. It is, therefore, possible that many of the observations made relate to an average of all the species present in this fraction, which may account for the discrepancies between the compositions reported by various groups.

Separation of the high molecular weight glycosylated fraction of ocular mucus by agarose gel electrophoresis (Berry *et al.* 2000) has demonstrated that many protein species are present in mucus layers.

1.4 METHODS OF PURIFICATION OF HIGH MOLECULAR WEIGHT GLYCOPROTEIN CONTAINING FRACTION

Much of the work which has been carried out on mucins has been performed using bovine sub-maxillary mucin (BSM) as a model. BSM is the mucin component used in the most common forms of artificial tear fluid described (Mirejovsky *et al.* 1991). This is largely due to the fact that it is easily obtainable from several commercial suppliers. BSM is probably not a good model for tear mucus as it is most likely a mixture of the bovine analogues for MUC-7 and MUC-5B. MUC-7 is the non-gel forming mucin found in human salivary glands (Mehrotra *et al.* 1998), and MUC-5B is often involved with sol. phases. As such, these mucins have substantially different physical and chemical properties to other gel forming mucins such as MUC-2 and MUC-6 (Veerman *et al.* 1992). BSM is only around 10-15% carbohydrate by weight when it is taken from a bottle (Cooper *et al.* 1994) and contains little or no sialic acid. This is possibly due to the commercial purification method used. Many methods of analysis used on BSM (such as types of enzymatic cleavage and oligosaccharide analysis) are inappropriate for the larger, more heavily glycosylated mucins (Cooper *et al.* 1994). Another commonly used mucin model is PGM. This is a better model for gel forming mucins as it is most likely an analog of MUC-6 and/or MUC-5AC (Turner *et al.* 1999; Nordman *et al.* 1998), but again there are several chemical problems associated with using it as a model. The solubility is greatly reduced when PGM is purified and lyophilised (being nearly 12 mg/ml when extracted fresh from porcine stomachs and only 2 mg/ml when taken from a bottle) (Bennett and McLean 1984). It also has a very low sialic acid content, being less than 1% sialic acid by weight (Nordman *et al.* 1998). It was, therefore, necessary to purify sufficient quantities of the high molecular glycoconjugates from ocular mucus to enable

analysis of the species present. In order to determine if either BSM or PGM were able to be used as a model for ocular high molecular weight glycoconjugates (OHMG), they were purified and analysed in tandem with mucus from bovine eyes.

1.4.1 Previous Methods of Purification

The reason for the lack of chemical analysis of mucins lies mainly in the fact that mucins are difficult to purify in sufficiently large amounts. This is especially true in the case of ocular mucus, where the volume of the tear film is only microlitres. Most previous work on human mucin glycoproteins has involved cancer-related mucins and tracheobronchial or intestinal mucins. Large amounts of these materials may be obtained by biopsies or the collection of sputum (Devine *et al.* 1991; Rose *et al.* 1989; Tseng *et al.* 1987). Because large amounts of crude material are available, the losses incurred during purification can be tolerated. A major disadvantage of these systems for the study of mucins is that the mucin may be degraded by stomach contents (Tabak *et al.* 1984) or it may occur in an altered state (e.g. mucins from tumour cells) (Devine *et al.* 1991). Also the purification of these mucins usually involves only the portion which was soluble in low salt and physiological buffers (Parker *et al.* 1993). This probably results in the purification of a sub-set of the total mucin complement. Methods of purification of mucins typically involve a size exclusion step followed by a caesium chloride (CsCl) density gradient centrifugation or *vice versa* (Corfield *et al.* 1997). Other gradients such as caesium trifluoro-acetate have also been used (Devaraj *et al.* 1992). Alternatively, a preparative scale electrophoresis unit using polyacrylamide gel may be used (Paszkiewicz-Gadek *et al.* 1995). A large amount of material is lost when removing the CsCl from the sample,

as the mucin binds to dialysis membrane and other supports (Ruggieri *et al.* 1992). Preparative scale electrophoresis is slow and has low recoveries.

Once mucin has been purified, it is also difficult to analyse, as only some mucins will enter into the lowest concentration sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (and even then not very well) (Tytgat *et al.* 1995A). Alternatively agarose gel electrophoresis can be used, but this is slow and has many problems of its own (discussed more fully in Chapter 3). Because mucins are so large and heavily charged, it is desirable to cleave them enzymatically for greater ease of analysis. This, however, is also very difficult to achieve. One of the functions of mucins is to protect the epithelial surface from degradation. Many of the cleavage sites are covered by oligosaccharides and, thus, are unavailable for proteolysis. Typically, large amounts of enzymes are required to remove the "naked" regions of the glycoprotein. This leaves large glycopeptides, which are still too large to analyse. In the past, proteases have proven to be unable to digest the glycopeptides any further (Chadee *et al.* 1990).

1.4.2 Previous Methods of Analysis

Most of the analysis of mucin glycoproteins has involved either the sequencing of the genes or the analysis of tissues or extracts by antibody staining of some kind (Tytgat *et al.* 1995B; Watanabe *et al.* 1995). As the technology becomes more accessible, mass spectrometry has been increasingly used to analyse peptides or oligosaccharides from mucins (Karlsson *et al.* 1995; Morelle *et al.* 1998). However, these methods of analysis have severe limitations. By sequencing the gene, little information about the authentic protein is gleaned, as the mucin has significant post-translational modifications. It is also

clear that at least one mucin (MUC-1) has several different forms, all stemming from the same gene (Hilkens *et al.* 1995). Each of these forms could be classed as a separate protein as they appear to serve different functions. There is a membrane bound form, which acts as part of the glycocalyx (Carraway and Hull 1991), a secreted form (Devine *et al.* 1991) and indications of a truncated form that acts as a receptor (Mall *et al.* 1992; Meerzaman *et al.* 2000). The use of mono-clonal antibodies to detect species of proteins is a powerful method of analysis, however it can produce misleading results. Similar oligosaccharide structures are present on many mucin species (and most antibodies to mucins are by necessity to the glycosylation). Therefore, the presence of an oligosaccharide found on one mucin species does not negate the possibility of another mucin species being present with similar glycosylation patterns. For example, antibodies for oligosaccharides present on the molecule CD24 were found to also stain both BSM and OSM (Mehmet *et al.* 1990). This problem is further compounded by the lack of accuracy in determining the size of mucin molecules. Mass spectrometry is a powerful technique for characterising proteins using peptide fingerprinting. In this process a protein is digested with specific proteases and the size and distribution of the resultant peptides is matched with a database entry (Karlsson *et al.* 1997A). This method has, however, limited value for the study of mucins. Mucin glycoproteins are much too large to "fly" - i.e. they will not ionise for analysis. Because of the nature of mucins (tandem repeat sequence), the glycosylated region is also large. Digestion of mucins with proteases results in glycopeptides that are still too large to ionise for mass spectrometry analysis. In addition, if oligosaccharides are attached to a peptide backbone, the ability to ionise is hindered so that, on the rare occasions that large glycoconjugates can be digested into small enough glycopeptides to fly, it is still difficult to get a result.

Past methods of purification and analysis have provided valuable insights into the composition of ocular mucus. However there still remains a discrepancy between that which has been observed through antibody staining and hybridisation techniques and the reported chemical analyses. Chemical characterisation was used in this thesis in an attempt to determine the nature of the high molecular weight glycoconjugate species present in bovine ocular mucus and to verify its unique composition.

1.5 HYPOTHESIS

That due to the unique nature of the tear film, high molecular weight glycoconjugates found in the mucus layer are different to that of mucins found in mucus layers from other epithelial surfaces.

That the mucus layer of the ocular epithelium contains one or more, as yet, uncharacterised glycoconjugate species that contribute to its unique properties such as visual clarity.

CHAPTER 2: DEVELOPMENT OF NEW METHODS FOR THE PURIFICATION OF OCULAR HIGH MOLECULAR WEIGHT GLYCOCONJUGATES (OHMG) AND THEIR SUB-UNITS

2.1 INTRODUCTION

Ocular mucins have a unique role in the body, which requires physical properties not necessary in mucins from other tissues, such as visual clarity, protection of a tissue directly exposed to the atmosphere and sustaining of ocular cells in an inhospitable environment (Holly and Hong 1982). This suggests that the components of ocular mucus will have different physical characteristics to accommodate these unique functions. The aim of this study is, as a first step, to compare the physical characteristics of high molecular weight ocular glycoconjugates to a commercially available large gel forming mucin (PGM) and a smaller soluble mucin (BSM). Sufficient quantities of the high molecular weight component of ocular mucus will then be purified to perform studies of chemical composition, so as to determine if ocular mucus is significantly different from mucus produced by other epithelial surfaces.

The first stage in the chemical analysis of any unknown protein is its extraction from the source tissue or solution followed by subsequent purification in large enough quantities for the required analytical techniques to be performed. Unlike the detection of epitopes by antibody staining, compositional analysis provides information on all components of a sample, without being able to distinguish between them. It is, therefore, important to remove all other proteins from a sample, as contamination will result in erroneous data. However, due to their size and charge characteristics, mucin glycoproteins are unable to

be purified by many of the techniques, such as electrophoresis and standard HPLC chromatography, which have proven useful in the separation of smaller (unglycosylated) proteins from other sources.

The propensity of mucins to form visco-elastic gels, or very viscous solutions at relatively low concentrations, has made them difficult to purify by physical means such as gel filtration, dialysis and electrophoresis (Paszkiewicz-Gadek *et al.* 1995). There is also evidence that the mucins interact with the separation matrices used in liquid chromatographic methods, interfering with isolation procedures (Tabak *et al.* 1984). Historically, the standard method for purification has involved partial purification by collection of the proteins eluting at the void volume of a size exclusion column followed by these excluded proteins being separated on the basis of density, using CsCl density gradient centrifugation (Davies *et al.* 1991). This method is time consuming and incurs relatively high losses during the removal of CsCl by dialysis (Ruggieri *et al.* 1992). Methods for single-step purifications have been described (Parker *et al.* 1993), but these have involved dissolving mucins in low salt buffers. The solubility of the gel forming mucins is quite low in these buffers, resulting in low yields and selectivity of the species (Davies *et al.* 1999). More recently anion exchange chromatography, using strong ionic buffers, has been used as a chromatographic purification step rather than size exclusion chromatography (SEC), resulting in separation of mucins from low molecular weight material, and fractionation into individual species (Thornton *et al.* 1995). Alternatively Paszkiewicz-Gadek *et al.* (1995) have described a procedure using preparative scale electrophoresis to purify mucin glycoproteins. The low molecular weight proteins migrate through the gel, while the heavily glycosylated mucins cannot enter the matrix. However

this process is also time consuming and the amount of material that can be loaded onto the gel is limited. The need for larger scale preparations of bovine high molecular weight glycoconjugates, which are required for protein characterisation, has resulted in the investigation of methods that are time efficient for the purification of glycoconjugates which are not dissolved into saline from a mucus gel.

2.2 EXTRACTION OF MUCINS FROM TISSUE

In many methods of purification, particularly from colonic scrapings, mucin glycoproteins are extracted in physiological buffers such as phosphate buffered saline (PBS) (Parker *et al.* 1993). However it has been shown by Herrmann *et al.* (1999) that the gel forming mucins do not readily dissolve in these buffers. To increase the extraction of the gel forming mucins from the eye, two extraction protocols were compared. Conjunctiva from bovine eyes (obtained fresh from an abattoir) were excised and extracted in PBS containing either 4 M urea, 1 mM DTT, 0.1% SDS or 6 M guanidinium HCl and 1 mM DTT. Both of these extraction buffers contained protease inhibitors (5 mM EDTA, 5 mM N-ethylmaleimide, 100 mM aminohexanoic acid, 5 mM benzamidine, 1 mM PMSF). The tissue (four excised conjunctiva per 10 ml buffer) was, in both cases, incubated at 4°C for 8, 16 and 24 hours.

Results:

The area of the excluded peak from an SEC column was larger when conjunctiva were extracted with urea than when the conjunctiva were extracted with guanidinium.

SDS-PAGE analysis of low molecular weight contaminants of the leading peak determined that there was a similar amount of low molecular weight proteins in each of the extractions (data not shown), therefore, the guanidinium extraction was discontinued. In addition an increase in the leading peak area was observed between 8 and 16 hour incubations but not between 16 and 24. It was therefore decided that 16 hour incubations would be used.

The solution extracted from bovine eyes using urea was viscous and deep straw in colour. Initially the extract was concentrated using Centriprep 100 kDa cut-off concentrator (Amicon, MA, USA), however, upon a 2.5X concentration of the protein in the extract (the buffer concentration remains constant using this method), the solution spontaneously formed a gel which then precipitated. As the extract formed gels at high concentration, it was used unconcentrated, only being centrifuged (10,000 g for one hour) to remove debris and the supernatant used for purification by SEC.

To confirm that the extraction of the excised conjunctiva was only removing the mucus layer that was being secreted by the ocular epithelium into the tear film and not glycoconjugates from the cells or stroma, bovine eyes were swabbed with cotton swabs soaked in extraction buffer. Analysis of these swabbings by SEC, SDS-PAGE and compositional analysis confirmed that similar material was being extracted (Figure 2.1).

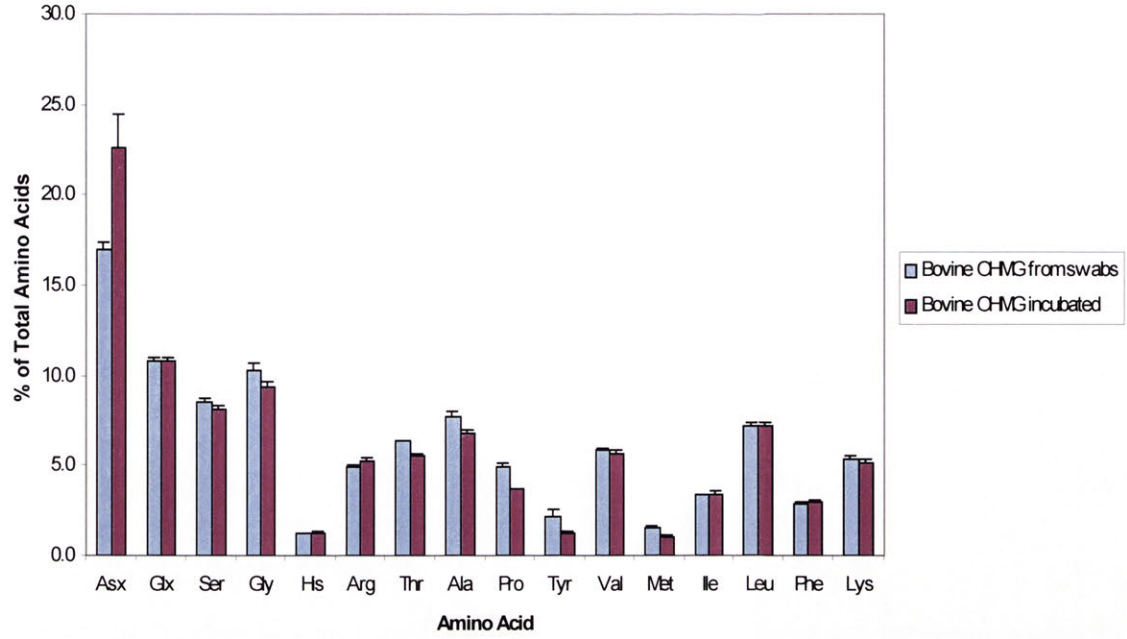

Figure 2.1: *Comparison of bovine conjunctival high molecular weight glycoconjugates purified by high salt SEC. It is clear that the material extracted by swabbing the surface of the eye with a swab dipped in extraction buffer (blue) was sufficiently similar to the material recovered by excising the conjunctiva and incubating for 16 hours in extraction buffer, to be called the same material. This suggests that the extraction protocol used was extracting the high molecular weight glycoconjugates from the mucus layer on the surface of the eye without introducing contamination from the stroma or epithelium*

2.3 PURIFICATION OF HIGH MOLECULAR WEIGHT GLYCOCONJUGATES

Traditionally mucins were purified using a two-step protocol involving SEC and one or more CsCl density gradient centrifugations. The protocols predominantly used size exclusion to remove low molecular weight proteins followed by CsCl density gradient centrifugation to remove DNA and the more tenaciously bound proteins (Davies *et al.* 1991). However, there are protocols that use a density gradient as the initial purification followed by SEC (Thornton *et al.* 1990). More recently, newer methods of purification have been described using anion exchange chromatography instead of SEC (Thornton *et al.* 1995) or even SDS-PAGE (Paszkiewicz-Gadek *et al.* 1995). Also matrixes other than

CsCl have been used to create density gradients such as sucrose (Axelsson *et al.* 1998) or caesium trifluoroacetate (Devaraj *et al.* 1992) or crude purifications have been performed by cetyl alcohol precipitations (Bhavanandan *et al.* 1999). However, the two-step methods involving SEC followed by CsCl still dominate the literature and are, therefore, used here for comparison.

2.3.1 Development of New Methods of Purification

There are several problems associated with the purification of mucins by density gradient centrifugation. Dialysis to remove the CsCl from the protein after centrifugation often results in large losses, due to protein binding to dialysis membranes (Ruggieri *et al.* 1992). This is not such a concern when purifying colonic or tracheobronchial mucins because of the large amounts of material which can be recovered from these tissues. Ocular glycoconjugates, however, are available in much smaller quantities. An entire eye produces only tens of milligrams of mucus, as opposed to the tens of grams produced in the gut and respiratory system (Meijer and van Haeringen 1993). It is also possible, that during dialysis, glycoconjugate species are binding preferentially to the membrane and one or more mucin species may be diminished. Of greater concern, however, is the extensive time required for a two-stage method of purification involving density gradients. The formation of a gradient can take as long as days in a conventional centrifuge (Dekker *et al.* 1989). In order to help reduce these losses and decrease the time involved, a second chromatographic purification step (replacing CsCl density gradient centrifugation of high molecular weight glycoconjugates) was developed to better

separate the high molecular weight components from low molecular weight contaminants.

2.3.1.1 Effects of SDS on Mucin Purification

SDS is used in PAGE to inhibit both inter- and intra-protein interactions, as well as conferring an overall negative charge coating to the molecule. A molecule of SDS has a highly negatively charged end and a hydrophobic tail at the other end. The tail binds to the proteins in a sample leaving the charge on the surface of the molecule. The number of SDS molecules that can bind to a protein is determined by the protein's size. The resultant negative charge overwhelms the charge characteristics of the protein itself and the sample is, therefore, separated due to size when placed in an electric potential. As SDS is an effective method for separating low molecular non-glycosylated proteins from mucins during SDS-PAGE, SDS was used in two buffer systems during chromatographic separation to determine if it could be used to separate the low molecular weight on the column. The crude urea extract (described in Section 2.2) was separated using either a solution of 0.5% (w/v) SDS in water or with SDS added to running buffer (50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.1) (low salt buffer) to a concentration of 0.5% (w/v). The sample was applied to a Superose 12 (Pharmacia, Uppsala, Sweden) HR 10/30 pre-poured column run on a Biologic system (Bio-Rad, California, USA), with detection set at 280 and 214 nm. The flow rate was 0.5 ml/min.

Results:

When the crude extract was purified using aqueous SDS, a single rise in absorbance (containing many peaks) was observed between 18 and 70 minutes (proteins in the range of 30-300 kDa as determined by SEC molecular weight markers) (Figure 2.2A). However, as the peaks were not resolved, it was determined that this method of purification would be unsuitable.

When low salt buffer was added to the SDS, the peaks began to resolve themselves (Figure 2.2B). The initial peak had a smaller area than that of the SDS without salt buffer (judged by relative peak areas for similar amounts of crude material applied). Analysis of the peak eluting at the void volume by SDS-PAGE demonstrated that low molecular weight proteins were still present in Peak 1 (Figure 2.2C). The fact that greater resolution was obtained with the addition of sodium phosphate and NaCl to the SDS suggested that the binding of the mucins to low molecular weight proteins might have been due to ionic interactions.

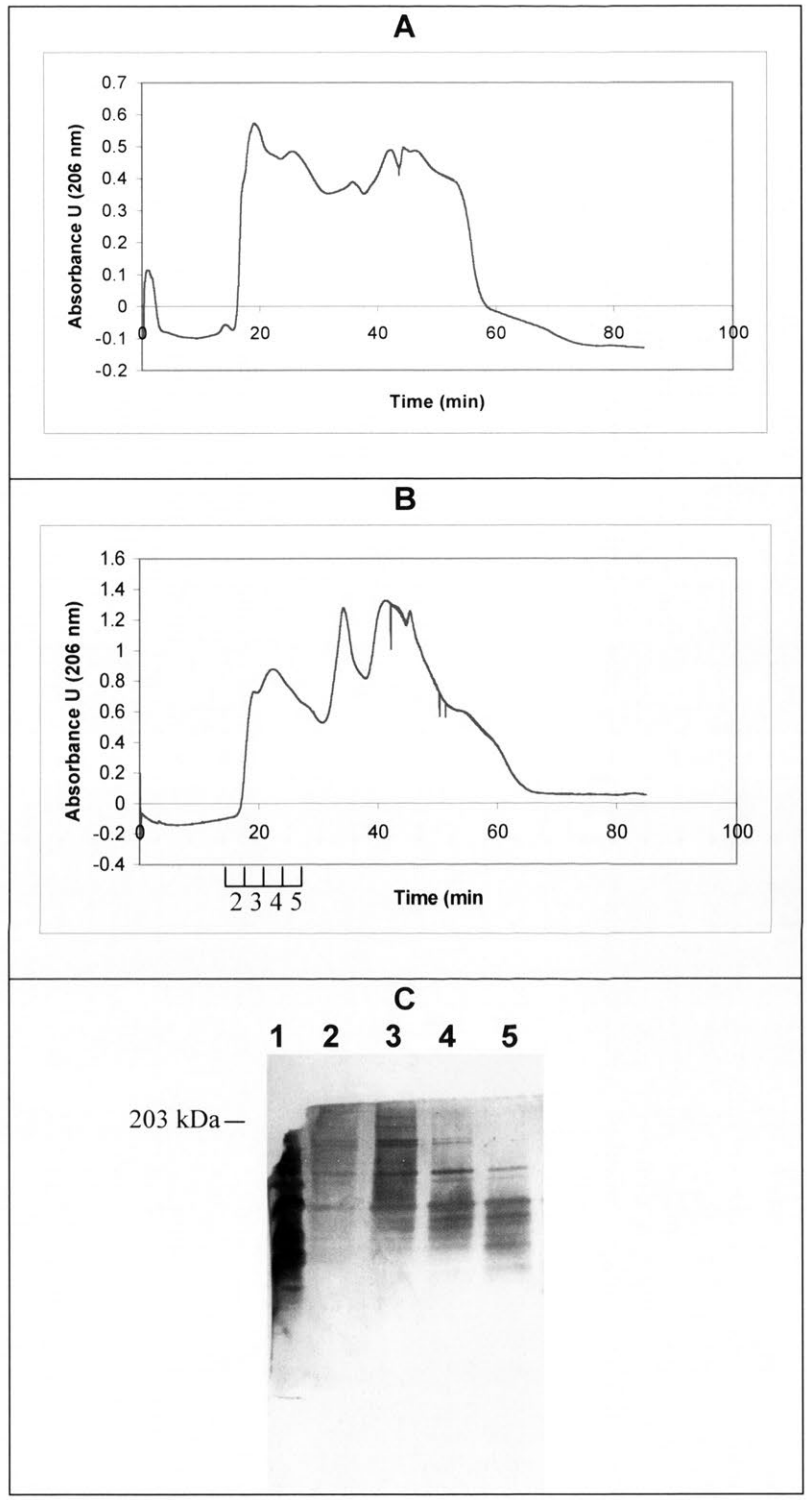

Figure 2.2: *Purification of bovine conjunctival extract in the presence of SDS. When separation was performed in 0.5% (w/v) SDS in water, a single broad peak containing many species was observed [**A**]. With the addition of low salt buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.1) to the SDS [**B**] the low molecular weight proteins are more distinct from the high molecular weight material. In addition, the peaks corresponding to the void volume have less area, suggesting that the high molecular weight material is more fully purified. However, analysis of the high molecular weight fraction by SDS-PAGE indicated that there was still a significant amount of material below 203 kDa in the peak [**C**]*

2.3.1.2 Effects of High Ionic Strength Buffers and Reduction and Alkylation on Mucin Purification

One possible explanation for the high amounts of low molecular weight components eluting with the peak containing mucin (> 200 kDa apparent molecular weight, when separated by Superose 12) was that the smaller proteins were associated with the mucin network by ionic interactions (Fullard 1988). Another possibility was that they were bound in some way that was broken by the conditions applied during SDS-PAGE. To explore these possibilities, two SEC protocols were developed. The first used reduction and alkylation to break disulfide bonds and prevent them from reforming prior to separation, the second used high ionic strength buffers to inhibit any ionic interactions during chromatography. Both these protocols used a first stage SEC purification in low salt buffer (50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.1). The crude extract was purified on a Pharmacia (Uppsala, Sweden) FPLC system using a Superose 12 HR 10/30 pre-poured column and a Uvicord II detector set at 206 nm.

a. Reduction and alkylation of partially purified mucin using 20 mM DTT and 80 mM iodoacetamide (Appendix B, Section B1.2.2) followed by SEC using a buffer of 3% (v/v) acetic acid titrated to pH 5.5 with triethylamine. This buffer system is commonly used for separating polysaccharides (Anumula and Dhume 1998). If the sugar component of the mucin was responsible for the binding of lower molecular weight proteins, this buffer may inhibit interactions between the high and low molecular weight proteins.

b. Separation of partially purified mucin using a high ionic strength eluting buffer (50 mM Na_2HPO_4/NaH_2PO_4, 1 M NaCl at pH 7.1).

Results:

a. The separation of reduced and alkylated bovine OHMG resulted in single peak eluting at the void volume and several peaks of low absorbance between the void and inclusion volumes of the Superose 12 column (Figure 2.3A). Mucins have a low absorbance at 280 nm due to the absence of aromatic amino acids in any quantity in their peptide chain (Moffatt *et al.* 2000). For this reason they must be detected using a shorter wavelength (214 nm or 206 nm were used) which detects the amide bonds present in the amino acid backbone. Triethylamine / acetate buffer systems are also UV absorbent at these wavelengths, making detection of the protein peaks difficult and quantitation by peak area impossible. However semi-quantitative analysis can be achieved by measuring the total amino acids recovered from the peak and this suggested losses had occurred, possibly in either the reduction and alkylation or the subsequent desalting step. Analysis of fractions taken within the void volume peak showed that it was free from low molecular weight material (as determined by SDS-PAGE analysis and silver stain, Appendix B, Methods - B3.1 and B4.1 respectively, Figure 2.3C). Even though the sample was shown to be free of low molecular weight proteins, this method was not continued due to the difficulties described above.

b. A second stage separation of the partially purified SEC void volume using a high salt buffer resulted in the further separation of low molecular weight proteins. A single peak at the void volume could be seen followed by several peaks between the void and inclusion volumes (Figure 2.3B). Fractions taken from the peak occurring at the void volume determined that the peak was free of low molecular weight proteins (analysis by SDS-PAGE and visualised by silver stain) (Figure 2.3D). As this purification method does not involve reduction and alkylation, loss of material is reduced. Additionally, using this method of purification, the OHMG was also recovered in an unmodified form (as opposed to reduced and alkylated).

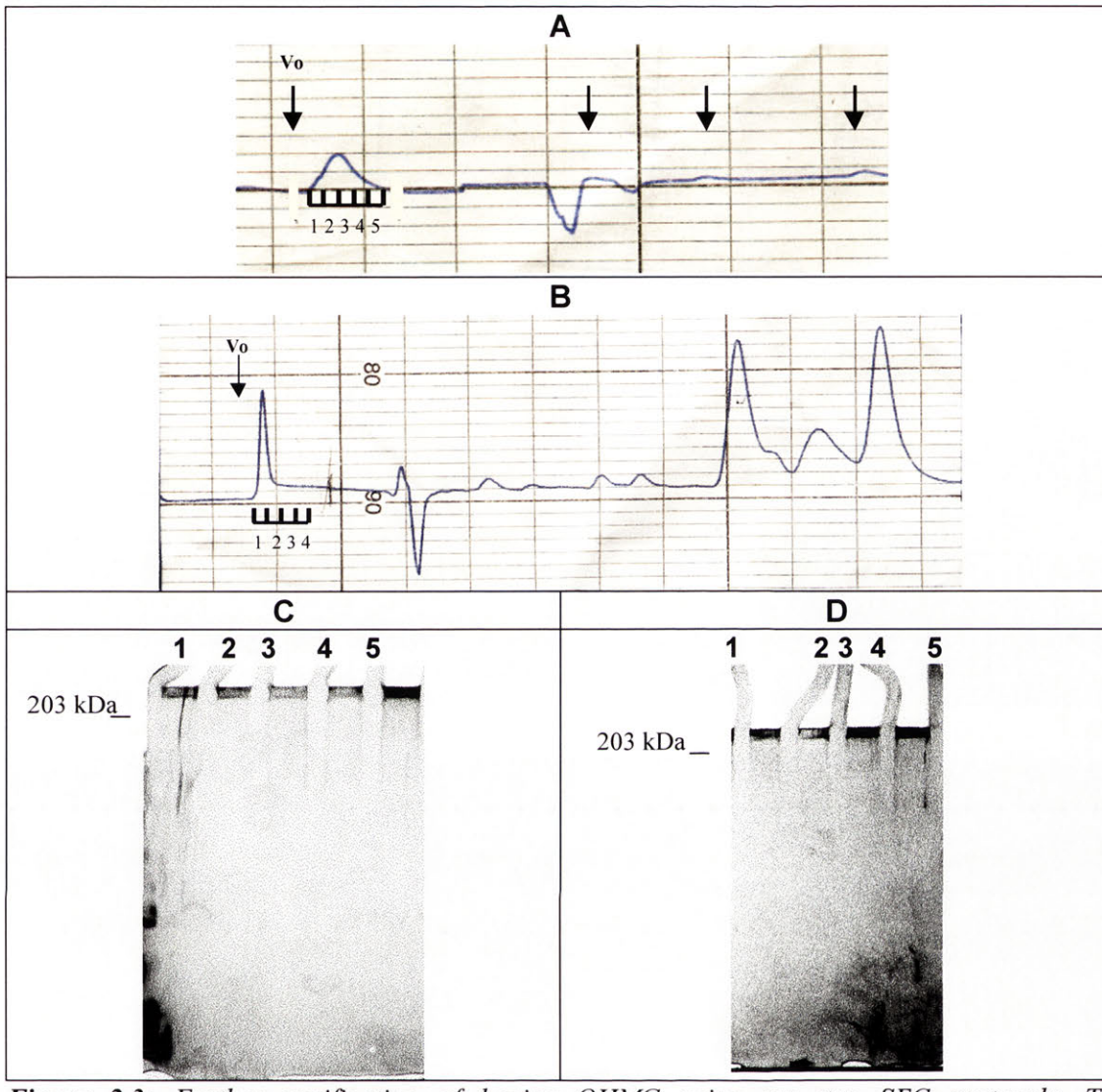

Figure 2.3: *Further purification of bovine OHMG using two-step SEC protocols. The conjunctival extract was purified using low salt buffer as described in Section 2.3.1.2, followed by [**A**] reduction and alkylation with iodoacetamide and separation by SEC a Superose 12 column using 3% (v/v) triethylamine buffer titrated to pH 5.5 with acetic acid. Alternatively, the low salt purification is followed by purification with high salt buffer (50 mM sodium phosphate buffer containing 1 M NaCl, pH 7.1) [**B**]. As can be seen, both methods result in further separation of low molecular weight material from the peak eluting at the void volume (separated peaks are marked on [**A**]). When fractions are taken across the void volume peak (as marked) and analysed using SDS-PAGE with silver staining, it is clear that both reduction and alkylation + triethylamine [**C**] and high salt buffer [**D**] have removed all proteins of molecular weight less than 203 kDa*

2.3.2 Comparison of the Standard Method of Mucin Purification with a New Single-Step Method

Both the addition of salts (increasing the ionic strength of the buffer) and the reduction and alkylation of proteins were shown to improve the purification of high molecular weight glycoconjugates from bovine conjunctival extract by separating low molecular weight components from the partially purified high molecular weight fraction. For this reason it was decided that a single-step purification protocol should be attempted using a high salt buffer under reducing conditions. To test the effectiveness of this method, comparison was made with the traditional two-step method described by Davies *et al.* (1991).

2.3.2.1 Traditional Two-Step Purification Procedure

As a control system, the ocular mucin was purified by SEC followed by CsCl density gradient centrifugation, based on the method described by Davies *et al.* (1991). The two-stage procedure was as follows:

a. Initial purification of the proteins was performed by separation on a Pharmacia (Uppsala, Sweden) FPLC system using a Superose 12 HR 10/30 pre-poured column and a Uvicord II detector set at 206 nm. Bovine conjunctival extract (as described in Section 2.2) was injected onto the column in 500 µl aliquots. The buffer system used was 50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl at pH 7 with a flow rate of 0.5 ml/min. Fractions (1 ml) were collected from the start of a rise in absorbance

(Peak 1). The total time of separation was 90 minutes. The samples were desalted by FPLC, as per Appendix B, Methods - B2.4 and lyophilised. The freeze-dried protein was then redissolved into PBS (to a concentration of approximately 2 mg/ml) for the second stage of purification (density gradient)

b. Solid guanidinium-HCl was added to a final concentration of 4 M to solutions containing partially purified bovine conjunctival mucin (as described above). CsCl was added to a density of 1.45 g/ml (determined by mass of the solution) and the solution was placed into Polyallomer Quick-Seal tubes (11 x 32 mm) (Beckman, California, USA) before being centrifuged at 120,000 rpm for 2½ hours in a Sorvall RC-M120 ultra-centrifuge using a RP 120VT rotor. Fractions (8 x 0.25 ml) were then taken from the bottom of the tube with a syringe and needle. The fractions from each tube (eight tubes per run) were combined and dialysed against water (four exchanges of 1 litre each over a period of 48 hours). Proteins were detected by silver stain after electrophoresis on Novex (California, USA) pre-cast (4-20%) SDS-PAGE gels (Appendix B, Methods - B3.1 and B4.1 respectively).

Results:

a. This initial SEC purification step resulted in three peaks which eluted with a retention time close to the void volume (approximate mass > 300-150 kDa, as determined by Mwt standards, Figure 2.4A) followed by several peaks eluting between the void and inclusion volumes. SDS-PAGE

analysis of the fractions corresponding to the first three peaks determined that each of the peaks contained both high and low molecular weight proteins (data not shown). The three initial peaks were collected into a single fraction, while the other peaks were discarded (as these contained proteins of apparent molecular weight less than 150 kDa). Multiple purifications were combined to acquire several milligrams of material for further purification.

b. SDS-PAGE analysis, with silver staining, of the fractions recovered from the CsCl gradient centrifugation showed that Fraction 2 of the gradient had a poorly staining band greater than 200 kDa molecular weight, with no evidence of proteins with molecular weights less than 200 kDa. Fractions 3, 4 and 5 of the gradient had protein bands in the range of 20-150 kDa (Figure 2.4C).

2.3.2.2 Single-Step Purification of High Molecular Weight Glycoconjugates from Bovine Conjunctiva

The reduction of disulfide bonds by dithiothreitol and inhibition of ionic interactions by high salt buffers were both shown to be effective methods of improving the separation of mucin from low molecular weight proteins (as described above in Section 2.3.1.2). A method of purification was therefore attempted using a high ionic strength running buffer (50 mM Na_2HPO_4/NaH_2PO_4, at pH 7.1 containing 1 M NaCl and 1 mM DTT) in a single SEC purification step. The high concentration of NaCl was included to inhibit ionic interactions of the high molecular weight glycoconjugates with themselves and with other proteins;

DTT was added to reduce the disulfide bonds. SEC of the crude extract was performed using a Pharmacia HR 10/30 pre-poured Superose 12 column run on a Pharmacia FPLC system using a flow rate of 0.5 ml/min for a total run time of 90 minutes. Detection was by UV absorbance using a Uvicord II detector set at 206 nm.

Results:

Separation of the crude extract by SEC with a high salt buffer under reducing conditions also resulted in three peaks eluting close to the void volume similar to that obtained by low salt SEC, but in different relative amounts (Figure 2.4B). The peaks were collected in 1 ml fractions and desalted individually, using a Pharmacia Fast Desalting HR 10/10 column at a flow rate of 1 ml/min, before being lyophilised. Analysis by SDS-PAGE and silver stain (Appendix B, Methods - B3.1 and B4.1 respectively) determined that Fraction A of this size exclusion purification had a poorly staining high molecular weight band (greater than 200 kDa) with no proteins below 200 kDa detected. Fractions B and C had several strongly staining low molecular weight bands (17-100 kDa) (Figure 2.4C).

2.3.2.3 Comparison of Purified Material By Polyacrylamide/Agarose Gel Electrophoresis

To ascertain whether the high molecular weight material recovered from a single-step SEC purification was identical to that from the traditional two-step (low salt SEC with a CsCl density gradient) purification method, two electrophoretic tests were performed. The presence of low molecular weight proteins was detected using standard SDS-PAGE with silver stain visualisation

(as described above in Figures 2.4C and 2.4D). In both cases, the final high molecular weight material was free of low molecular weight proteins (< 200 kDa). However this method of analysis is inadequate for the direct comparison of high molecular weight glycoconjugates such as mucins, as these large glycoproteins will not enter the matrix of standard (3-20%) SDS-PAGE gels. To overcome this problem, polyacrylamide/agarose composite gels were used to compare the high molecular weight glycoconjugates prepared by the two methods, as this method has been shown to allow penetration of high molecular weight glycoconjugates such as giant myofibrillar proteins (Tatsumi and Hattori 1995).

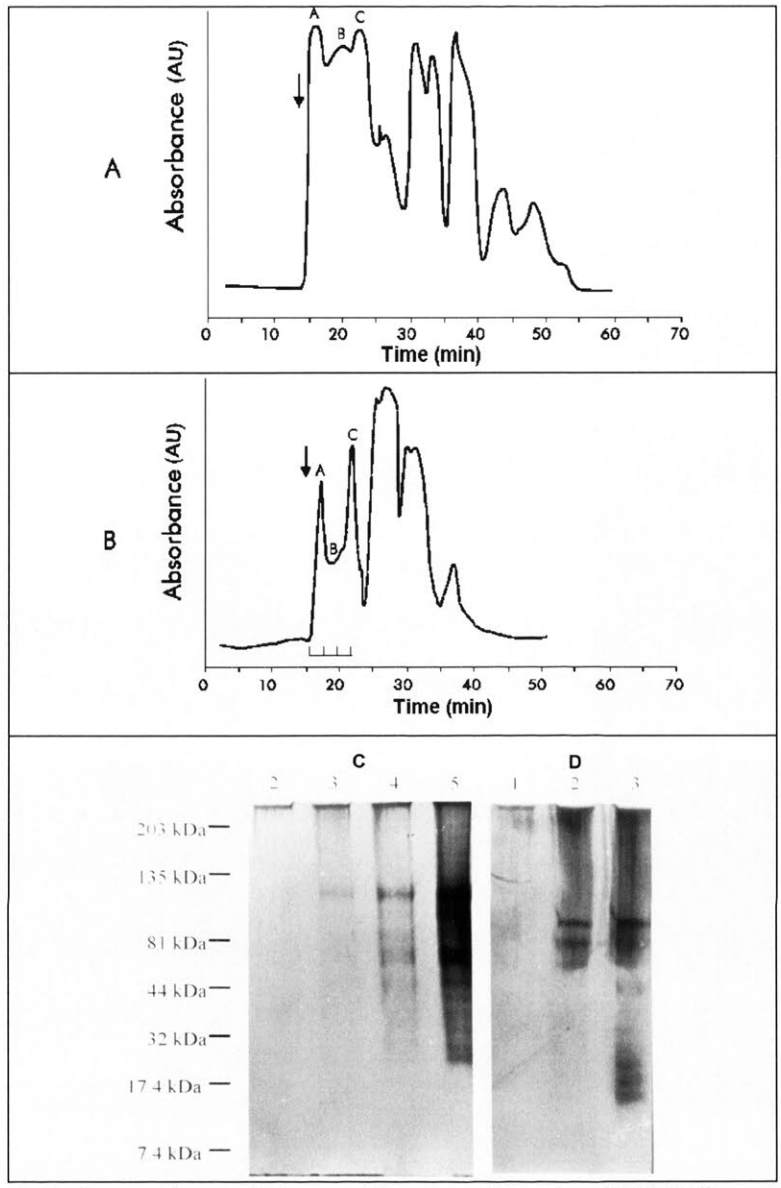

Figure 2.4: *Comparison of the purification of bovine OHMG by a single-step SEC protocol using high salt buffer under reducing conditions to a traditional two-step procedure using low salt SEC followed by CsCl density gradient centrifugation. When bovine conjunctival extract is purified by SEC using PBS [**A**] the high molecular weight glycosylated material forms a triple peak (A,B,C) with low molecular weight contaminants. When these peaks are combined and separated by CsCl density gradient (1.35-1.55 g/ml) fractions are taken from the bottom to top of the tube (most–least dense). Analysis of these fractions by SDS-PAGE with silver stain detection [**C**] indicates that the high-density fraction (Lane 2) is free of low molecular weight protein, while the less dense fractions (Lane 3-5) contained low molecular weight proteins. In contrast, separation of the extract by high salt SEC under reducing conditions [**B**] the relative proportions of the void volume triplet of peaks to each other and the peaks corresponding to low molecular weight proteins is different. Analysis of fractions taken across these peaks by SDS-PAGE with silver stain detection [**D**] shows that the void volume peak (Peak A, Lane 1) does not contain proteins less than 200 kDa molecular weight. Increasing amounts of low molecular weight proteins are observed in Peak B and C (Lanes 2 and 3 respectively)*

Method:

Slab gels (15 cm x 15 cm) of 0.6% (w/v) agarose/1.2% (w/v) polyacrylamide were prepared essentially by the method of McDevitt and Muir (1971). Agarose (0.9 g) was placed into a flask and 40 mM Tris-acetate buffer containing 1 mM EDTA and 0.1% SDS (w/v), pH 8.0 (150 ml) was added. The solution was heated to 80°C to dissolve the agarose then allowed to cool to 45°C in a water bath. Acrylamide (3.42 g) and bis-acrylamide (0.18 g) were added to a separate flask and dissolved into the same buffer (150 ml). This solution was also placed into the water bath and heated to 45°C. The solutions were combined and mixed. TEMED (30 µl) and 10% ammonium persulfate (150 µl) were added and the solution quickly mixed. The mixture was placed into a horizontal gel tray (15 x 15 cm) and allowed to set for several hours at 4°C. All materials were purchased from Bio-Rad (California, USA). 40 mM Tris-acetate buffer containing 1 mM EDTA and 0.1% SDS, pH 8.0 was used as the running buffer for the procedure (as described by Thornton *et al.* 1989 for use with agarose gels). The gels were run at 50 mA constant current for 16 hours at 4°C and were blotted onto nitrocellulose by capillary action (Appendix B, Section B3.3). Glycoproteins were visualised on the blot using the DIG glycan detection kit (Roche Diagnostics, Mannheim, Germany).

Results:

Figure 2.5 depicts the polyacrylamide/agarose gel band pattern of the high molecular weight fraction obtained by the separation of the bovine OHMG, purified by both SEC in low salt buffer, followed by CsCl gradient centrifugation (Lane 2) and by the single-step SEC purification in high salt buffer under

reducing conditions (Lane 3). BSM can be seen in Lane 1. In both OHMG lanes, three diffuse bands can be seen, one occurring just above the buffer front, one with approximately the same migration as BSM (400-500 kDa estimated Mwt, Payza *et al.* 1970), and one with only slight migration into the gel (corresponding approximately to PGM [not shown on this blot]) of estimated Mwt approximately one million Daltons (Paszkiewicz-Gadek *et al.* 1997).

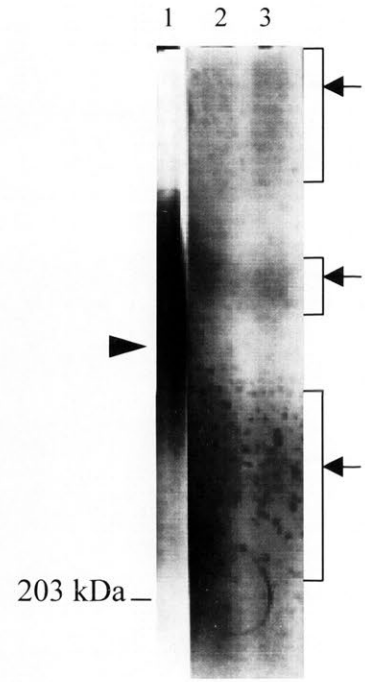

Figure 2.5: *Comparison of bovine OHMG purified by either low salt SEC and CsCl density gradient centrifugation or high salt SEC, by polyacrylamide/agarose composite gel electrophoresis. This gel was used as a comparison of the purification methods to confirm that the same material was being recovered. The gel was blotted onto nitrocellulose and stained with the Roche Diagnostics DIG glycan detection kit for visualisation purposes. Lane 1 contains BSM (with an estimated Mwt of 400-500 kDa) used as a marker protein (203 kDa was determined using Bio-Rad kaleidoscope molecular weight marker [lane not shown]). Lane 2 contains mucin purified using the traditional two-step protocol described in Section 2.3.2.1 and Lane 3 contains mucin purified using the single-step SEC purification using high salt described in Section 2.3.2.2. As can be seen the same species are present in both samples*

Kaleidoscope markers (17-203 kDa, from Bio-Rad, California, USA) were used in an attempt to calibrate the gels and the 203 kDa standard runs just above the buffer front in this gel system. However, these are non-glycosylated proteins and have been shown to have a non-linear migrational relationship with mucins (Tytgat *et al.* 1995A). Therefore an accurate determination of the molecular weights of the three bands could not be made.

As similar species are present in the final fractions and there are no low molecular weight proteins found in either preparation, it is possible to use a single-step purification of OHMG, using high salt SEC under reducing conditions to obtain the high molecular weight fraction of ocular mucus. It should be noted, however, that the relative amounts of each species of glycoconjugate differ between the two preparations, for example, the single-step purification of OHMG appeared to enrich the largest of the three species present. It is still possible that one or both of the preparations is only recovering a sub-set of the total glycoconjugate content.

2.4 ENZYMATIC DIGESTION OF OHMG TO GLYCOPEPTIDES AND COMPARISON WITH GLYCOPEPTIDES RECOVERED FROM PGM AND BSM

2.4.1 Introduction

The single-step purification method allowed purification of the high molecular weight component of ocular mucus in sufficient quantities for further studies, and analysis of the physical and chemical characteristics of bovine OHMG could now be performed.

Mucin sub-units usually have several functional domains including regions high in cysteine (that may allow cross-linking between molecules in the formation of the gel matrix), lightly glycosylated regions and a region that is densely glycosylated (Nordman *et al.* 1997) (Figure 2.6).

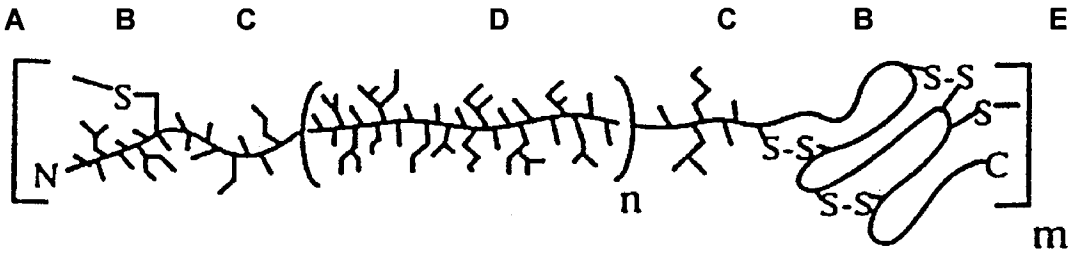

Figure 2.6. *Diagrammatic representation of a mucin molecule which depicts the predominant regions (taken from Nordman et al. 1997)*

A: N-terminal of protein
B: Cysteine-rich domains responsible for cross-linking of sub-units and possible binding of other complementary proteins
C: Non-tandemly repeated glycosylated region possibly containing N-linked oligosaccharides
D: Tandem repeat glycosylated regions, which are heavily glycosylated with O-linked oligosaccharides
E: C-terminal of molecule

It seems likely that the oligosaccharide chains are presented to the environment and are responsible for many of the characteristics of mucus, such as their water retention and ability to form gels (Neiuw *et al.* 1998). For this reason there has been a focus on the region of the sub-units that contain the glycosylation sites (both in the heavily and lightly glycosylated regions). To date, much of the data on the glycosylated regions has involved the analysis of the genes for the amino acid sequence with some attempts at sugar analysis by antibody staining and mass spectrometry (Karlsson *et al.* 1995). Many of the mucins (such as MUC-1, MUC-2, MUC-4, MUC-5AC, MUC-5B, MUC-6, MUC-7 and MUC-8) contain a tandem repeat sequence in their large glycosylated region. A tandem repeat is a sequence of amino acids that is repeated with no other sequence in between

repeat units (i.e. GTTVAPGSSNT GTTVAPGSSNT GTTVAPGSSNT GTTVAPGSSNT would be a tandem repeat sequence of GTTVAPGSSNT containing four repeats). This is the sequence reported for BSM (Gerken 1993). The tandem repeat sequences of mucin species vary in size from 6-81 amino acids (Table 1.1 lists the known repeat sequences). Since the glycosylated regions of mucins are regarded as the most important for function, the glycopeptides for PGM, BSM and OHMG were recovered in order to determine whether ocular mucus conforms to the accepted view of mucus structure.

2.4.2 Protocols for the Digestion of Mucins with Proteases

In order to chemically analyse the glycosylated regions of OHMG, the other parts of the backbone (i.e. the non-glycosylated regions) must be removed by enzymatic cleavage. However, resistance to protease digestion is one of the defining characteristics of mucin glycoproteins. Thus, the recovery of glycopeptides is often a difficult task. Historically, methods for digestion require large amounts of trypsin (Herrmann *et al.* 1999) or non-specific proteases such as pronase (Paul *et al.* 1998) or papain (Baldwin *et al.* 2000). These digestions typically require 1:1 or higher concentrations of enzyme to mucin (Shankar *et al.* 1991). Such large amounts of protease cause problems in separating the mucin glycopeptides from the enzyme and the use of large amounts of enzyme is costly. In addition, even such large amounts of enzyme do not digest the large tandem repeat sequences, which are still several hundred kDa in size. As the resultant glycopeptides are also high molecular weight glycoproteins, purification and analysis by conventional means, such as SDS-PAGE, is still difficult (Thornton *et al.* 1994). There is little data on

the composition of these regions reported in the literature other than that which is derived from genome or antibody analysis.

A non-specific serine protease has been cloned from a thermophilic fungus found in New Zealand hot springs (*Thermus sp.* strain Rt41A) (Munro *et al.* 1995). This protease is used in the preparation of DNA for polymerisation chain reaction (PCR) enrichment. The enzyme is added to blended whole cells and results in all proteins being digested, allowing the commencement of PCR (Murray *et al.* 1995). This enzyme has many advantages as a protease. It has a very high activity (a low amount of enzyme is required) and this, coupled with its ability to stay active in even the harshest of conditions (the enzyme will still function in 6 M urea 0.1% SDS, 1 mM DTT), suggested its usefulness in the digestion of mucins into glycopeptides. For comparative purposes, mucins were digested with both Rt41A and pronase.

2.4.2.1 Pronase Digestion

Pronase digestion was chosen as a comparative test for the Rt41A protocol as it has been previously described for use in the release of glycopeptides from mucin sub-units (Paul *et al.* 1998).

1 mg of mucin was dissolved into 1 ml of 50 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl and 1 mg pronase in 100 µl of the same buffer was added. The solution was well mixed and incubated for 24 hours at 37°C. A further 1 mg of pronase, dissolved into 100 µl buffer, was added and the digest incubated for an additional 24 hours at 37°C. The digest was centrifuged at

10,000 g for five minutes to remove any precipitate and the supernatant separated by SEC as described for the purification of the whole glycoconjugate fraction (see Section 2.3.2.3).

Results:

When separated by SEC, the undigested PGM was found to have a single peak corresponding to the void volume of the column and several peaks around the inclusion volume (Figure 2.7A). After digestion with pronase, a new peak of intermediate size appeared (approximately 200-250 kDa) (Figure 2.7B). Also present was a large amount of lower molecular weight material (less than 100 kDa), i.e. probably from the pronase self-digesting. Figure 2.7C shows considerable material in this molecular weight range when a pronase blank is separated under the same conditions.

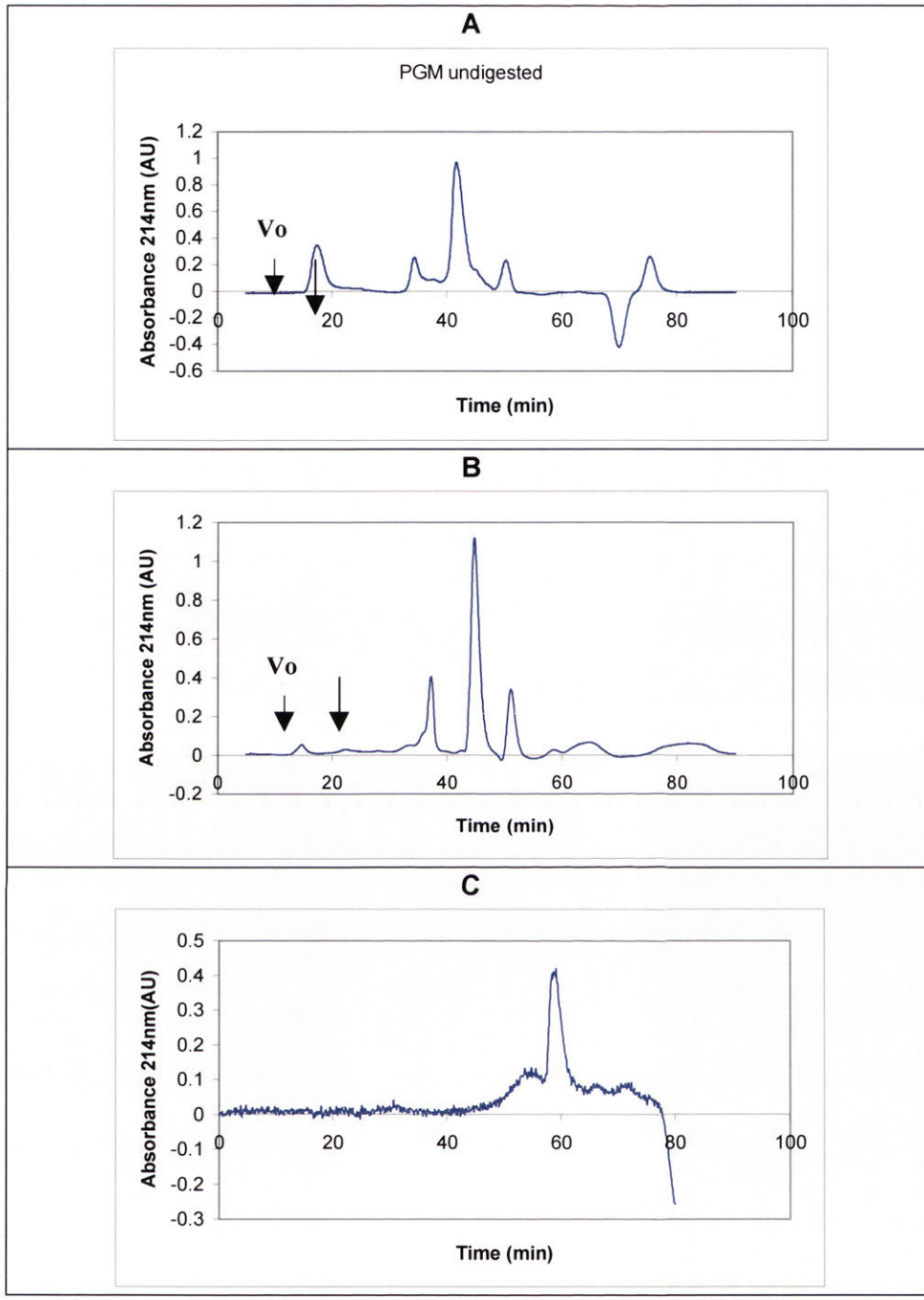

Figure 2.7: *Pronase digestion of PGM. SEC analysis of the starting material [**A**] shows a single peak at the void volume (with a slight trailing edge, marked with an arrow) followed by three peaks corresponding to low molecular weight material. After digestion with pronase [**B**] the void peak has lost area and the trailing edge has become a distinct peak. Also in evidence is an increase in the amount of low molecular weight material. This can be accounted for by the digestion of the PGM as well as the low molecular weight material contributed by the enzyme (as can be seen in the enzyme blank [**C**])*

When the digested PGM was compared to the starting material by polyacrylamide/agarose gel electrophoresis, the largest band disappeared and two smaller glycosylated proteins (still above 203 kDa, stained with the DIG glycan detection kit) appeared (Figure 2.8).

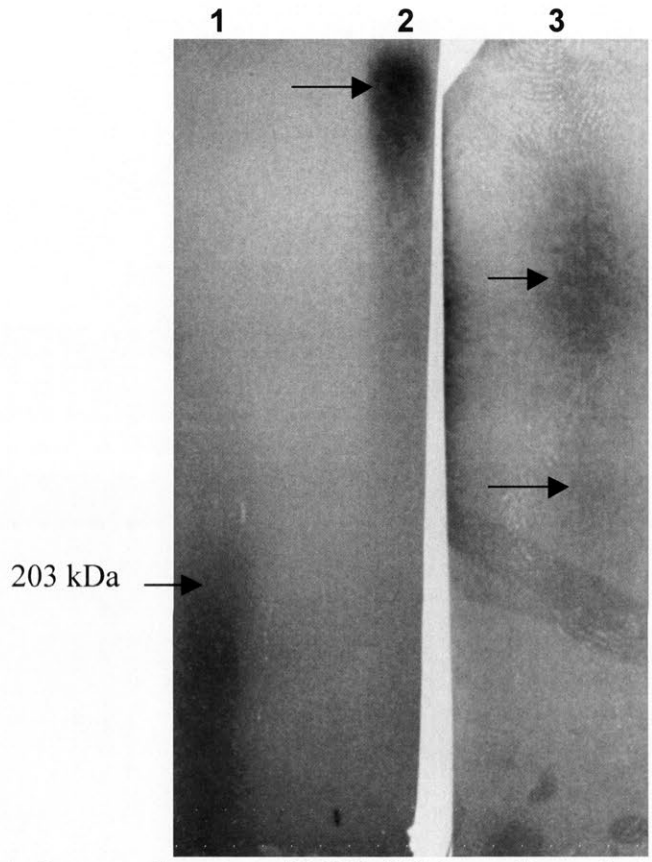

Figure 2.8: *Pronase digestion of PGM. PGM was digested with pronase for 16 hours before being separated by polyacrylamide/agarose gel electrophoresis, blotting onto nitrocellulose by capillary action and staining with the DIG glycan detection Kit (Roche Diagnostics, Mannheim, Germany). As can be seen, the single band observed in undigested PGM (Lane 2) is digested into two smaller glycosylated bands, one well staining at high molecular weight and one poorly staining band at a lower molecular weight (Lane 3). Both these bands are still a significantly higher molecular weight that 203 kDa (as can be observed from the molecular weight markers in Lane 1)*

Digestion of both BSM and OHMG with pronase resulted in the disappearance of the mucin peak (at the void volume). However no intermediate peaks were detected (Figures 2.9A and 2.9B).

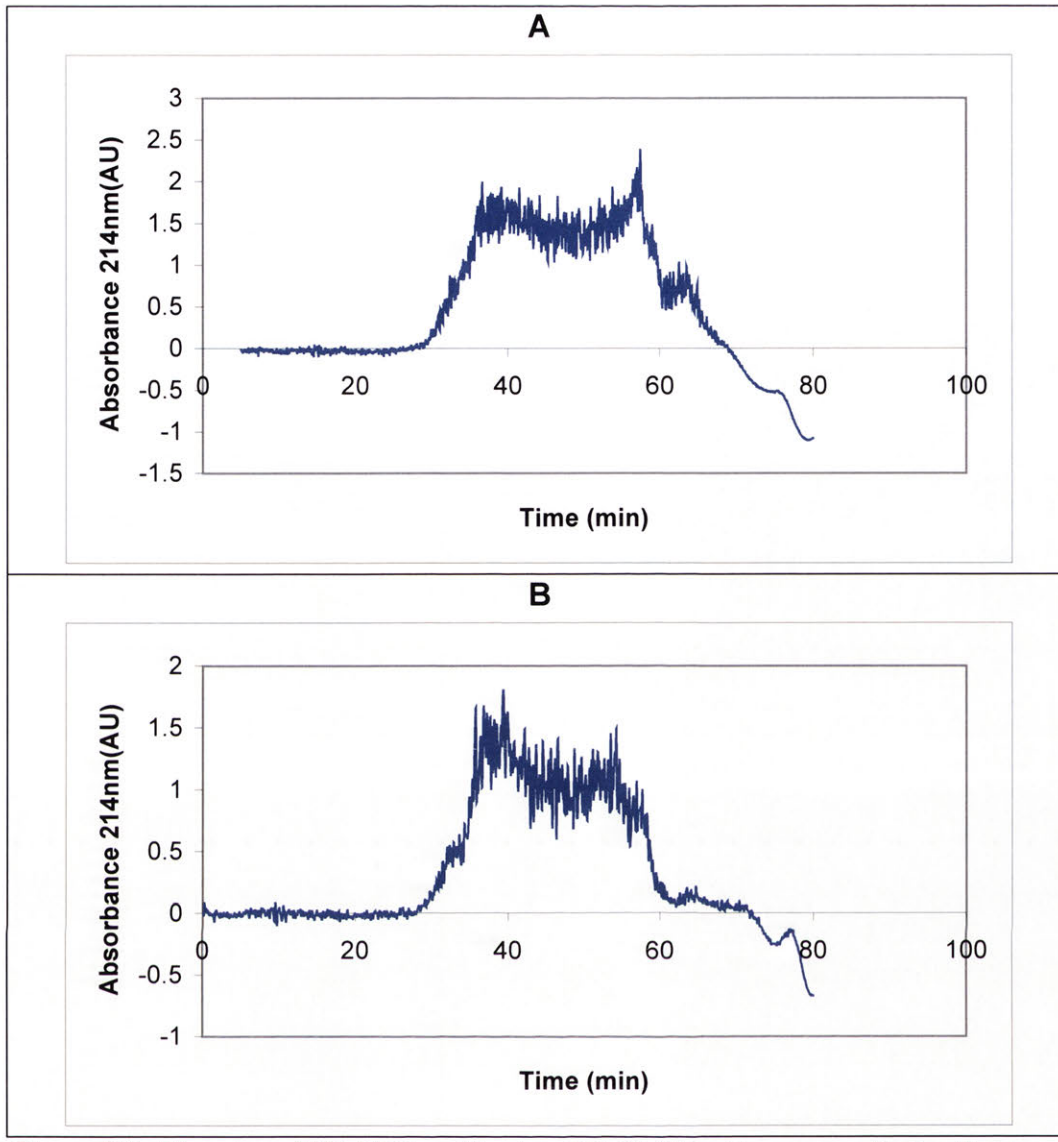

***Figure 2.9**: Digestion of BSM [**A**] and bovine OHMG [**B**] with pronase. Notice that in both cases the peak at void volume (approximately 20 minutes) is no longer in evidence. However, due to the large amount of smaller peptides generated by the self-digestion of the pronase, it was not possible to recover the glycopeptide containing fraction free of other peptides*

It is believed that BSM is more lightly glycosylated than PGM. This may also be true for OHMG. It is, therefore, possible that the pronase digested the glycoproteins into smaller glycopeptides, which may have been small enough to co-elute with the fragments of self-digested pronase. This would make purification of the glycopeptides by SEC impossible. For this reason digestion of mucins by pronase was not pursued further.

2.4.2.2 Rt41A

The efficiency of digestion and non-specific nature of Rt41A suggested it as a viable alternative to other methods of protease digestion of mucins into glycopeptides. It was also possible that Rt41A would be able to penetrate the highly glycosylated regions of PGM and allow digestion into smaller glycopeptides than were possible with pronase. In addition, as such small amounts are required for digestion, the self-digested enzyme should not interfere with the purification of glycopeptides of BSM by SEC.

2.4.2.2.1 Time Course Digestion of Mucins:

Rt41A (Pacific Enzymes Ltd, Auckland New Zealand) digests proteins and lightly glycosylated glycoproteins in minutes (Murray *et al.* 1995) but, as mucins are highly resistant to enzymatic degradation, a time course of digestion was performed to determine the optimum digestion period. The evidence from pronase digestion suggested that PGM would be more highly resistant to degradation than BSM and OHMG. For this reason three hour intervals were chosen with 3 U being added to the digest at each interval (1 U is defined as the quantity of enzyme required to released sufficient trichloroacetic acid soluble peptides from azocasein to produce a change in absorbance (at 410 nm) of 1 AU in one hour at 75°C. This is equal to 22 pmol enzyme (Peek *et al.* 1992). As the enzyme is thermally activated, the solutions were heated to 80°C (the reported optimum temperature) (Munro *et al.* 1995).

1 mg of each of the samples (PGM, BSM and OHMG) was dissolved into 500 µl of PBS pH 8.0 and 3 U Rt41A added. The solution was then heated to 80°C for 24 hours with and without the addition 3 U enzyme added at 3, 6 and 9 hours. Aliquots were taken at 0, 3, 6, 9 and 24 hours and separated using the SEC protocol developed in Section 2.3.2.3. Glycosylation was detected by dot blotting fractions onto nitrocellulose and using the Bio-Rad (California, USA) Immuno-Blot Kit for glycoprotein detection (see Appendix B, Section B4.2.1).

Results:

Figures 2.10, 2.11 and 2.12 depict the results of the SEC separations. At t=0, PGM can be separated into a mucin peak at the void volume with a tail suggesting another peak and several peaks close to the inclusion volume (Figure 2.10A). After being treated with Rt41A for six hours, the mucin peak has decreased in area with a corresponding increase in the tail edge peak (Figure 2.10B). After treatment for a total of 24 hours, the initial peak has separated into two distinct peaks (with a further decrease in area) and a corresponding increase in the lower molecular weight material. When the PGM was heated without the presence of Rt41A a small increase in the low molecular weight material was observed. However no peak of intermediate size was observed (data not shown). The intermediary peak was collected for compositional analysis. BSM and OHMG were digested in a similar fashion.

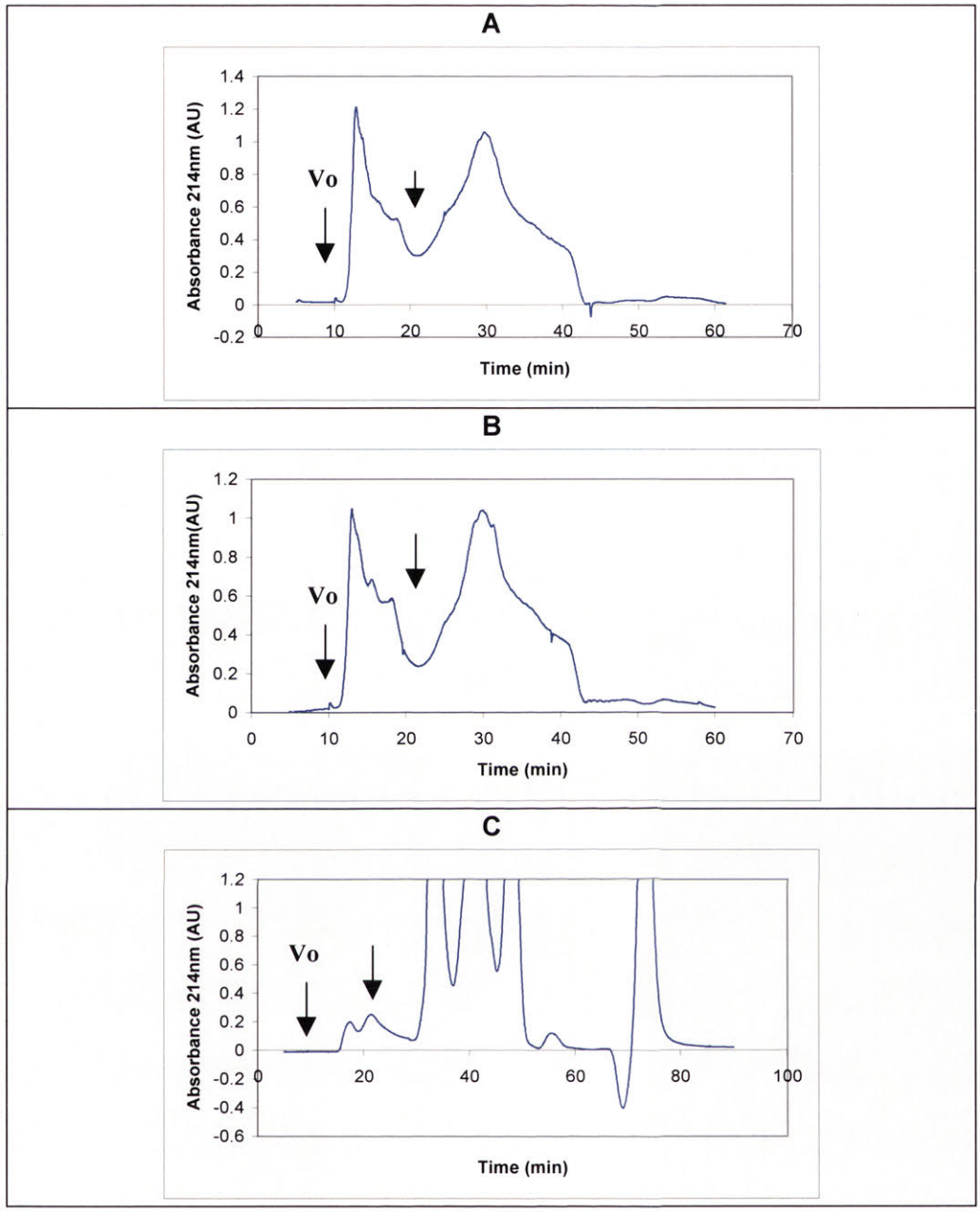

Figure 2.10: *Time course digestion of PGM with Rt41A. At t=0 [**A**] a large peak at the void volume with a smaller peak in the trailing edge can be seen (marked with an arrow) (note that as the digestion was performed on PGM (from Sigma) without further purification, there is also a large amount of low molecular weight proteins in the sample). After six hours at 80℃ [**B**] the void peak has begun to become smaller with a corresponding increase in the following peak. Following a further 18 hours at 70℃ [**C**] the void volume peak has been greatly reduced and a new distinct peak has become apparent (at approximately 20-25 minutes). This new peak was collected for compositional analysis*

Figure 2.11 follows the course of the digestion of BSM. At t=0 (Figure 2.11A) a large peak is present at the void volume - corresponding to mucin. Also present is a large peak at the inclusion volume. After three hours the void volume peak has been significantly reduced and a peak eluting in the intermediate range (100-200 kDa) can be seen (Figure 2.11B). By nine hours both the void volume and intermediate peaks have disappeared, leaving only low molecular weight peptides (Figure 2.11C).

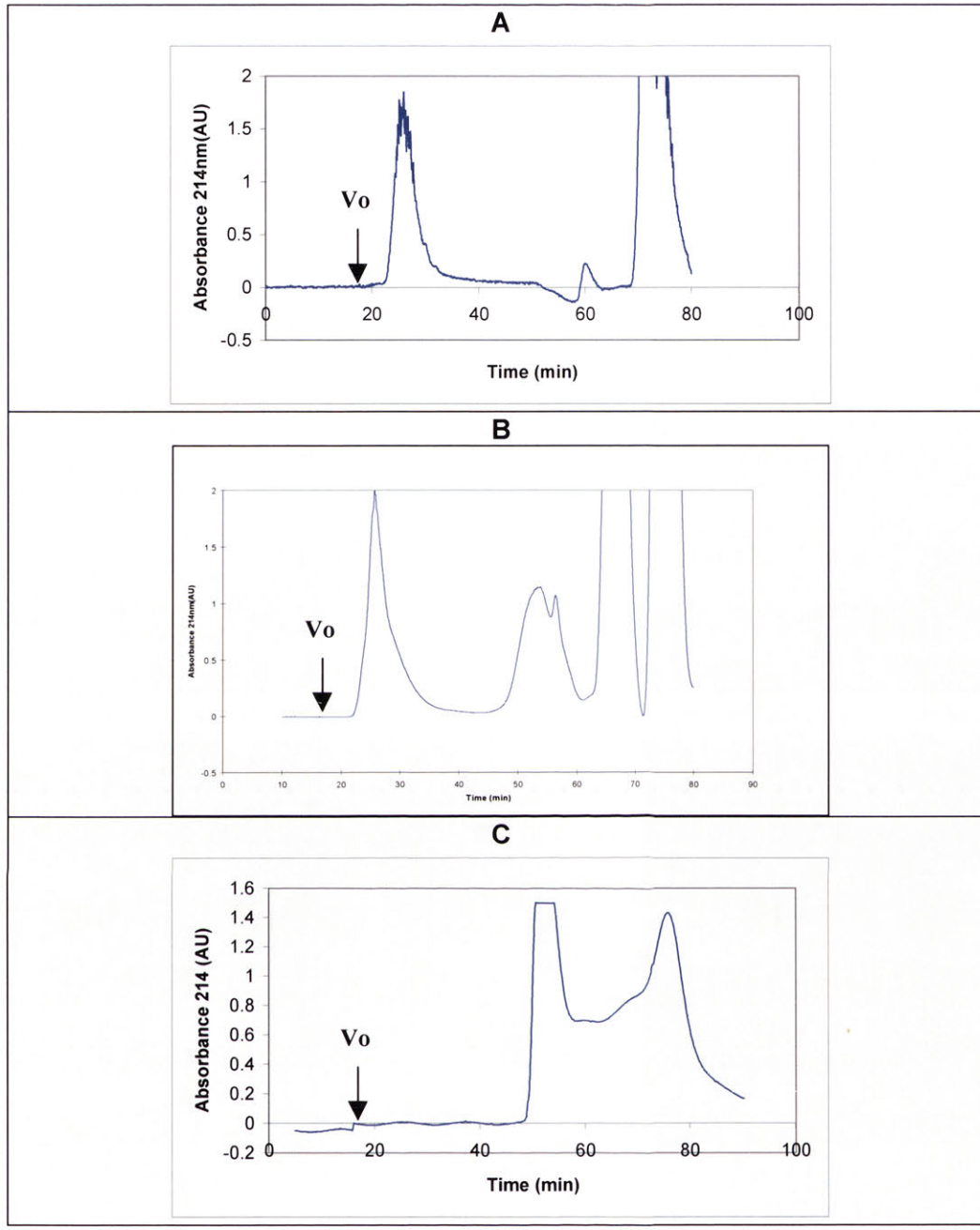

Figure 2.11: *Time course of digestion of BSM with Rt41A. At t=0 [**A**] a large peak can be observed at the void volume with another at inclusion. After digestion for three hours at 80℃ [**B**] an intermediary peak can be observed (at approximately 50 minutes). After a further six hours [**C**] only low molecular weight material is in evidence*

OHMG was also completely digested by nine hours and so peptides were recovered from the intermediary peak, which was still present after digestion for three hours (Figure 2.12). The Rt41A treatment apparently resulted in digestion to smaller components that are glycosylated, and different conditions were optimal

for PGM and BSM and OHMG. Two protocols for the digestion of mucins were used for later analysis of the digested products of the different glycoconjugates.

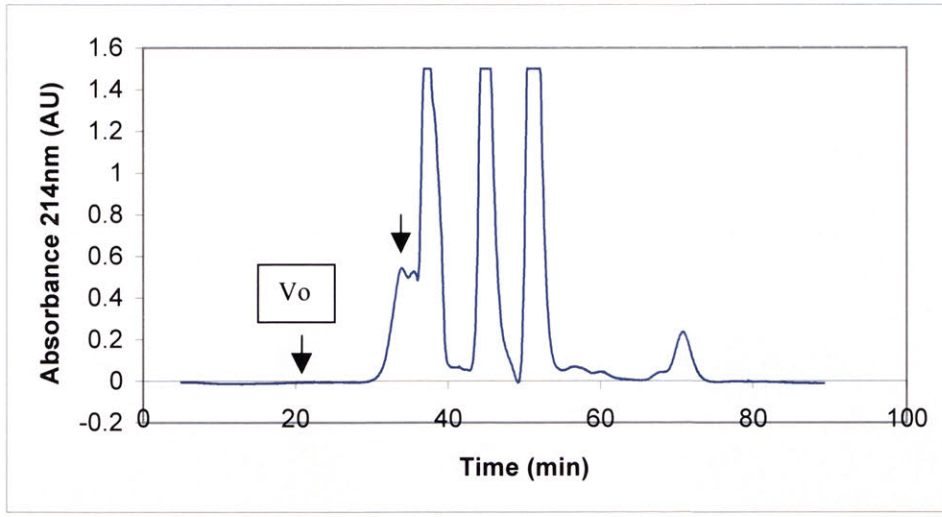

Figure 2.12: *Digestion of bovine OHMG with Rt41A. Treatment of the bovine high molecular weight glycoconjugate fraction with Rt41A resulted in a similar digestion pattern to that of BSM. After treatment for three hours at 80°C (above) the peak corresponding to the void volume (Vo) had completely disappeared, and a peak at an intermediate molecular weight (marked) was in evidence. This peak was collected and found to contain sugar by dot blotting fractions onto nitrocellulose and testing with the DIG glycan detection kit (Roche Diagnostics, Mannheim, Germany)*

a. Digestion of PGM (Heavily Glycosylated Glycoconjugates):

Mucin (0.5 mg purified by high salt SEC - see Section 2.3.2.2) was dissolved in 500 µl PBS (pH 8.0) and 6 U Rt41A was added. The solution was heated to 80°C for eight hours. A further 8 U Rt41A was added to the sample and allowed to digest overnight. The digest was purified using SEC (see Section 2.3.2.2) and the peak eluting after the void volume was collected, desalted and lyophilised for compositional analysis.

b. Digestion of BSM and OHMG (Lightly Glycosylated Glycoconjugates):

To recover the glycosylated regions of less glycosylated species such as BSM or OHMG intact, mild digest conditions were used. Mucin (0.5 mg purified as per

Section 2.3.2.2) was dissolved in 500 µl PBS buffer (pH 8.0). Rt41A (3 U) was added and the sample heated to 80°C for three hours. The digest was purified using SEC (see Section 2.3.2.2) and the peak corresponding to the largest peptide (i.e. that which eluted first) collected, desalted and lyophilised. After purification, amino acid (see Section 4.2.2) and monosaccharide and sulfate/phosphate analysis (see Section 4.2.3) were performed on the freeze-dried fractions, which were then compared to the starting material.

2.5 CONCLUSIONS / DISCUSSION

Initial extraction of the conjunctival proteins has shown that OHMG are secreted into the tear film, as they can be removed from the surface of the eye by swabbing. In addition, if the extract is concentrated 2.5X, the solution spontaneously forms a gel matrix, suggesting that the gel-forming component is present in the extract. This preliminary observation suggests that OHMG is a member of the gel forming mucin group. In the literature the gel forming mucins are generally purified via a two-step protocol involving a SEC step and a CsCl density gradient centrifugation (Corfield *et al.* 1997). This method is unsuitable for purifying the high molecular weight glycoconjugates from ocular mucus, however, as it results in high losses.

The development of a single-step method for the purification of conjunctival glycoconjugates has several advantages. By eliminating the necessity of a CsCl gradient, not only has the amount of time required to purify the mucins been drastically reduced

(several hours as opposed to several days), but the steps where large amounts of losses occur i.e. the desalting, drying and dialysis stages have been removed. Large amounts of crude material are available when purifying mucins from other tissues, such as the gastrointestinal or the respiratory tract, so losses incurred in a two-stage separation can be tolerated (Devine *et al.* 1991; Rose *et al.* 1989; Tseng *et al.* 1987). These losses, however, became unacceptable when dealing with the low amounts of material found in the eye (typically four conjunctiva will yield only 4-6 mg of purified glycoprotein). In the past, purification of mucins usually involved only the portion that was soluble in low salt, physiological buffer (Parker *et al.* 1993). This may have resulted in the purification of a sub-set of the total mucin complement.

The loss of mucin due to the protein sticking to supports and tubes, and mucins co-purifying with other proteins can be overcome by separating the mucin in high ionic strength buffer under reducing conditions. These conditions break the inter- and intra-molecular di-sulfide bonds of the complex, causing them to separate from other proteins. High salt conditions inhibit ionic interactions with other proteins and the separation matrix, as it out competes the glycoprotein ionic interactions.

The use of Rt41A to digest high molecular weight glycoconjugates, both heavily glycosylated from the colon (PGM) and lightly glycosylated from eyes (OHMG) or sub-maxillary glands (BSM) has several advantages. While Rt41A appeared to be unable to penetrate into the glycopeptide regions of PGM, it was able to produce large glycopeptides more efficiently than pronase. Only 1.2 ng (22 pmol) of Rt41A was required to digest 300 μg of mucin - a ratio of 2.5×10^{-5}:1 (enzyme:substrate) as opposed

to 1:1 for pronase. It was possible to recover the larger glycopeptides from PGM and the smaller glycopeptides of BSM and OHMG by changing the time course and digestion conditions. One disadvantage of Rt41A compared to specific proteases such as trypsin is the production of "ragged ends" i.e. the enzyme is not specific for the cleavage of a unique residue or sequence and amino acid sequencing was not possible. Therefore, if the glycopeptides were required for sequencing for using the fragments for gene analysis, some method of enriching one fragment would be required. If the cut is near a K or R then one way to enrich the peptides may be to redigest the ends with trypsin, clipping of all the longer ends, or by using other specific enzymes. Alternatively, reversed phase or ion exchange chromatography could be used to separate the fragments. However, as the focus of this thesis was compositional analysis of the fragments, the ragged ends produced by Rt41A were not a significant problem. Heating of the mucin samples to 80°C is required to digest the protein with Rt41A, this may cause some problems with the analysis of post translational modifications such as glycosylation. For example sialic acid is particularly vulnerable to hydrolysis in mildly acidic conditions at these temperatures.

The Rt41A digestion of PGM for 24 hours resulted in the mucin being digested into two large glycopeptide fractions; one at the void volume of Superose 12 (greater than 200-300 kDa apparent molecular weight) and several eluting after the void (approximately 150-200 kDa apparent molecular weight). Other similar mucins such as human gastric and intestinal mucins, e.g. MUC-6 (human gastric mucin) or MUC-2 (found in the intestines), are large gel forming mucins. MUC-2 has two distinct glycosylated regions – a large tandem repeat domain (labelled Glycopeptide A ["Gly A"]) and a smaller less glycosylated region that contains imperfect repeats (labelled

Glycopeptide B ["Gly B"], Figure 2.13) (Karlsson *et al.* 1997B). The smaller fragment collected from PGM may correspond to a smaller glycosylated region similar to the "Gly B" region reported for MUC-2. This can be confirmed using compositional analysis and is addressed in Chapter 4. The larger peak, which may contain the "Gly A" equivalent, was not collected as it co-eluted with non-digested mucin.

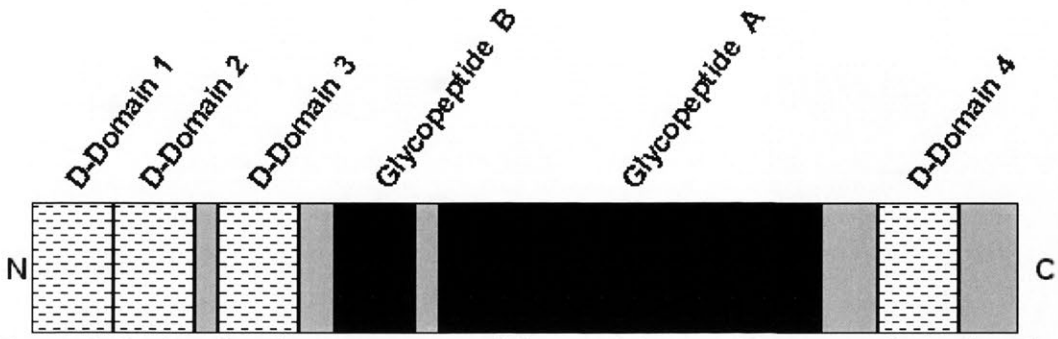

Figure 2.13: *Graphical representation of the MUC-2 gene. Note particularly the Gly A and Gly B regions. Gly A contains the large heavily glycosylated tandem repeat region (approximately 900 kDa with oligosaccharides attached), while Gly B is a smaller region that does not contain a tandem repeat (approximately 350 kDa) (taken from Karlsson 1997)*

BSM digested into a small glycosylated peptide fraction (approximately 75-120 kDa, as determined by molecular weight markers) when treated for only three hours with Rt41A. Further time resulted in the total digestion of the glycoprotein into small peptides. The tandem repeat sequence reported for BSM has few attachment sites for O-linked oligosaccharides present. It is possible that the oligosaccharides present on BSM may not be able to sufficiently inhibit the activity of Rt41A (it is reported that the majority of oligosaccharides present on BSM are 1-4 monosaccharide units in size) (Chai *et al.* 1992).

Recovery of a glycosylated peptide fraction from OHMG was also possible after three hours of digestion with Rt41A. Continuation of the digest resulted in total digestion of the molecule by nine hours. This suggests the possibility that the structure of OHMG may

be similar to that of BSM. This then poses an interesting problem for the development of a model for ocular mucus. The mucus of the eye is similar in characteristics to that of other epithelial surfaces. It provides protection from physical and bacterial invasion in a similar way to the mucus of the gut or respiratory tract. The glycoprotein component of the mucus is secreted (can be removed from the surface of the eye by non-invasive means) and spontaneously forms gels when the correct concentration is reached. However, when the high molecular weight component of the extract is purified and analysed for its physical properties, it has characteristics more closely related to smaller non-gel forming mucins such as BSM. Some of the mucins that have been reported to be found in the eye (MUC-2 and MUC-5AC) (Berry *et al.* 2000; Gipson *et al.* 1995; Tei *et al.* 1999) would be expected to have characteristics similar to those of PGM. The others species reported in the eye (MUC-1 and MUC-4 which are membrane bound (Perez-Villar and Hill 1999) and MUC-7 which is non-gel forming (Wickstrom *et al.* 2000) may not be a part of the mucus gel, rather linked to it while attached to the cell membrane of the epithelium. It is therefore possible that they were not extracted by the procedures used. Compositional analysis of the OHMG fraction, and the glycosylated fractions recovered after protease digestion, could further understanding of these discrepancies.

This chapter has described improvements in the protocols of purification and digestion of sufficient quantities of the high molecular weight glycosylated fraction of ocular mucus, to enable these analyses to be performed.

CHAPTER 3: FRACTIONATION OF OCULAR HIGH MOLECULAR WEIGHT GLYCOCONJUGATES (OHMG)

3.1 INTRODUCTION

Traditionally, analysis of mucus has involved the whole high molecular weight fraction (Chao and Butala 1986). However, several species of mucins have been reported in the mucus layers of epithelial surfaces (Corfield *et al.* 1997). The physical properties of the OHMG fraction, such as gel-like properties and ability to be digested by proteases, were shown in Chapter 2 to be substantially different to those of PGM and BSM. In order to characterise this fraction, it was further separated into its components by two methods. A novel method of SDS-PAGE was developed for electrophoretic analysis and a previously described method of ion exchange chromatography (Thornton *et al.* 1994) was modified to recover species for chemical analysis.

Electrophoresis of mucin glycoproteins is made difficult by their large size, charge density and three-dimensional structure. The heavily glycosylated gastric mucins, such as PGM and the human mucin coded by the MUC-2 gene, have sub-units with molecular weights in the order of millions of Daltons (Corfield *et al.* 1997). The high density of oligosaccharide chains on the amino acid backbone of mucin sub-units gives the protein a rigid structure, causing it to have an apparent molecular weight much higher than that of a globular protein (Sheehan *et al.* 1991). These rigid structures make it difficult for the molecule to pass through a polyacrylamide gel matrix, or to enter into a SEC pore. Finally, the high negative charge density, resulting from either sialic acid and/or sulfate substitution of the molecules, inhibit the correct binding of sodium dodecyl sulfate,

making the analysis of mass by conventional SDS-PAGE ineffective. Mucins will not migrate through any but the lowest percentage polyacrylamide gels (typically only 2-3% gels can be used) (Wells *et al.* 1986). In fact, some groups have used the inability of mucins to migrate through conventional PAGE as a method of purification (Paszkiewicz-Gadek *et al.* 1995). In addition, even if low percentage gels are used, the migration of the mucin species is not a linear relationship with non-glycosylated proteins. This makes conventional molecular weight markers unusable to determine molecular weight. Polyacrylamide gels of such low concentrations have the added difficulty of low physical stability (the critical acrylamide concentration to form a gel is just under 2%) (Tatsumi and Hattori 1995), making handling these gels without damaging them difficult.

Several strategies have been used to overcome these difficulties. The most successful of these has been the adoption and modification of electrophoresis techniques used for other large molecules, such as agarose gel electrophoresis developed for DNA/RNA (Thornton *et al.* 1989) and polyacrylamide/agarose composite gel electrophoresis developed for the analysis of poly-amino glycans (McDevitt and Muir 1971). In 1993 Wenisch and her co-workers described a method for producing macroporosity in polyacrylamide gels using polyethylene glycol (PEG). The PEG sequesters the water in the solution and forms tiny micelles which then force the acrylamide to polymerise around them. This causes the acrylamide to form long strings rather than a tight mat when polymerising, thus, pores of orders of magnitudes larger are formed (Figure 3.1). The paper describes several concentrations and sizes of PEG resulting in the gel having different pore sizes and physical characteristics. The advantage of this system is that, in most other respects, the gels can be treated as normal polyacrylamide gels. This method was adapted to try to sub-fractionate the high molecular weight ocular glycoconjugates.

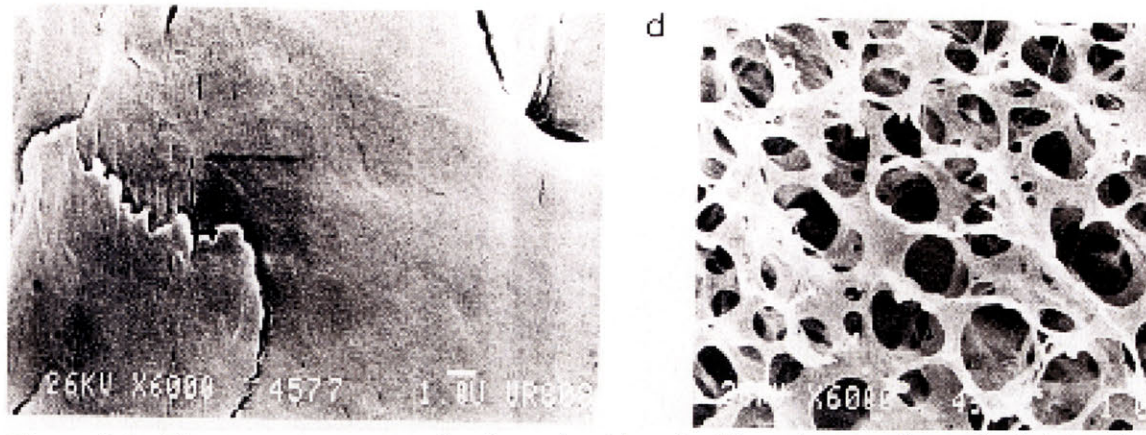

Figure 3.1: *Electron microscopy of polyacrylamide gels. The micrograph on the left is of a typical 5% acrylamide gel containing 7% bis-acrylamide. The right micrograph represents the same gel with 5% PEG 10000 added before polymerisation. The increase in pore size by the addition of the PEG is clearly visible. Taken from Righetti et al. 1992*

As another approach, Thornton *et al.* (1994) have described a method for the separation of mucin species from a purified high molecular weight fraction using anion exchange chromatography (IEC). Mucins are generally highly charged due to the presence of sialic acid and/or sulfate modifications. The high negative charge means that buffer systems traditionally used for anion exchange chromatography are not effective in eluting the molecules from an IEC column, as the molecules remain bound to the column media. In order to elute the mucin species from an anion exchange column, a more highly charged buffer is required and lithium perchlorate has been found to be suitable (Thornton *et al.* 1994). This method was applied to the high molecular weight fraction obtained by high salt SEC of bovine conjunctival mucus.

3.2 ELECTROPHORESIS

3.2.1 Materials and Methods

3.2.1.1 Agarose Gel Electrophoresis

The method used was taken from Thornton *et al.* (1994).

1% (w/v) DNA grade agarose (Sigma, MO, USA) was prepared in 40 mM Tris-acetate buffer pH 8 containing 1 mM EDTA and 0.1% SDS. Gels were poured into 15 cm x 15 cm slab gels for use in a Pharmacia (Uppsala, Sweden) horizontal gel apparatus. Mucin was loaded onto the gel (25 µl per lane) and the gel run at 35 mA constant current at ambient temperature overnight (approximately 16 hours). The gels were blotted by capillary action (see Appendix B, Section B3.3) and the membranes stained using a DIG glycan detection kit (Roche Diagnostics, Mannheim, Germany).

3.2.1.2 Polyacrylamide/Agarose Composite Gels

The high molecular weight peak of bovine conjunctival extract from high salt SEC (see Section 2.3.2.2) was separated into two fractions (leading and trailing edge of the peak). Slab gels (15 cm x 15 cm) consisting of 0.6% agarose/1.2% polyacrylamide were prepared essentially by the method of McDevitt and Muir (1971) using 40 mM Tris-acetate buffer containing 1 mM EDTA and 0.1% SDS, pH 8.0. 30 µl of sample was loaded per well and the gels were run at 50 mA constant current for 16 hours at 4°C and were blotted onto nitrocellulose by capillary action (see Appendix B, Section B3.3). Glycoproteins were visualised using the DIG glycan detection kit (Roche Diagnostics, Mannheim, Germany).

3.2.1.3 Polyethylene Glycol (PEG) Modified SDS-PAGE

Mini gels were poured using the Bio-Rad Mini Protean II system (California, USA). Gels were poured with concentrations of PEG 8000 between 1% (w/v) and 4% (w/v) as per Wenisch *et al.* (1993). PGM was separated using these gels in order to determine the optimum PEG concentration. A concentration of 2.5% (w/v) was optimal as it produced the tightest bands while allowing migration of the mucin species. Acrylamide stock was 40% (w/v) containing 7% (w/v) PDA and the final concentration in gel was 5% (w/v) acrylamide. The gel buffer used was 24 mM Tris-acetate, pH 6.4 containing 0.06% (w/v) SDS. Gels were set for several hours at room temperature or overnight at 4°C. A diphasic system was used with the anode buffer being 45 mM Tris-acetate, pH 6.6 containing 0.04% (w/v) SDS and the cathode buffer 80 mM Tricine titrated to pH 7.1 with Tris base, containing 0.06% (w/v) SDS and 2.5% (w/v) PEG 8000. Gels were run using a Bio-Rad (California, USA) Mini Protean II Gel System using the following program:

- 2 mA gel constant current - one hour
- 5 mA per gel constant current - one hour
- 10 mA per gel - one hour
- 20 mA per gel one hour (the buffer front runs off the gel at approximately three hours running time).

The resulting gels were visualised in one of three ways:

- The gels were loaded with reduced glycoprotein that was alkylated with a fluorophore containing agent (coumarin maleimide) (see Appendix B, Section B1.2.2.) and visualised by placing the gel on a UV light box;

- The gels were blotted onto an immobilising membrane (nitrocellulose or PVDF) and visualised with the Bio-Rad Immuno-Blot glycan detection kit; or
- The gels were stained using the periodic acid Schiff's reagent stain as per Thornton *et al.* (1994) (Appendix B, Section B4.2.2).

3.2.2 Results

3.2.2.1 Agarose Gel Electrophoresis

When using the agarose gels it was found that the molecular weight markers all migrated at the buffer front with the exception of the 203 kDa marker which was slightly retarded. Staining for glycoproteins with the DIG glycan detection kit resulted in large smears for all species. Figure 3.2 depicts a DIG stained nitrocellulose membrane. Lane 1 contains PGM. As can be seen, the lane has a continuous smear of staining from the well to the buffer front with some areas of higher density at the high (just below the well) and intermediate molecular weight ranges. Three distinct bands were observed in the lanes containing bovine OHMG (Lanes 2 and 3). One band was observed in the high molecular weight region (just below the area of high staining in PGM), one intermediate (with similar migration to the intermediate staining for PGM) and one at the low molecular weight range (just above 203 kDa). The agarose gels proved difficult to stain, often resulting in

high background staining. This, coupled with the diffusion of the glycoproteins through the gel and the smearing of the samples, resulted in the discontinuation of this method.

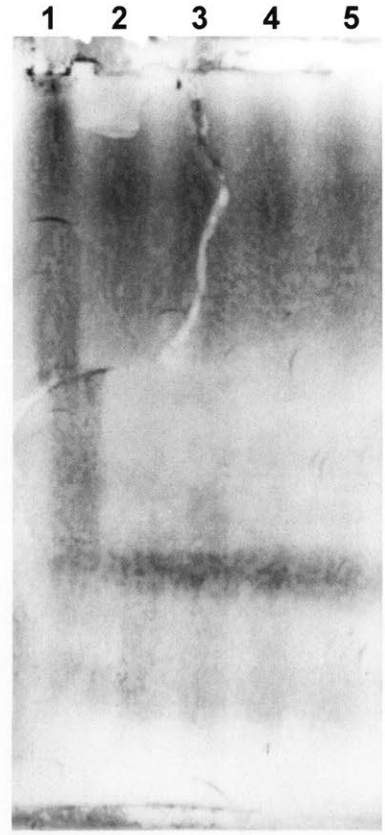

Figure 3.2: *Agarose gel electrophoresis of PGM and bovine OHMG. When agarose gel electrophoresis was used to analyse PGM (Lane 1) and bovine OHMG (Lanes 2-5), large smears were in evidence. One continuous smear running down the entire lane for PGM and three wide diffuse bands were observed for OHMG. Because the ability of agarose gel electrophoresis was determined to be insufficient, the method was not continued*

3.2.2.2 Polyacrylamide Agarose Composite Gels

Polyacrylamide/agarose composite gel electrophoresis of the high molecular weight (> 200 kDa) material, purified from bovine conjunctiva, showed that this fraction contained three distinct glycosylated bands (Figure 2.5). When the whole fraction was separated into leading and trailing edge of the V_o peak, it was observed that the leading edge of the peak contained the high and intermediate species, while the trailing edge contained the intermediate and lower band

(Figure 3.3). This suggests that the three bands are distinct when separated by high ionic strength SEC and not associated in any way. This method of electrophoresis still results in broad diffuse bands and, as such, was not continued.

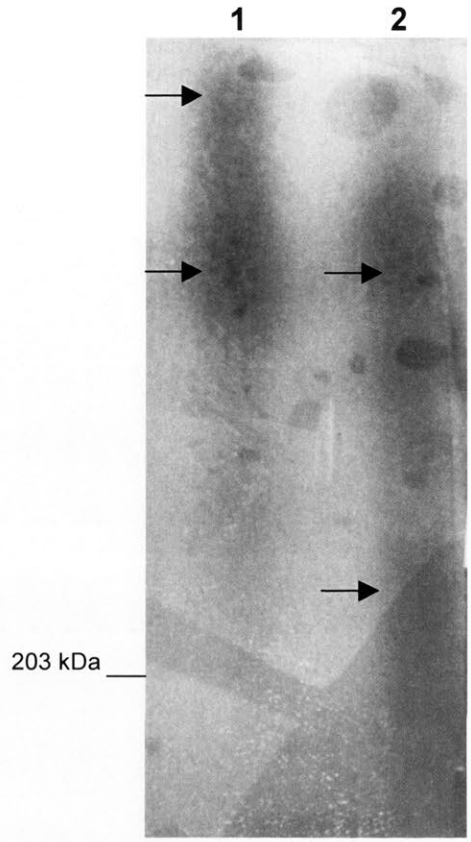

Figure 3.3: *Polyacrylamide/agarose composite gel electrophoresis of the high molecular weight glycoconjugate fraction of bovine conjunctival mucin. When the void volume peak from high salt SEC purification of extract (see Chapter 2, Section 2.3.2.2) is split into the leading and trailing edge and analysed by polyacrylamide/agarose composite gel electrophoresis, it can be seen that the three bands observed in the whole fraction are split. The leading edge of the peak (Lane 1) contains the very high molecular weight material and some of the intermediate material, while the trailing edge has intermediate and lower molecular weight material (Lane 2). As the bands are diffuse and produce smears on these gels as they did for agarose gels, the method was not continued*

3.2.2.3 PEG Modified SDS-PAGE

Several mucin types were separated using PEG modified SDS-PAGE, including PGM, BSM, Gly A (from rMUC-2, supplied by Ingmar Carlstedt) and bovine OHMG prepared by high salt SEC. It was found that proteins of less than 200 kDa ran with the buffer front (by loading Bio-Rad Kaleidoscope pre-stained markers

onto the gel), but that separation of high molecular glycoconjugates occurred. It is known that Gly A is a glycoprotein of approximately 900 kDa (Carlstedt *et al.* 1993), so this protein was used as a molecular weight marker for the other glycoconjugates. When PGM was separated, it was found to consist of two heavily and one lightly staining bands - one above Gly A, one approximately the same size as Gly A, and one lightly staining band smaller than Gly A (stained with PAS as per Appendix B, Section B4.2.2) (Figure 3.4).

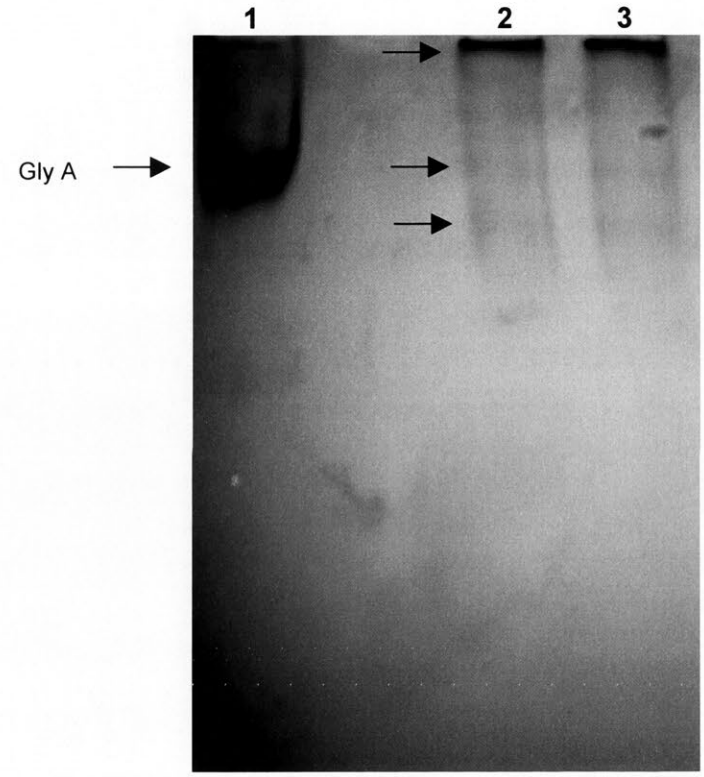

Figure 3.4: *Separation of PGM on PEG modified SDS-PAGE gel. Lane 1 contains Gly A made from digestion of human MUC-2 gene product with trypsin (supplied by Ingmar Carlstedt). This peptide is reported as being between approximately 900 kDa (Carlstedt et al. 1993). Lanes 2 and 3 contain repeat runs of PGM. Visible in each of these lanes are three bands. One that has not completely entered the gel, one with approximately the same migration as Gly A and one below it. As no further analysis was performed, it is not possible to determine what these bands represent. Staining was with PAS stain as per the method described by Thornton et al. 1994 (Appendix B, Section B4.2.2)*

It is not clear whether they represent distinct species or glycosylation variants or even aggregates. Bovine OHMG and BSM also separated into three major bands. The lower of the bands corresponded to just over 200 kDa, an intermediate band,

and the highest band, which migrated only slightly on a 2.5% PEG gel (Figure 3.5).

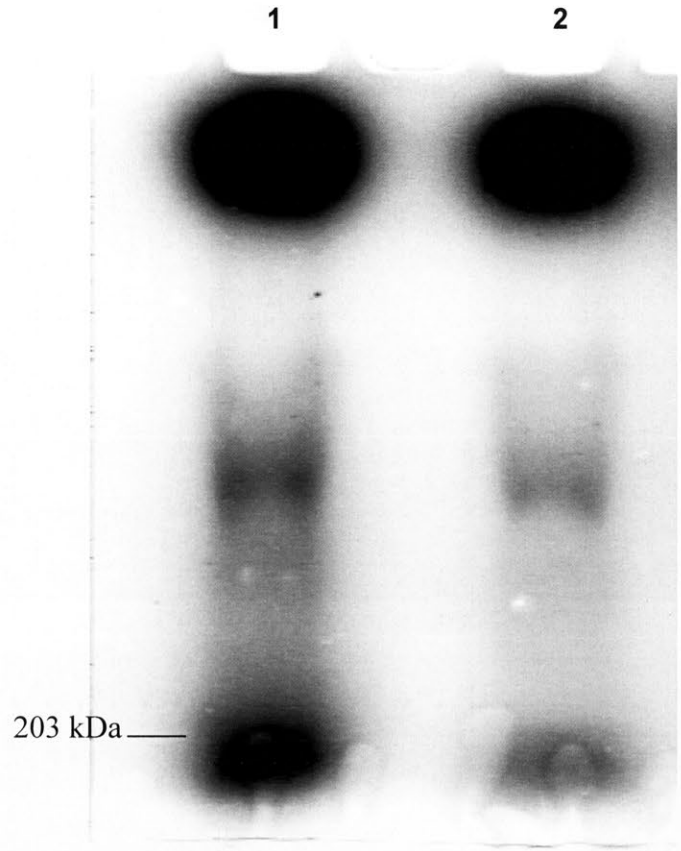

Figure 3.5: *Separation of BSM and OHMG species using PEG modified SDS-PAGE after fluorescent alkylation. Lane 1 contains bovine OHMG, while BSM is in Lane 2. For OHMG, three bands are clearly visible with several less distinct bands. It is not known whether the three major bands correspond to those observed on polyacrylamide/agarose gels or if a new division has occurred. Interestingly the single smear that was observed for BSM on composite gels has also been resolved into three bands. Samples were visualised by reducing and alkylating with a fluorophore (coumarin maleimide) (see Appendix B, Section B1.2.2)*

Close inspection of repeated analysis of the band pattern of the two glycoconjugates indicates that the migrations of each of the species are slightly different. Furthermore, the relative amounts of each of the bands is different. OHMG has a larger amount of the intermediate and low molecular species than that of BSM. Most interesting, however, is the tendency of mucus to have three major species analysed by gel electrophoresis. This was observed for PGM, BSM

and BCM when analysed by PM SDS-PAGE. Furthermore, three major species have been observed in such diverse mucus secretions as human airways (Thornton *et al.* 1990), saliva (Versura *et al.* 1986), and amphibian egg coat mucin (Morelle *et al.* 1998). On occasion, when bovine OHMG has been analysed by PM SDS-PAGE, several other bands have also been observed (as many as four to five, ranging from just above the buffer front to just below the largest of the prominent bands - contrast too low to photograph). It is unclear whether or not these three bands are similar to the three bands observed in the polyacrylamide/agarose composite gels (see Figure 3.3) or if they are a further division of one of the bands. However, the amount of material that could be loaded onto the gels was only suitable for analytical analysis. It was not possible to recover sufficient material from the bands to perform further characterisation.

3.3 ION EXCHANGE CHROMATOGRAPHY

A method of mucin purification using anion exchange chromatography which was first described by Thornton *et al.* (1989) was modified to try to obtain preparative amounts of the OHMG species.

3.3.1 Materials and Methods

Proteins were extracted from bovine conjunctiva and the high molecular weight fraction of the extract was separated from low molecular weight by high salt SEC (see Sections 2.2 and 2.3.2.2). This fraction was desalted and lyophilised. 2 x 2 mg of the dried sample was re-dissolved into 10 mM piperazine buffer containing 6 M urea and 0.02% (w/v) CHAPS, one sample at pH 5.0 and one at pH 7.0 (1 ml each). These solutions were then separated on a Bio-Rad Biologic System using a Pharmacia Mono Q (HR 5/5) column using the following:

- Buffer A = 10 mM piperazine buffered to either pH 5.0 or 7.0 with HCl, containing 6 M urea and 0.02% (w/v) CHAPS.

- Buffer B = 10 mM piperazine buffered to either pH 5.0 or 7.0 with HCl, containing 6 M urea, 0.02% (w/v) CHAPS and 0.5 M lithium perchlorate.

A linear gradient of 0-80% buffer B over a period of 50 minutes was used (for the complete program, see Appendix B, Section B2.1). Fractions were collected at 0.5 ml intervals. The fractions were slot blotted onto nitrocellulose using a Bio-Rad Slot Blot apparatus and glycoprotein fractions were detected using a Bio-Rad Immuno-Blot Kit for Glycoprotein Detection. Fractions found to contain glycoproteins were dialysed against water (six changes over 72 hours) and lyophilised for further analysis. For comparative purposes, similar purifications were performed on PGM and BSM.

3.3.2 Results

As mucin-like glycoproteins do not significantly absorb UV light at 280 or 253 nm (the standard wavelengths used to detect proteins), 214 nm is usually used. However urea, which is required for the solubilisation of the glycoconjugates, absorbs UV light at this wavelength and, as such, the UV detector was close to saturation. For this reason fractions were collected throughout the separation for slot blotting and detection by a glycoprotein stain for the presence of glycosylation.

Figures 3.6, 3.7 and 3.8 depict the separation of PGM, BSM and OHMG at pH 5.0 and 7.0. Some differences can be seen in the UV absorption between the species, the most notable being the broad peak eluting later in the program in the BSM separation (30-42 minutes). Fractions were collected, blotted onto nitrocellulose and stained for glycosylation by the Bio-Rad Immuno-Blot Glycan detection Kit (Figures 3.6C, 3.7C and 3.8C). Four distinct series of fractions were stained in PGM (Fractions 2-6, 9-12, 21-30 and 35-41). This was also the case for BSM (Fractions 1-4, 7-9, 13-17 and 31-39). Three peaks were present when OHMG was separated by high ionic strength anion exchange chromatography at pH 5.0. The separation of the peaks appears to be more distinct in the pH 7.0 buffer IEC, as the OHMG preparation was split into five glycosylated peaks (Fractions 1-4, 9-11, 13-18, 22-30 and 34-38) and this buffer was used for preparative scale separations. The five peaks of glycosylation were then collected for further analysis (see Chapter 4).

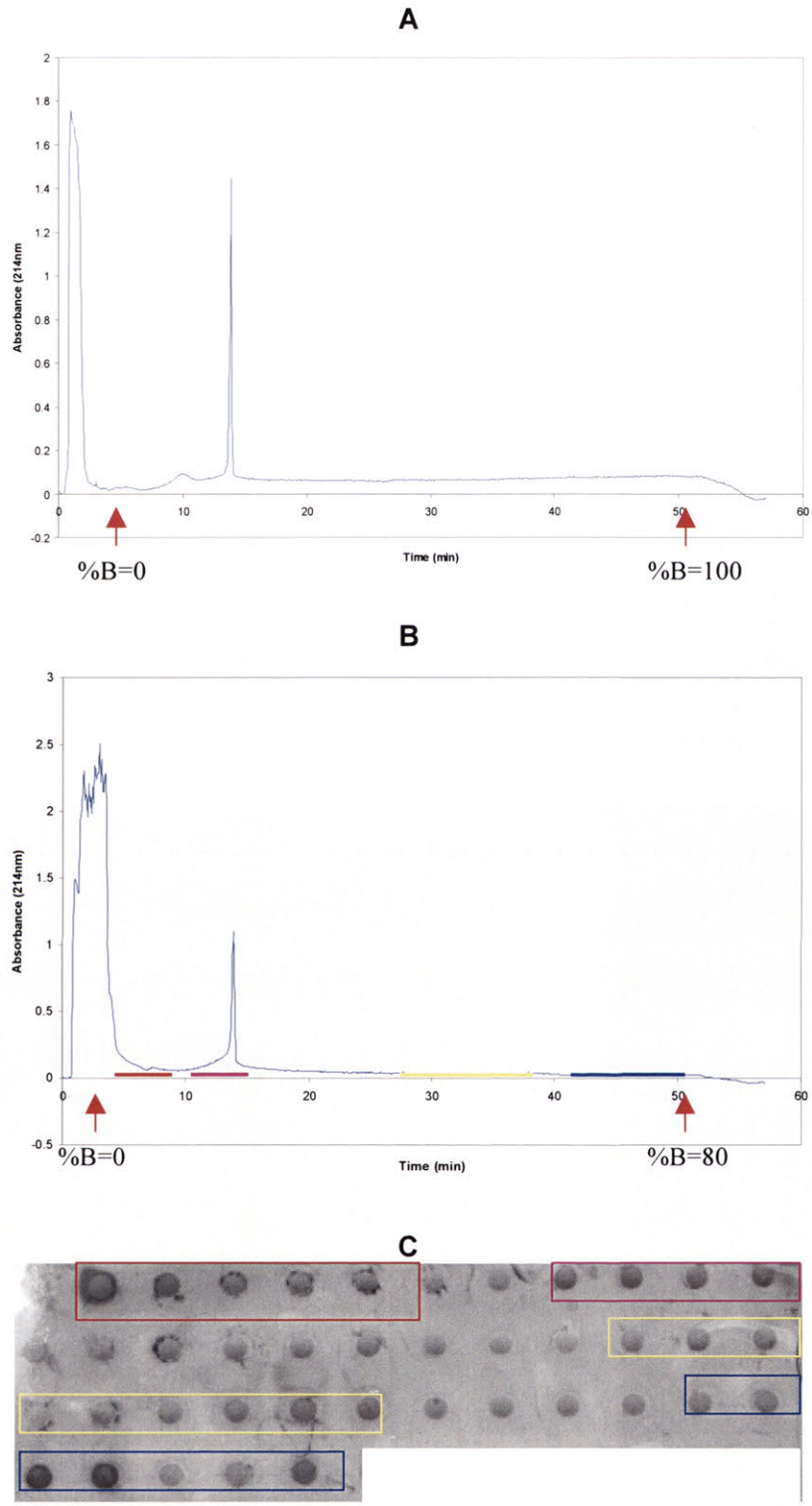

Figure 3.6: *High ionic strength anion exchange chromatography of PGM. Separations were performed at pH 5.0 [**A**] and 7.0 [**B**]. Note that this buffer is highly absorbent at 214 nm and, as such, there may be peaks that are masked in the trace. Using pH 7.0 buffer resulted in better separation of species and resulted in four distinct glycosylated regions being recovered [**C**]. Fractions were collected at %B=0 and stopped at %B=80.The fractions were blotted onto nitrocellulose and glycosylation detected for using the DIG glycan detection Kit (Roche Germany) The spots were then compared to a negative control and fractions that stained more heavily were collected. These fractions could be divided into four groups as indicated by the colored Boxes*

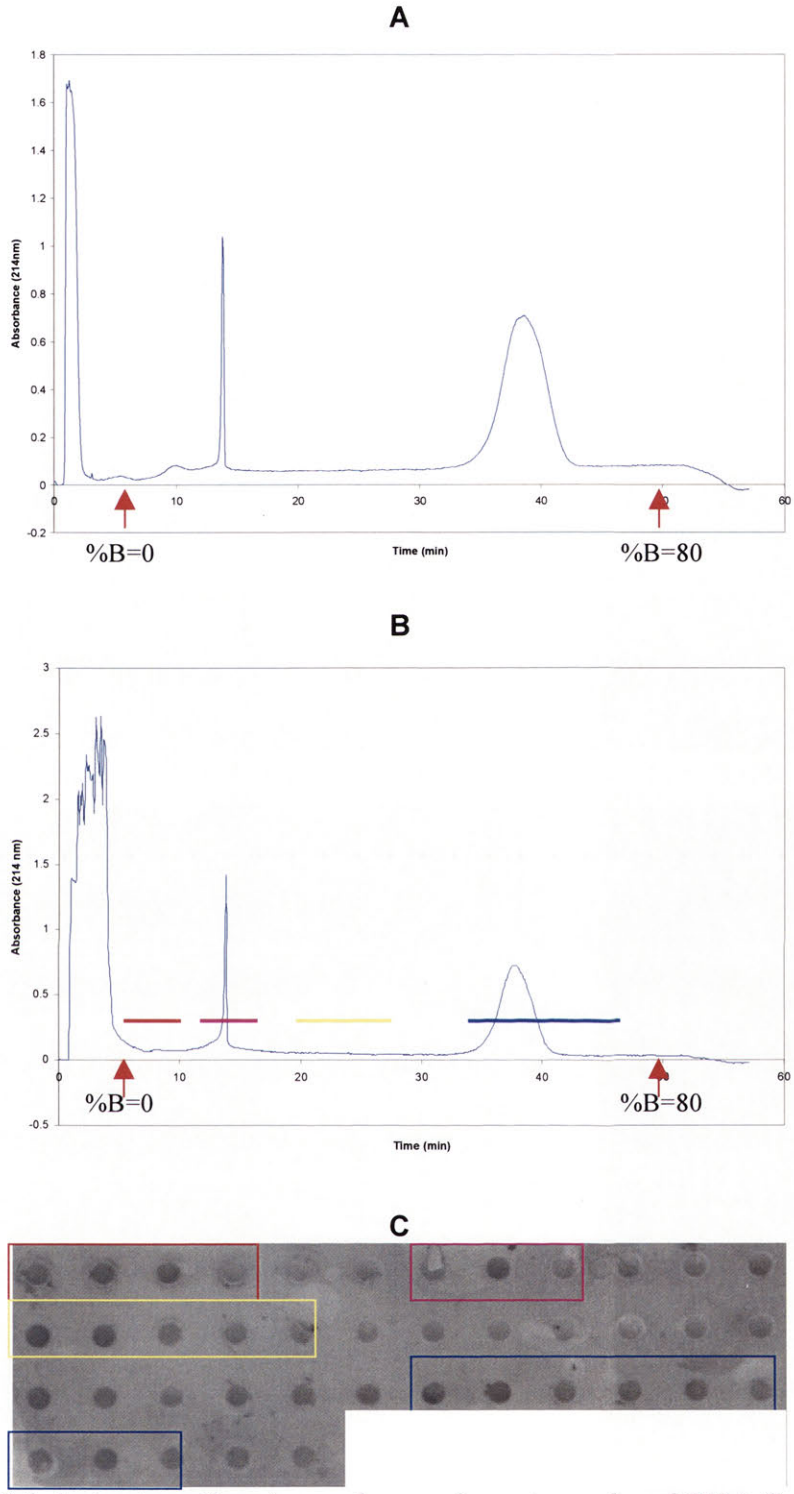

Figure 3.7: *High ionic strength anion exchange chromatography of BSM. Separations were performed at pH 5.0 [**A**] and 7.0 [**B**]. Note that this buffer is highly absorbent at 214 nm and, as such, there may be peaks that are masked in the trace. Using pH 7.0 buffer resulted in better separation of species and resulted in four distinct glycosylated regions being recovered [**C**]. Fractions were collected at %B=0 and stopped at %B=80. .The fractions were blotted onto nitrocellulose and glycosylation detected for using the DIG glycan detection Kit (Roche Germany) The spots were then compared to a negative control and fractions that stained more heavily were collected. These fractions could be divided into four groups as indicated by the colored Boxes.*

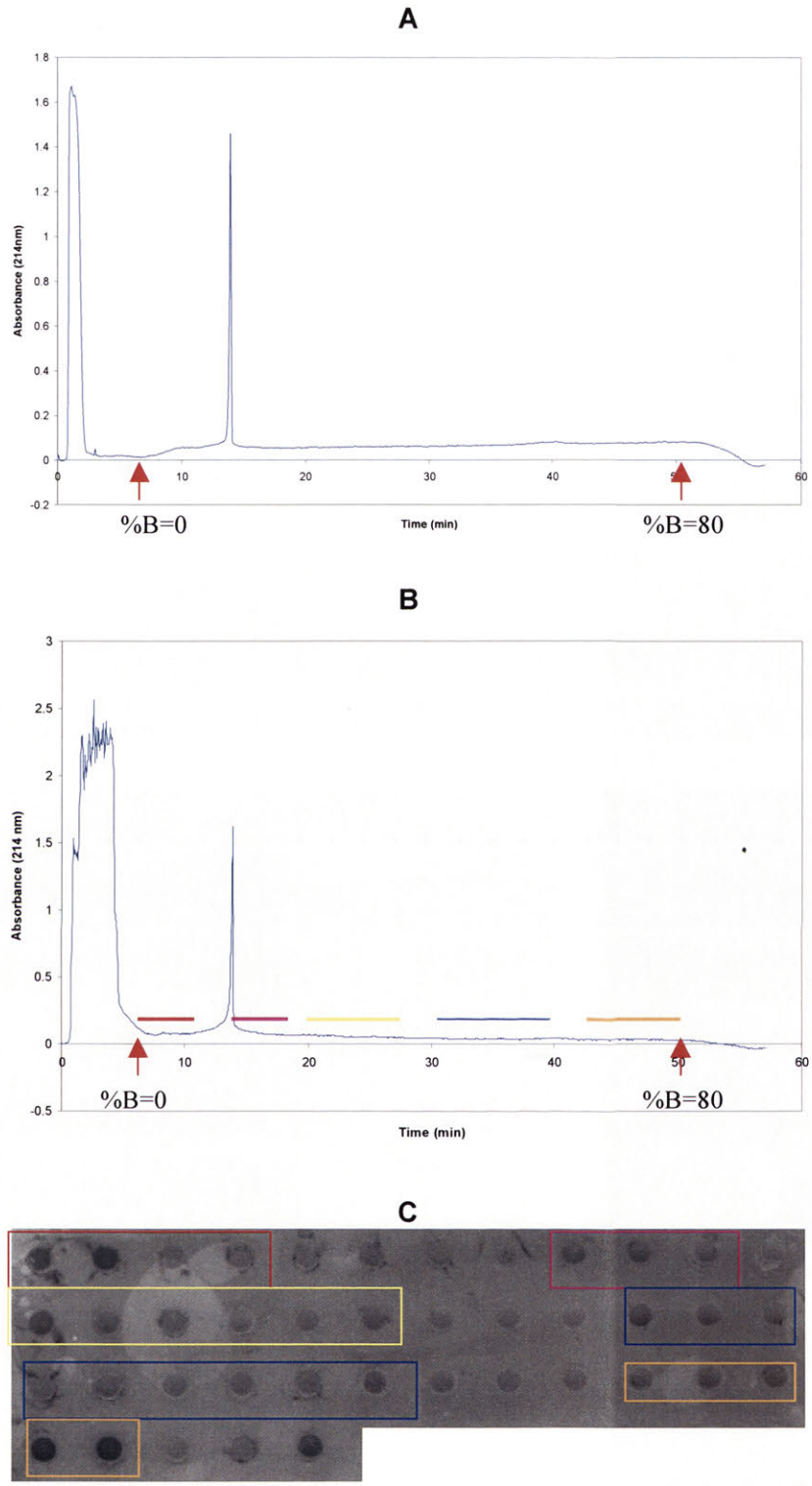

Figure 3.8: *High ionic strength anion exchange chromatography of bovine OHMG. Separations were performed at pH 5.0 [**A**] and 7.0 [**B**]. Note that this buffer is highly absorbent at 214 nm and, as such, there may be peaks that are masked in the trace. Using pH 7.0 buffer resulted in better separation of species and resulted in five distinct glycosylated regions being recovered [**C**]. Fractions were collected at %B=0 and stopped at %B=80. .The fractions were blotted onto nitrocellulose and glycosylation detected for using the DIG glycan detection Kit (Roche Germany) The spots were then compared to a negative control and fractions that stained more heavily were collected. These fractions could be divided into five groups as indicated by the colored Boxes*

3.4 CONCLUSIONS / DISCUSSIONS

While there are several methods of separation of the large heavily glycosylated mucin sub-units, each has many disadvantages. Agarose gel electrophoresis works for the larger, more glycosylated mucins, such as the gastrointestinal mucins (e.g. MUC-2). However, because of the large amount of dispersion through the gel, the bands become difficult to visualise when only a small amount of material is loaded. This makes agarose gels unsuitable for the analysis of the bovine OHMG's, which are much harder to obtain in large amounts. The gels also require large DNA slab gel apparatus to run and take several hours for the mucins to migrate sufficiently.

Polyacrylamide agarose gels are more successful in reducing diffusion sufficiently to allow the analysis of ocular mucins, however, significant diffusion still occurred. This system also uses the large DNA style slab gels that must be run for several hours to get migration. The gels are very unstable physically, as the agarose is present to stabilise a polyacrylamide matrix, which is only just barely solid. If the gels are allowed to heat up too much (in the process of passing current through them) then the polyacrylamide will destabilise which makes the gel useless. Physical handling of these gels is difficult as they have the consistency of gelatine.

By using PEG to produce large pores in otherwise normal SDS-PAGE gels, it is possible to overcome many of the problems of the other two methods. The gels are poured into mini gel systems, making handling of them much easier. The PEG modified mini-gels are also much less physically stable than normal (having a consistency similar to the polyacrylamide/agarose gels). This is not such a problem, however, due to their small size

(80 x 100 x 1 mm as compared to the 150 x 150 x 5 mm slab gels); the weight of the gel does not make them break up as the polyacrylamide/agarose gels do. The gels also run at a similar rate to standard SDS-PAGE gels taking only 1.5-4 hours to run, as opposed to the 16 hours required for an agarose or polyacrylamide/agarose gel. Another advantage of the mini-gel unit is that the temperature can be kept constant using an ice bath, rather than having to run the apparatus in a cold room. The mucin bands are much tighter on PEG modified SDS-PAGE gels and there seems to be much less diffusion of sample. However, the PEG-modified (PM) gels do have some problems that require special handling. Firstly, the gels have a lower loading capacity than normal SDS-PAGE gels of the same size. This means that collecting preparative amounts of material from the gels is difficult. Secondly, as PEG is used to precipitate proteins from solution, the PEG in the sample buffer and gel could reduce the solubility of protein samples. The most significant problem with PM gels is that they cannot be stained by many of the conventional protein methods, such as silver stain. These stains either stain the whole gel or stain nothing at all. This problem can be overcome by using the periodic acid Schiff's reagent stain which is specific for glycosylation or by blotting the gel onto nitrocellulose or PVDF and using commercially available glycoprotein stains such as DIG (Roche Diagnostics, Mannheim, Germany) or Immuno-Blot (Bio-Rad, California, USA).

Some possible modifications to PM gels, which were not attempted due to their non-preparatory nature, to improve their separation properties would be to pour a gel with a gradient of PEG concentrations rather than acrylamide concentrations, thus, altering the macroporosity characteristics. There is also some evidence to suggest that SDS interferes with the separation of mucins in gels (Tytgat *et al.* 1995A). This is possibly due to the fact that the charge characteristics of the mucins make them very hydrophilic and the

binding of SDS is, therefore, inefficient. As mucins can possibly be thought of as sugars held together by amino acids, then perhaps the removal of SDS and addition of borate (which is often used for sugar separations) (Stellwagen *et al.* 2000) may improve the separation of the mucin species. Initial attempts of using a Tris-borate buffer system had shown an improvement in separation characteristics and reduction of smearing, but more work in this area is required.

High ionic strength anion exchange chromatography has proven useful for preparative recovery of individual species from the high molecular weight component of mucus samples (Thornton *et al.* 1994). By using a highly ionic counter ion (such as perchlorate), it is possible to remove mucins from the column matrix. When separating PGM and BSM with this method, three species were recovered with increasing charge densities. This may or may not correspond to the three species detected using the PEG modified SDS-PAGE gels. It is possible that the bands on the PEG modified SDS-PAGE gels contain more than one species. As many as five have been observed on gels on occasion (data not shown). By altering the elution program of the ion exchange chromatography, it may be possible to further divide the recovered peaks. One species of the bovine OHMG fraction (Peak 1) began elution prior to the start of the gradient, when a buffer of pH 7.0 was used (Figure 3.8). This may be due to overloading of the column, though this is unlikely as similar amounts of sample were loaded in all three separations. If overloading was occurring then it would be expected that peaks eluting before the gradient would be present for PGM and BSM separations also. Another possible reason for the early eluting peak is that the post translational modifications are altered in some way (desialated or desulfated or possibly deglycosylated). Deglycosylation is unlikely as the deglycosylated species should have been removed in the SEC purification. However loss of the

negatively charged groups may account for this. Monosaccharide analysis of the species does not seem to support this however. This suggests that there is a species in the bovine OHMG fraction that may have a net positive charge at pH 7.0. This is not consistent with it being a mucin. Mucins usually have an overall negative charge due to their low positive amino acid content and high sialic acid/sulfate. This presents the possibility that there is a non-mucin-like component present in the high molecular weight glycoconjugate fraction of bovine ocular mucus.

CHAPTER 4: COMPOSITIONAL ANALYSIS OF THE HIGH MOLECULAR WEIGHT COMPONENT OF OCULAR MUCUS

4.1 INTRODUCTION

The purification and fractionation protocols described in Chapters 2 and 3 demonstrated some physical differences between the ocular glycoprotein components of bovine eyes and mucin from porcine stomachs and bovine sub-maxillary glands. mRNA analysis and antibody binding studies of the human eye have reported many of the same components in the eye as those found in the stomach, airway and intestinal tract mucus secretions. MUC-1, MUC-2, MUC-4, MUC-5AC and MUC-7 have tentatively been identified as being present on the ocular surface (Morris *et al.* 2000; Corfield *et al.* 1997; Tei *et al.* 2000) using the above techniques. MUC-2 is found in the intestinal tract (Toribara *et al.* 1993), MUC-5AC in the stomach and lungs (Loomes *et al.* 1999), MUC-1 and MUC-4 are found in the airways (Gum *et al.* 1992) and MUC-7 in the salivary glands (Mack and Hollingsworth 1994). As MUC-2 and MUC-5AC are the only mucins found in the eye that are secreted and gel forming, it may be expected that the mucus layer of the eye would have similar physical properties to that of gastric and/or airway mucins. Ocular mucin was found to have characteristics of both the heavily glycosylated gel forming mucins of the stomach, in that it can spontaneously form gels in solution, and has been reported to make a structured gel in the tear film (Saso *et al.* 1999A); as well as of the non-gel forming mucins such as MUC-7 found in saliva, in that it can be completely digested by proteases in a matter of hours. Analysis of mucin glycoproteins has, to date, consisted largely of studies characterising antibody epitopes and identifying mucin genes (Tytgat *et al.* 1995B; Watanabe *et al.* 1993). These techniques have proven powerful

analytical tools to determine the amino acid backbone of the different mucin molecules. However, it has been argued that the amino acid chain is merely a scaffolding to hold the post-translational modifications of mucins, and there is no doubt that the glycosylation of mucins plays an important role in their functionality (Eckhardt *et al.* 1987). Antibody binding studies have proven to be useful when analysing proteins as most proteins have unique amino acid sequences (Drakopoulou *et al.* 2000). However, the same oligosaccharide structure may be attached to many glycoproteins. In mucins, glycosylation accounts for a large proportion of the molecule and, as such, oligosaccharides are what is presented to an antibody. This results in cross-reactive binding to different mucin molecules (Montagne *et al.* 2000). There are antibodies that have been raised to the amino acid cores, but in many cases these were raised using the apomucins (the core protein with the sugars removed) and as such will not react with the native proteins (Corfield *et al.* 1997). One alternative to these approaches is to determine the composition of the high molecular weight fraction and its components. By determining which amino acids are present and their post-translational modifications (such as neutral and acidic sugars, and sulfate/phosphate) a total picture of the mucus components can be obtained. This has the advantage of analysing the expressed protein directly by chemical means as opposed to indirectly by antibody reactivity or through predicted amino acid sequences extrapolated from genes.

As a first approach, the general similarities and differences between glycoconjugates can be determined. Since mucin species differ substantially in amino acid composition and extent of glycosylation (Corfield *et al.* 1997), information can be obtained quickly and easily. To this end, compositional analysis was performed on commercially available

BSM and PGM, as representatives of a lightly glycosylated non-gel forming mucin (BSM) and a heavily glycosylated gel forming mucin (PGM), as well as the glycosylated fraction recovered after SEC purification of protease digested bovine OHMG. These compositions were compared to the composition of the high molecular weight glycoconjugate fraction recovered by the high salt SEC purification of bovine conjunctival mucus.

Amino acid composition provides information on the protein backbone of the three glycoconjugates. Information on the oligosaccharides attached to the protein core was obtained by monosaccharide analysis (comprising neutral, amino and sialic acid monosaccharides). Total sulfate content was determined and combined with acidic monosaccharide analysis to establish the relative charge of the oligosaccharides present on the molecules. Finally, the extent of O-glycosylation of Ser and Thr residues (determined by β-elimination of the oligosaccharides and re-analysis of the Ser and Thr content after acid hydrolysis) was determined. From these base level analytical procedures, several calculations can be made:

a. The percentage of carbohydrate by mass.

b. The Ser/Thr - GalNAc ratio, which is an indicator of the level of glycosylation.

c. The percentage by mass of the acidic monosaccharide content (which, when combined with the content, gives a gross picture of the charge density of the protein).

d. The likelihood of a tandem repeat sequence (if the mucin is enriched in only a few amino acids, then it is likely that a tandem repeat sequence exists). These types of

characteristics can provide an informative comparison of bovine OHMG to BSM and PGM.

To account for any possible contamination caused by the extraction process and, as an initial investigation into how appropriate a model bovine conjunctival mucus is for later human studies, compositional analysis was also performed on human tears.

4.2 METHODOLOGY

4.2.1 Amino Acid Compositional Analysis

Two separate analyses were performed to determine the amino acid composition of the samples. The first was a strong acid hydrolysis of the glycoconjugates. Glycoprotein samples were lyophilised into pre-fired glass hydrolysis tubes (approximately 100 picomoles of sample). The tubes were placed into a hydrolysis vessel and 250 µl of constant boiling (6 M) HCl added. A crystal of phenol was placed onto the wall of the vessel and a vacuum applied. The vessel was flooded with O_2 free N_2. This process was repeated twice more and a final vacuum applied to the chamber. The samples were hydrolysed at 110°C for 22 hours and any HCl removed by drying. 30 µl of 2:2:1 ethanol: triethylamine: water was added to each tube and the samples re-dried. Each sample was dissolved into 10 µl of 250 mM borate buffer and derivatised by automated F-moc coupling. The derivatised amino acids were then separated on a GBC Scientific (Illinois, USA) Aminomate system with a Keystone Scientific (Thermo Hypersil, Pennsylvania,

USA) 150 x 4.6 ODS Hypersil reversed phase column. Complete run conditions are described in Appendix B, Section B5. This analysis gave a precise determination of the majority of the amino acids. However, strong acid hydrolysis removes the amine groups of some amino acids, converting Gln to Glu and Asn to Asp. The amino acids are therefore combined in the results as Glx and Asx. Cysteine and tryptophan are labile in strong acid and the hydrolysis process therefore destroys these amino acids. An alkylating group may be used to protect cysteine residues, so second analysis was performed on mucins reduced with DTT and alkylated by iodoacetamide (as described in Appendix B, Section B1.2.2). This enabled the percentage composition of Cys in each of the glycoconjugates to be determined.

To determine the extent of O-linked glycosylation present in the samples alkaline β-elimination of the oligosaccharides from the glycoprotein was used. Protein samples were treated with 0.1 M NaOH for 16 hours at 45°C. This treatment removes the oligosaccharides from the amino acid backbone, and in the process converts Ser to dehydroalanine and Thr to 2-amino butanoic acid (Cooper *et al.* 1994). After strong acid hydrolysis and F-moc derivatisation these residues do not have the same retention time as their parent residues, thereby reducing the peak area of Ser and Thr. By taking the protein and performing amino acid analysis before and after analysis, it is possible to estimate how many Ser and Thr residues were not glycosylated. The number of Ser and Thr residues that were O-glycosylated can then be calculated from the reduction of peak area from the original hydrolysis.

All analyses were performed a minimum of three times.

4.2.2 Monosaccharide Analysis

To correctly estimate the total monosaccharide composition of the glycoproteins, each sample was divided into thirds and three hydrolyses were used (taken from Packer *et al.* 1998A):

a. **Neutral Monosaccharides:** 100 µl of sample was diluted with 100 µl 4 M TFA and hydrolysed at 100°C for four hours.

b. **Amino Sugars:** 100 µl sample was diluted with 100 µl 8 M HCl and hydrolysed at 100°C for four hours.

c. **Sialic Acid:** 100 µl of sample was diluted with 100 µl of 0.2 M TFA and hydrolysed for 40 minutes at 80°C.

In each case the sample was lyophilised to remove the acid and re-suspended in 100 µl water containing 4 nmol of internal standard. For methods (a) and (b) the internal standard used was deoxy-glucose, while the standard for method (c) was lactobionic acid. Samples (a) and (b) (25 µl) were loaded onto a Dionex (California, USA)CarboPac PA10 column, separated isocratically in 15 mM NaOH and detected using a pulsed amperometric detector. Analysis of sialic acids were performed on a Dionex (California, USA) CarboPac PA1 column using an acetate gradient in 250 mM NaOH with pulsed amperometric detection (for complete protocol, refer to Appendix B, Section B6).

All analyses were performed a minimum of three times.

4.2.3 Sulfate Analysis

Analysis of the sulfate composition of the samples was performed as follows. The same sample that was used for amino sugar composition (4 M HCl hydrolysis 100°C four hours) was injected (25 µl) onto a Dionex IonPac AS11 column and separated using a NaOH gradient (for complete protocol, see Appendix B, Section B6). The hydroxide ions were neutralised, post-column, using a Dionex Anion Micromembrane suppressor (AMMS-1) (using 50 mM H_2SO_4 as the neutraliser), ion suppressor (50 mM H_2SO_4 as the neutraliser) and the SO_4 ions detected using a ED40 conductivity detector (taken from Packer and Harrison 2000).

All analyses were performed a minimum of three times.

4.3 COMPARISON OF THE COMPOSITION OF OHMG AND ITS GLYCOPEPTIDE FRACTION TO PGM AND BSM

4.3.1 Whole Glycoprotein Fraction

4.3.1.1 Amino Acid Composition

A summary of the amino acid composition of the three glycoconjugates purified by high salt SEC (PGM, BSM and OHMG) is listed in Table 4.1 and shown in Figure 4.1. Ser and Thr are the amino acids that are the attachment sites for O-linked oligosaccharides in glycoproteins and constitute just over 32% of the total amino acids in PGM approximately 27% of BSM and 14% OHMG.

Interestingly, the Thr to Ser ratio of PGM was high (2.3:1) which was not true for BSM (0.9:1) or bovine OHMG (0.6:1). The published data for BSM from genomic information indicates a higher Thr than Ser content (1.8:1). The data from the present study indicates roughly the same amount of Ser and Thr in total (26.7% from amino acid analysis 27.9% from genomic sequence) (SWISS-PROT protein database, http://expasy.proteome.org.au/sprot/). It is possible that as the bovine OHMG fraction contains many species, and other mucin glycopeptides may account for the low Thr:Ser ratio. This is unlikely, however, as no mucin gene so far described has higher levels of Ser than Thr.

Table 4.1: *Amino acid composition of PGM, BSM and bovine OHMG*

	PGM		BSM		Bovine OHMG	
A. Acid[1]	% Comp.	SD	% Comp.	SD	% Comp.	SD
Asx	3.2	0.09	2.5	0.06	22.6	1.83
Glx	5.7	0.29	6.5	0.03	10.8	0.17
Ser	10.0	4.19	13.8	0.03	8.1	0.26
Gly	6.0	0.42	17.5	0.03	9.3	0.35
His	1.4	0.74	0.2	0.00	1.3	0.09
Arg	2.3	0.06	4.9	0.00	5.2	0.17
Thr	22.6	4.68	12.9	0.03	5.5	0.15
Ala	9.1	0.70	13.4	0.00	6.7	0.23
Pro	19.0	1.50	13.1	0.09	3.7	0.07
Tyr	0.5	0.20	0.2	0.00	1.3	0.09
Val	6.0	0.49	7.7	0.06	5.7	0.12
Met	3.2	1.27	0.2	0.00	1.0	0.09
Ile	1.6	0.12	1.9	0.03	3.4	0.12
Leu	3.8	0.48	4.4	0.00	7.2	0.20
Phe	0.8	0.06	0.4	0.03	3.0	0.10
Lys	1.4	0.10	0.4	0.03	5.2	0.13
Cys*	1.6	0.15	0.5	0.08	1.1	0.25

1: figures are presented as percentage of total amino acid composition

**Cys residue composition was determined using protein reduced and alkylated and bound to PVDF membrane*

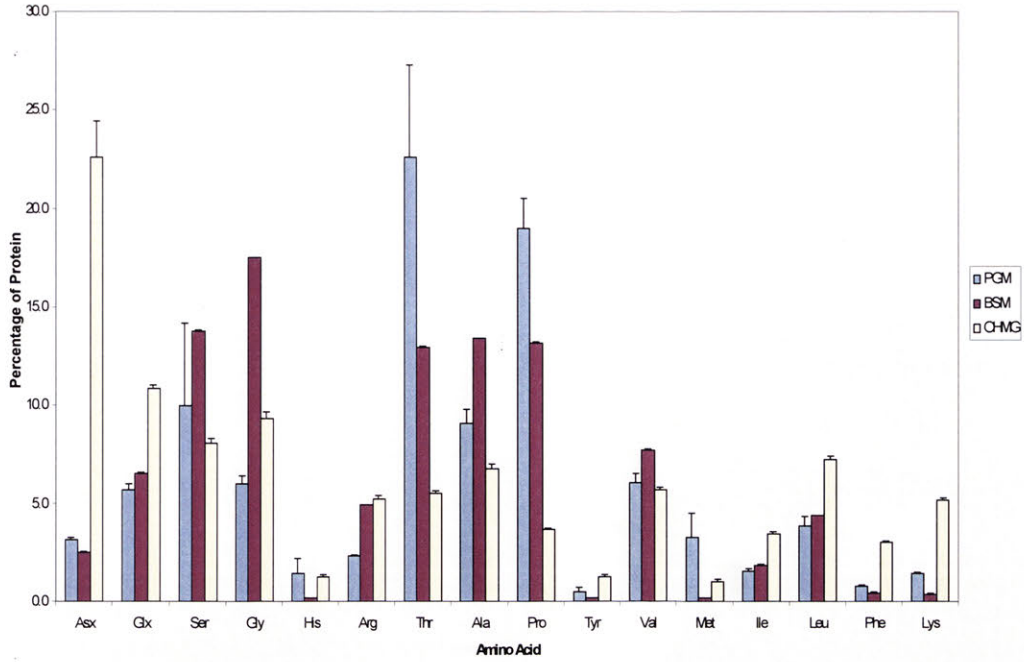

Figure 4.1: *Amino acid composition of PGM, BSM and bovine OHMG. Note the high levels of Asx and Glx present in the OHMG as compared to that of the mucins. Also note the lower levels of Ser and Thr*

Bovine OHMG was found to contain significantly higher Asx and Glx content than either PGM or BSM. Asx/Glx made up 33.4% of the total amino acids in OHMG while they constituted only 8.9% in PGM and 9.1% in BSM. This suggests a different composition of the amino acid backbone of the OHMG protein, potentially containing more charge and possible N-linked oligosaccharide attachment sites (N-linked sugars attach to asparagine).

Many mucins are reported to have tandem repeat sequences. A tandem repeat sequence is a series of amino acids that are duplicated a number of times in succession with no amino acids in between (e.g. PTVTPTVTPTVT is a tandem repeat sequence of PTVT containing three repeats). Tandem repeat sequences vary between mucins, but all so far reported are rich in Val, Ala, Pro, Ser and Thr, occasionally interspersed with other amino acid residues (Table 1.1 lists some

known tandem repeat sequences for mucins from human and animal sources). These amino acids totalled almost 67% of the amino acid content in PGM, suggesting a high probability of a tandem repeat sequence. The reported tandem repeat sequence of BSM is GTTVAPGSSNT. These amino acids account for 41.6% of the total amino acids in the protein. However, BSM is a mixture of mucin species, all of which contain tandem repeats. Val, Ala, Ser, Thr, and Pro comprise approximately 61% of the total amino acid composition of BSM. The bovine OHMG is unlikely to have a repeat sequence containing amino acids (they account for only 30% of the protein). However a repeat sequence containing Asx and Glx residues (which make up 33% of the protein) is a possibility.

The percentage amount of cysteine present in the protein does not correlate with the gel forming properties of these mucins, as PGM (gel forming) and BSM (non-gel forming) have a similar cysteine content (1.6% as opposed to 1.1%). The cysteine content of OHMG was even lower (0.5%) even though it is gel forming. The presence of several species of glycoconjugates in the whole OHMG fraction could account for this.

4.3.1.2 Monosaccharide and Sulfate Composition

The monosaccharide analysis of the three glycoconjugates analysed is presented in Table 4.2 and Figure 4.2.

Table 4.2: *Monosaccharide composition of PGM, BSM and bovine OHMG. Shown is both the nmols of each monosaccharide per nmol amino acid (nmol/AA) and the values normalised to GalNAc (Norm)*

	PGM			BSM			Bovine OHMG		
	nmol/AA	SD	Norm.	nmol/AA	SD	Norm.	nmol/AA	SD	Norm.
Fucose	0.80	0.16	0.38	0.04	0.01	0.05	0.02	0.12	0.17
GalNAc	2.11	0.53	1.00	0.80	0.91	1.00	0.12	0.61	1.00
GlcNAc	5.13	1.62	2.42	0.29	0.35	0.37	0.14	0.28	1.15
Galactose	2.99	0.53	1.41	0.10	0.04	0.12	0.07	0.04	0.62
Mannose	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.16	0.38
NAcSial	0.00	0.00	0.00	0.09	0.04	0.11	*	*	*
NGlySial	0.00	0.00	0.00	0.04	0.00	0.05	*	*	*
Sulfate	0.05	0.06	0.02	0.00	0.03	0.00	0.05	0.09	0.39

** denotes that the monosaccharide was detected but at levels too low to quantify*

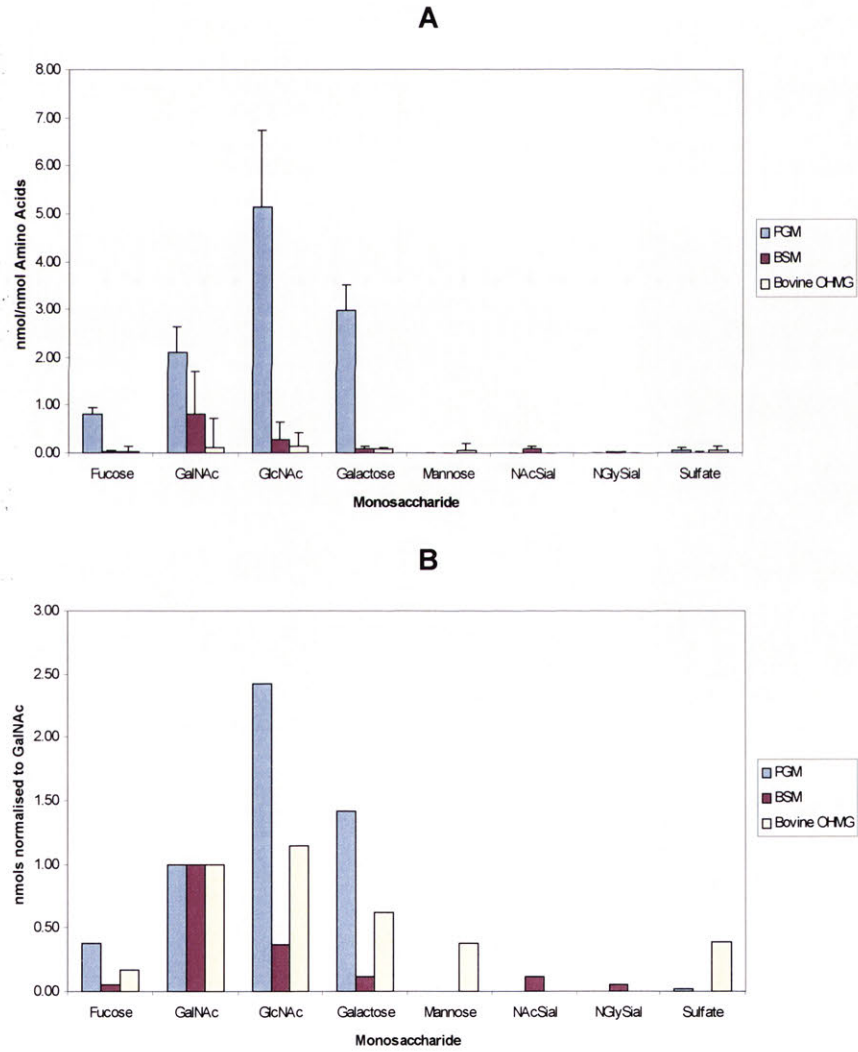

Figure 4.2: *Comparison of monosaccharide composition for PGM, BSM and bovine OHMG. Numerical values of each monosaccharide (represented in nmol oligosaccharides per nmol of amino acids) are tabulated. Graph A presents a graphical representation of this data while graph B is the nmols of sugar normalised to GalNAc*

The sulfate content of each of the glycoconjugates is also included, as sulfate modification can be as an addition to either the oligosaccharide chains or attached directly to the Ser and Thr residues (to date only sugar bound sulfate has been reported in mucins) (Degroote *et al.* 1999). Whether attached to the sugar or amino acid backbone, sulfate contributes to the negative charge of the mucin molecule. The table includes both the amount of each sugar (nmol of each sugar per nmol of total amino acids) found in the sample and the ratio of sugars normalised to the amount of galactosamine present. This is a common practice to allow the rough comparison of glycoprotein monosaccharide content, as GalNAc is the attachment sugar of O-linked oligosaccharides. The monosaccharide profiles of the three glycoconjugates were found to be substantially different. BSM contained predominantly the amino sugars GalNAc and GlcNAc with small amounts of fucose, galactose, N-acetyl neuraminic acid and N-glycolyl neuraminic acid. As sialic acid is only found as the terminal substituent in O-linked oligosaccharides of mucins, the ratio of monosaccharides to GalNAc suggests small O-linked oligosaccharides that are lightly charged, as there is a 1:5 ratio of sialic acid to galactosamine. Complex O-linked structures have been reported for BSM (Chai *et al.* 1992) but these structures must make up only a small percentage of the total oligosaccharide content for these ratios to result. Sulfate was not detected in the sample to a significant extent (less than 0.1 nmol sulfate per 12 nmol amino acids).

PGM had a higher glucosamine than galactosamine content (2.42 mol to 1 mol) and contained a higher percentage of galactose than BSM. The sialic acid content

was low, having no detectable sialic acid at this level of analysis. The reported levels of sialic acid on PGM are low (being below the limit of detection) (Karlsson *et al.* 1997A) when freshly extracted. It is, therefore, possible that this small amount was removed in the purification process. The Sigma commercial purification method involves an acid precipitation step, which might have removed acid labile sialic acids (Fu and O'Neill 1995). PGM from another commercial source (Fluka, California, USA) also had no detectable sialic acid (data not presented). A small amount of sulfate was detected in PGM (0.02 mol for every mol of GalNAc). Many sulfated oligosaccharide species have been described in the literature (Karlsson *et al.* 1995), however, as carbohydrate accounts for the majority of the molecular mass of PGM, this may still translate to a small total sulfate content. Sulfate is also acid labile, therefore the acid precipitation step used in the commercial purification may have also removed sulfate modifications.

Bovine OHMG had a different monosaccharide profile to both PGM and BSM. The most significant difference was the relatively large amount of mannose present, which is usually associated with N-linked oligosaccharides in mammals (mannose accounted for 12.5% of the monosaccharides). Mannose is a potential contaminant in this method of analysis, as high levels of glucose can isomerise into mannose, leading to a false reading. To test for this, a lower amount of sample is loaded onto the column (if the mannose value is real then the drop in mannose will be proportional to that of the other sugars). When this test was performed the mannose in the bovine OHMG preparation was determined to be

real. There was a relatively high amount of sulfate (0.39 mol:1 mol GalNAc). This would give the conjunctival glycoconjugates a high negative charge density. Sialic acid (both N-acetyl and N-glycolyl neuraminic acid) was present in trace amounts in bovine OHMG (both monosaccharides were detectable but not quantifiable by the method used). Conjunctival glycoconjugates have a monosaccharide composition that suggests a more complex oligosaccharide profile than that normally associated with mucins, as there are significant amounts of all the neutral and amino sugars present. While mucins have a large amount of heterogeneity of the oligosaccharides attached to the amino acid backbone, the monosaccharide composition of these oligosaccharides is reasonably consistent. The unusual monosaccharide composition, coupled with the relatively low amount of Ser/Thr present in the high molecular weight fraction, suggests that the major component of bovine OHMG is not a mucin in the traditional sense.

4.3.1.3 Overview of Composition of High Molecular Weight Glycoconjugates

Table 4.3 and Figure 4.3 depict the calculation of a number of useful attributes which can be obtained from compositional data.

Table 4.3: *Some comparative calculations of PGM, BSM and bovine OHMG*

	PGM	BSM	Bovine OHMG
1) % by wt Carbohyd.	94.6	71.1	38.7
2) % by wt Sialic Acid	*	9.9	*
3) % by wt Sulfate	0.2	*	1.9
4) % total Ser + Thr	32.5	26.7	13.6
5) GalNAc: Ser + Thr	1:1.32	1:4.11	1:9.41
6) % Ser Glycosylated[1]	56.2	54.2	7.9
7) % Thr Glycosylated[1]	78.9	83.8	46.7

** denotes that the component was below the limit of detection*

1: determined by β-elimination of glycoprotein and calculating loss of serine and threonine residues

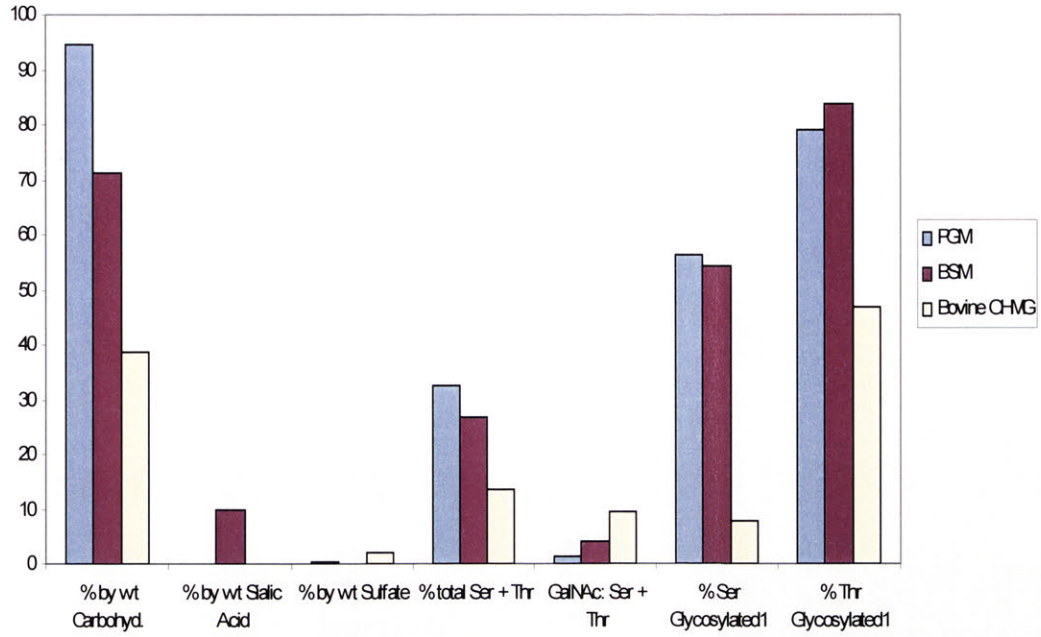

Figure 4.3: *Comparative calculations of PGM, BSM and bovine OHMG and their graphical representation.*

1) *The percent of total mass of the glycoprotein made up of carbohydrate, calculated by multiplying the nmol sugars by their Mwt and the nmol amino acids by 115 (average mass of an amino acid)*
2) *The percent of total mass of the mucin that is comprised of the monosaccharides N-acetyl neuraminic acid and N-glycolyl neuraminic acid*
3) *The percent of total mass of the mucin made up of sulfate residues*
4) *The percent of the total composition of the protein that are Ser and Thr residues i.e. for PGM out of every 100 residues of protein 32.5 of them will be either Ser or Thr*
5) *The number of Ser and Thr residues in the amino acid backbone for every residue of GalNAc (the monosaccharide attached to Ser/Thr in mucins) in the oligosaccharides*
6) *The percentage amount of all the Ser residues in the protein backbone that are glycosylated i.e. in PGM just over half the Ser residues have a sugar attached to them*
7) *The percentage amount of all the Thr residues in the protein backbone that are glycosylated i.e. in PGM just over three quarters of the Thr residues have a sugar attached to them*

The percent by weight of carbohydrate is an estimate of the extent of glycosylation of the glycoconjugates. The estimate was calculated by using the formula:

% by weight carbohydrate =

$$\frac{\text{(the residue mass of each sugar x nmol of each sugar)}}{\text{(residue mass of each amino acid x nmol each amino acid)} + \text{(residue mass of each sugar x nmol of each sugar)}}$$

The residue mass is defined as the mass of a linked amino acid or monosaccharide. PGM was the most heavily glycosylated, with carbohydrate accounting for 94.6% of its total mass. BSM was moderately glycosylated containing 71.2% carbohydrate by mass, while bovine OHMG was the most lightly glycosylated being only 38.7% carbohydrate. Literature reports that BSM is only lightly glycosylated (in the order of 10-15%) (Cooper *et al.* 1994), however, BSM and PGM from commercial sources contained a significant amount of low molecular weight contaminant proteins (data not shown). Therefore, the reported carbohydrate content of BSM in the literature may be artefactual (if no purification step was used). Amino acid analysis of the mucin before and after purification resulted in essentially the same composition (data not shown). This suggests that the low molecular weight contaminant may be, in fact, apomucin and would explain the reports of low levels of glycosylation (before purification the BSM sample tested as only 16.6% carbohydrate by weight). BSM was the only glycoconjugate tested that had significant sialic acid content (almost 10% by weight), however the charge density of bovine OHMG would also be high, due to the large amount of sulfate present (almost 2% of the protein by weight).

Two methods of deducing the oligosaccharide distribution from the compositional analysis are:

1. Determining the ratio of GalNAc to Ser and Thr;
2. Detecting the number of Ser/Thr sites that are glycosylated by β-elimination of the sugars attached to Ser and Thr residues.

GalNAc is the monosaccharide at the reducing terminal of an O-linked oligosaccharide in mucin glycoproteins. If we make the assumption, usually but not necessarily true, that GalNAc residues are only at the site of attachment to the protein, then the ratio of Ser/Thr residues to GalNAc can give an indication of the number of oligosaccharides present e.g. if there is 1 mole of GalNAc per mole of Ser + Thr, there is probably a high site occupancy. The number of Ser and Thr residues that are glycosylated gives an indication of the "thickness" of the oligosaccharide post-translational modifications on the peptide backbone. The Ser and Thr residues of PGM are highly glycosylated (79% of Thr and 56% of Ser residues, as determined by β-elimination), which is to be expected for a mucin with a high carbohydrate content by weight. BSM has similar percentages of O-linked glycosylated amino acids, 84% Thr and 54% Ser glycosylated. However the GalNAc to Ser + Thr ratios are different for the two mucins (1:1.32 for PGM and 1:4.11 for BSM). This suggests that some of the GalNAc in PGM is not only directly attached to an amino acid, but is contained in the oligosaccharide chains. This could show that there are large chain oligosaccharides on PGM compared to BSM. This deduction is supported in the literature (Bertolini and Pigman 1969; Chai *et al.* 1992).

The number of Ser and Thr attachment sites is quite small for OHMG (only 46.7% of Thr and 7.9% of Ser were found to be glycosylated). The GalNAc:Ser + Thr ratio of OHMG (1:9.41) also suggests a low site occupancy. The number of attachment sites is much less than the weight of carbohydrate would suggest i.e. there is about half the carbohydrate by mass of PGM but there are much less than half the attachment sites occupied. This, together with the different monosaccharide composition data compared to the two mucins studied, suggests that there are either some larger oligosaccharide structures (high

carbohydrate content at each site), or that the bovine OHMG fraction contains a significant amount of sugar attachments that are not mucin O-linked oligosaccharides (i.e. N-linked sugars or novel O-linked oligosaccharides present).

4.3.2 Comparison of the Protease Derived Glycosylated Fractions

Analysis of the whole OHMG fraction has led to the conclusion that bovine ocular mucus may have other high molecular weight glycoconjugates as a significant component. A simple way to support this theory is to analyse the glycopeptides recovered after the high molecular weight fraction is digested with proteases. The majority of knowledge concerning the chemical composition of mucin species involves the "tandem repeat" glycosylated region. By digesting bovine OHMG with proteases and comparing the resulting glycosylated peptide fraction with similar fractions recovered from PGM and BSM, it will be possible to further investigate whether mucins are the major component of bovine ocular mucus.

4.3.2.1 Amino Acid Composition

The results of the amino acid compositional analysis of the glycosylated fraction resulting from the protease (Rt41A) digestion of PGM, BSM and OHMG are listed in Table 4.4 and graphically represented in Figure 4.4.

Table 4.4: *Amino acid composition of the glycopeptide fraction recovered by high salt SEC after the digestion of PGM, BSM and bovine OHMG with Rt41A*

	PGM Glycopeptide Fraction		BSM Glycopeptide Fraction		Bovine OHMG Glycopeptide Fraction	
A. Acid[1]	% Comp.	SD	% Comp.	SD	% Comp.	SD
Asx	4.6	1.23	6.0	0.45	12.5	0.40
Glx	7.9	1.78	10.3	4.32	14.0	0.06
Ser	14.7	1.11	14.6	1.28	6.3	0.25
Gly	16.9	1.16	17.9	0.68	9.1	0.32
His	0.0	0.00	0.2	0.40	1.6	0.10
Arg	4.5	0.79	3.8	0.15	4.1	0.47
Thr	11.6	1.44	10.4	2.05	6.4	0.31
Ala	10.0	0.72	9.1	1.18	8.0	0.58
Pro	9.1	0.95	9.1	0.46	7.2	0.25
Tyr	0.1	0.10	0.1	0.10	0.7	0.31
Val	5.8	0.15	5.9	0.44	7.1	0.15
Met	1.4	1.47	2.0	0.87	1.0	0.32
Ile	2.4	0.23	2.5	0.45	4.3	0.06
Leu	8.1	1.65	5.5	0.25	9.6	0.29
Phe	1.4	0.38	1.5	0.67	3.6	0.12
Lys	1.5	0.21	1.2	0.29	4.7	0.15

1 Results are reported as percentage of total amino acids

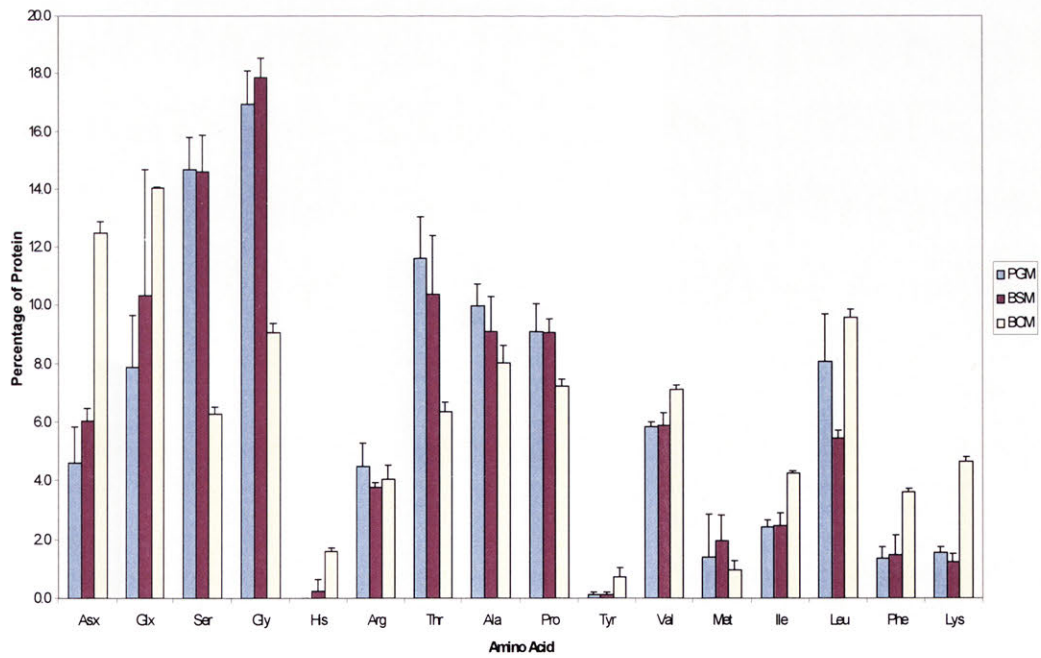

Figure 4.4: *Amino acid composition of the glycopeptide fraction recovered from the Rt41A digestion of PGM, BSM and bovine OHMG. Note that, as the whole glycoconjugate fraction, the glycopeptide fraction of OHMG is high in Asx, Glx, Lys, and Leu., while low in Ser, Thr compared to the glycopeptide fractions of BSM and PGM*

The fragments recovered from PGM and BSM both have higher ratios of Ser than Thr than that of the whole mucins (14.7% Ser / 11.6% Thr for PGM and 14.6% Ser / 10.4% Thr for BSM respectively). The fragment recovered from OHMG has approximately the same Thr and Ser (6.3% Ser, 6.4% Thr). The large tandem repeat sequences of all mucins reported in the literature have a higher Thr than Ser content (Table 1.1). The higher Ser than Thr in the PGM glycopeptide suggests that the fragments obtained by protease digestion may not be a large, heavily glycosylated tandem repeat sequence. Rather it may be a smaller non-repeated glycopeptide similar to the "Gly B" region reported in MUC-2 (Carlstedt *et al.* 1993).

The amino acids Pro, Val, Ala, Thr, Ser, and Leu are most often associated with tandem repeat sequences in the literature (SWISS-PROT database, http://expasy.proteome.org.au/sprot/). These amino acids are enriched in the PGM glycopeptide making up 59.3% of the amino acids in the fraction. Very little is reported in the literature about the amino acid composition of PGM and the genes responsible for expression have not yet been characterised. It is possible that the smaller glycopeptide region of PGM also contains a repeat. The BSM glycopeptides were also found to predominantly contain the amino acids associated with its tandem repeat (67% of the glycopeptide fraction was made up of these amino acids). The glycopeptide fraction recovered from OHMG was enriched in the amino acids associated with tandem repeats in mucin glycoproteins though to a lesser extent (46.6% of the peptide is made up of these amino acids). The glycopeptide fraction recovered from the protease digestion of

bovine OHMG shows a drop in the total Asx + Glx content when compared to the whole OHMG fraction. However, they comprise significantly more total amino acid content of the OHMG peptide fraction than either PGM or BSM. Asx and Glx comprise just over a quarter of the total amino acids in the fragment of OHMG (26.5%). This is approximately twice as much as found in the other two mucin species (PGM glycopeptides contain 12.5% and BSM glycopeptides contain 16.3% Asx + Glx). Also of note is the relatively high amount of the hydrophobic amino acids His, Phe, Lys and Tyr in the glycopeptide fraction of OHMG compared to that of PGM and BSM glycopeptides. The values for these amino acids are approximately twice as high in the bovine glycoconjugates.

4.3.2.2 Monosaccharide and Sulfate Composition

Table 4.5 lists and Figure 4.5 shows the monosaccharide content of the fractions obtained after Rt41A digestion of PGM, BSM and bovine OHMG, both as a proportion of the total protein (nmols monosaccharide per nmol total amino acids) and normalised to N-acetyl galactosamine.

Table 4.5: *Monosaccharide composition of the glycopeptide fraction recovered by high salt SEC after the digestion of PGM, BSM and bovine OHMG with Rt41A. Shown is both the nmols of each monosaccharide per nmol amino acid (nmol/AA) and the values normalised to GalNAc (Norm)*

	PGM Glycopeptide Fraction			BSM Glycopeptide Fraction			Bovine OHMG Glycopeptide Fraction		
	nmol/AA	SD	Norm.	nmol/AA	SD	Norm.	nmol/AA	SD	Norm.
Fucose	0.09	0.01	0.10	0.06	0.01	0.09	0.03	0.01	0.59
GalNAc	0.94	0.00	1.00	0.73	0.00	1.00	0.05	0.00	1.00
GlcNAc	0.57	0.03	0.60	0.60	0.02	0.82	0.19	0.01	4.16
Galactose	0.62	0.02	0.66	0.25	0.01	0.34	0.19	0.01	4.02
Mannose	0.28	0.39	0.30	0.37	0.02	0.51	0.12	0.04	2.49
NAcSial	0.00	0.00	0.00	0.06	0.02	0.08	0.00	0.00	0.00
NGlySial	0.00	0.00	0.00	0.02	0.00	0.03	0.00	0.00	0.00
Sulfate	0.10	0.00	0.11	0.19	0.00	0.27	0.08	0.00	1.77

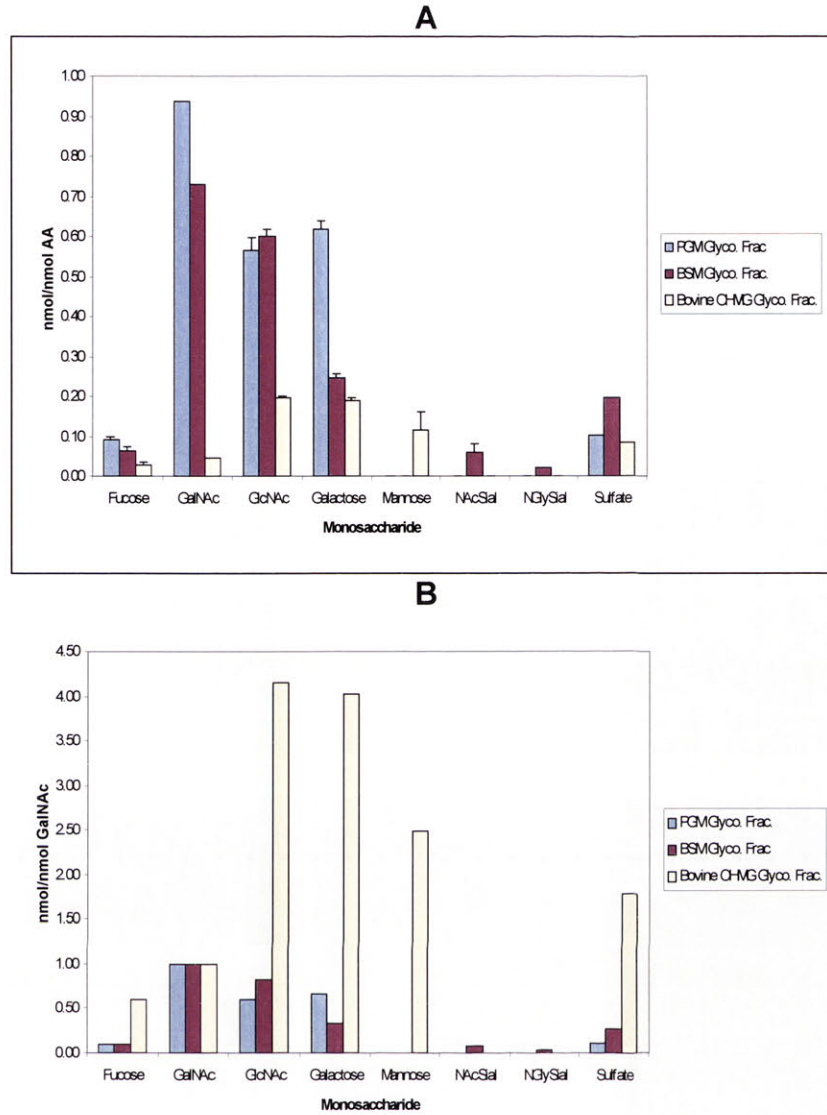

Figure 4.5: *Monosaccharide composition of the glycopeptide fractions recovered by high salt SEC from the Rt41A digestion of PGM, BSM and bovine OHMG. Total monosaccharides [**A**] and values normalised to GalNAc [**B**] are presented. Note the dramatically different composition of OHMG to the mucins. The glycopeptide fraction recovered from bovine OHMG has high ratios of GlcNAc, Gal, Man and sulfate to GalNAc*

There was a marked difference between the monosaccharide profile of PGM and OHMG glycopeptides compared to their parent glycoproteins. However this was not the case for BSM. The ratio of GlcNAc to GalNAc in the fragment of PGM was much lower than that of the whole protein (0.58:1 as opposed to 2.42:1). Similarly the ratio of Gal to GalNAc had dropped (0.61:1 compared to 1.41:1).

Fucose to GalNAc was also very low in the fragment compared to the whole mucin (0.09:1 compared to 0.38:1). The total nmol sugar per nmol amino acid has also dropped from 11.03 to 2.25. This is further evidence that a lightly glycosylated region of PGM was recovered after digestion rather than the heavily glycosylated tandem repeat domain. The mannose that was detected in the glycopeptide fraction of BSM and PGM was shown, in a similar manner to be a artefact of glucose contamination. However it is possible that there is some mannose present, but it is impossible to separate the actual mannose from the contamination.

On the other hand, the fragment fraction of bovine OHMG had more sugar than the starting material (0.58 nmol sugar per nmol amino acids compared to 0.37 nmol for the whole OHMG fraction). The ratios of fucose, GlcNAc and Gal to GalNAc were all much higher in the fragment than in the whole OHMG (fuc 0.56/0.17, GlcNAc 4.02/1.15, Gal 3.73/0.62) suggesting that the sugars that were found in this fraction may be larger and more highly substituted. The ratio of mannose to GalNAc was also higher (2.43:1 for the fragment, 0.38:1 for the whole protein). This combined with the high Asx content suggests that the glycopeptides of OHMG may contain a significant amount of N-linked oligosaccharides, as asparagine is the amino acid to which N-linked oligosaccharides are attached.

In contrast to the PGM and bovine OHMG, the monosaccharide content of the glycopeptide recovered from BSM is similar to that of the whole protein. There is

1.68 nmol of sugar per nmol amino acids in the fragment and 1.37 for the whole protein. The ratios of fucose to GalNAc are similar in both (0.08:1 in the fragment, 0.05:1 in the whole mucin). However the ratios of GlcNAc and Gal to GalNAc are both slightly higher (0.79/0.37 and 0.31/0.12 respectively) suggesting that BSM may not contain a heavily glycosylated region. Rather it may have evenly spaced oligosaccharide attachment along the whole molecule, and digestion with Rt41A resulted in an initial digestion into glycopeptides of roughly the same molecular weight. This would agree with the published description of non-gel forming mucins such as MUC-7 (which should be an analog of the major component of BSM) (Seregni *et al.* 1997).

The sulfate content of all the fragments was much higher than the whole mucins when normalised to GalNAc. PGM glycopeptides contained five times the sulfate of the whole glycoconjugates (0.11 compared to 0.02 respectively) while BSM had only trace amounts of sulfate, the glycopeptide fraction had significant sulfate (0.27 nmols of sulfate per nmol GalNAc). The normalised sulfate content of the fragment of OHMG was about four and a half times higher (1.77:1 in the fragment, 0.39:1 in the whole mucin). This suggests that the fraction collected after the digestion of bovine OHMG was highly sulfated and, coupled with the increase in carbohydrate by mass of this fraction, indicates that a heavily glycosylated, highly charged region exists in the high molecular weight components of the bovine conjunctival mucus.

4.3.2.3 Overview of Composition of Protease Derived Fractions of the High Molecular Weight Glycoconjugates

As with the whole high molecular weight glycoconjugate fractions, comparison of the computation of the different components of the glycopeptides can provide invaluable information about the characteristics of the species present. By comparing the protease derived fragments to the whole glycoconjugate from which they are derived, insight can be made into their structure. This is listed in Table 4.6 and shown in Figure 4.6.

Table 4.6: *Some calculated comparisons of the glycopeptide fractions of PGM, BSM and bovine OHMG*

	PGM Glycopeptide fraction	BSM Glycopeptide fraction	Bovine OHMG Glycopeptide fraction
% by wt Carbohyd.	79.1	74.4	45.8
% by wt Sialic Acid	0.0	1.5	0.0
% by wt Sulfate	1.4	3.1	3.0
% total Ser + Thr	26.3	25.0	12.6
% Ser Glycosylated	35.0	30.1	18.1
% Thr Glycosylated	71.0	63.8	51.3

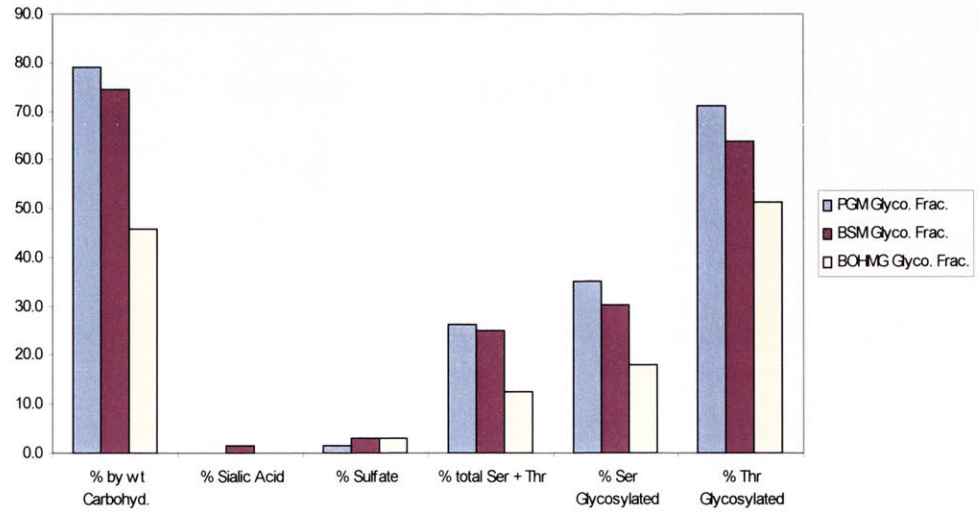

Figure 4.6: *Some calculated comparisons of the glycopeptide fractions of PGM, BSM and bovine OHMG. Note that, as is the case of the whole OHMG fraction, the glycopeptides have lower Ser + Thr and higher sulfate than that of the mucins studied*

The fragment derived from PGM, by protease digestion, had a lower total carbohydrate content than the whole mucin fraction (76.7% / 94.6%), however the fragment of OHMG had more carbohydrate by weight than the whole OHMG fraction (45.6% / 38.7%). BSM had a similar carbohydrate content of both the fragment and whole mucin fraction (70.9% / 71.2%). The sulfate content of the fragments was higher for all three species when compared to the whole mucin. PGM glycopeptides were 1.4% by weight sulfate while the whole mucin fraction was only 0.2%. BSM had no detectable sulfate, the fragment, however, contains 3.1% by weight sulfate. The limit of detection for sulfate is approximately 1 nmol, so the whole mucin molecule may contain levels of sulfate that are below the limit of detection with the amount of protein analysed. OHMG is almost 2% by weight sulfate but its glycopeptide is 3%. The protease digestion, and subsequent purification by SEC, appears to have recovered a sub-set of the proteins that contain sulfate, while only bovine OHMG was enriched in total carbohydrate.

The Ser and Thr content of the PGM glycopeptide is lower than that of the whole protein (32.5% of the whole mucin is Ser and Thr while only 26.3% of the fragment is composed of these amino acids). In addition, less of these amino acids were glycosylated (56.2% of Ser and 78.9% of Thr in the whole protein compared to 35% and 71% in the glycopeptide). In BSM the content of Ser and Thr was about the same (26.7% of BSM and 25% of the glycopeptide) but fewer were glycosylated (83.8% / 63.8% Thr, 54.2% / 30.1% Ser). In contrast, bovine OHMG glycopeptides had a slightly lower Ser/Thr content (12.6% to 13.6%). More of these amino acids were glycosylated (18.1% Ser was glycosylated in the

glycopeptide fraction compared to only 7.9% in the whole OHMG fraction and 51.3% / 46.7% of Thr residues were glycosylated glycopeptide fraction/whole OHMG). Interestingly OHMG glycopeptides still contained a high content of Asx and Glx, though slightly less than the whole mucin (26.5% of the glycopeptide is Asx + Glx compared to 33.4% of the whole mucin). In comparison, the content of these amino acids in the glycopeptide fractions of PGM and BSM was 12-16%.

The compositions of both the whole high molecular weight glycoconjugate fraction of bovine ocular mucus and the glycopeptides recovered after protease digestion with Rt41A have shown that the ocular glycoconjugates are significantly different from those of a large gel forming mucin (PGM) and a non-gel forming mucin (BSM). It is clear that one or more species in ocular mucus is not a mucin in the traditional sense. As the mucins selected have proven to be incomparable in composition to bovine OHMG, it was decided that further comparison between these glycoconjugates was unnecessary, and the OHMG fraction was more thoroughly examined on its own merit.

4.3.3 Composition of Species of OHMG Separated by Ion Exchange Chromatography of the Whole High Molecular Weight Fraction

Generally characterisations of mucins have involved the purification of the whole high molecular weight component, followed by analysis or protease digestion and analysis of the undigested glycopeptides (as performed in the two sections above) (Paul *et al.* 1998; Chao *et al.* 1983B). However all data collected so far of the high molecular weight

fraction of ocular mucus has suggested it does not conform to the behaviour of the standard mucins. To obtain further information on the apparent differences between this high molecular weight fraction, the ocular mucus species were further separated by ion exchange chromatography (Section 3.3) and their compositions determined. The peaks were determined by detection of those fractions that contained carbohydrate (determined by slot-blotting the fractions and staining with the DIG glycan detection kit (Roche Diagnostics, Mannheim, Germany). These fractions were combined to form five fractions and these were analysed for amino acid and monosaccharide content as described for the whole fraction (Section 4.2).

4.3.3.1 Amino Acid Composition

Figure 4.7 shows the amino acid compositions and of each of the peaks collected from the ion exchange chromatographic separation of the high molecular weight glycoconjugate fraction of bovine conjunctival mucus (the values are listed in Table 4.7).

Table 4.7: *Amino acid composition of fractions recovered from the high ionic strength anion exchange chromatography of the high molecular weight glycoconjugate fraction of bovine OHMG*

	Peak 1		Peak2		Peak3		Peak 4		Whole Bovine OHMG	
A. Acid	**% Comp.**	**SD**	**% Comp.**	**SD**	**% Comp.**	**SD**	**% Comp.**	**SD**	**% Comp.**	**SD**
Asx	3.5	0.33	3.4	0.34	5.8	0.00	7.4	0.36	22.6	1.83
Glx	12.3	0.58	12.4	0.52	5.5	0.13	6.5	0.3	10.8	0.17
Ser	24.6	2.39	22.4	2.03	5.3	0.33	6.6	0.22	8.1	0.26
Gly	5.8	0.46	5.4	0.31	6.0	0.07	6.8	0.32	9.3	0.35
His	0.8	0.00	0.8	0.03	5.5	0.53	3.6	0.12	1.3	0.09
Arg	3.3	0.09	3.4	0.03	6.3	0.2	7.0	0.20	5.2	0.17
Thr	8.0	0.5	7.8	0.33	5.6	0.17	5.2	0.24	5.5	0.15
Ala	3.8	0.15	3.6	0.15	5.8	0.17	4.6	0.23	6.7	0.23
Pro	0.2	0.03	0.1	0.03	5.7	0.57	3.3	0.35	3.7	0.07
Tyr	16.5	0.64	16.8	0.55	6.1	0.07	6.9	0.25	1.3	0.09
Val	0.1	0.00	0.1	0.00	6.4	0.07	8.0	0.35	5.7	0.12
Met	3.9	0.03	4.0	0.2	6.5	0.33	7.0	0.22	1.0	0.09
Ile	3.5	0.12	3.7	0.23	6.4	0.23	7.3	0.26	3.4	0.12
Leu	11.3	3.50	13.1	2.7	5.8	0.17	5.6	0.09	7.2	0.2
Phe	2.4	0.15	2.8	0.19	5.5	0.3	2.8	0.93	3.0	0.1
Lys	0.1	0.06	0.1	0.07	11.7	0.57	11.4	1.81	5.2	0.13

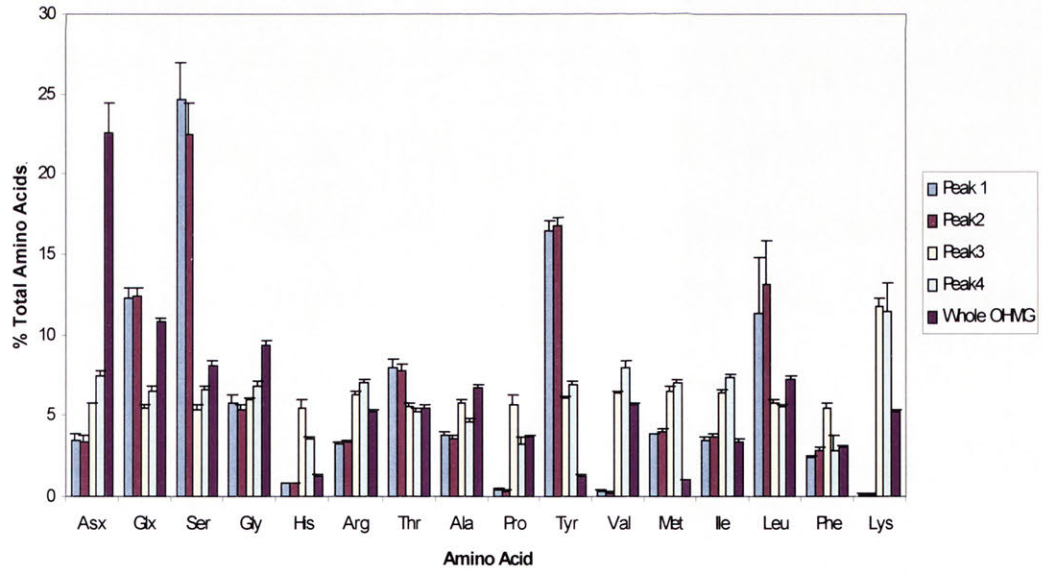

Figure 4.7: *Amino acid composition of fractions recovered from the high ionic strength anion exchange chromatography of the high molecular weight glycoconjugate fraction of bovine OHMG. Note that Peaks 1 and 2 have similar amino acid compositions as do Peaks 3 and 4. Peaks 1 and 2 have a high level of Ser and Thr while Peaks 3 and 4 have roughly equal amounts of most of the amino acids*

The peaks were designated in order of elution from the column (that is Peak 1 eluted first [least ionic], Peak 5 eluted last [most ionic]). Peak 5 was found to contain no significant protein, even though it stained positive for sugar. When graphed alongside each other it can be seen that Peaks 1 and 2 are not significantly different in amino acid composition. Peaks 3 and 4 are also substantially similar differing only significantly in His, Pro and Phe content. Peaks 1 and 2 are high in Glx (13%) like the whole OHMG fraction (11%) but have a low Asx content (4% compared to 20% for the whole OHMG fraction). Interestingly the total Ser/Thr content is what would be expected for a large gel forming mucin-like glycoprotein (around 30% as is observed in PGM), however, it is unusual to find higher total Ser than Thr content in mucins. The Ser content is approximately 25% while the Thr is only 8%. This is not a species-specific phenomenon, as BSM has roughly equal total Ser and Thr content (14% Ser, 13% Thr). Peaks 1 and 2 are also high in tyrosine (16%) and leucine (13%) with no appreciable enrichment of the other amino acids usually associated with tandem repeat sequences in mucins (valine, proline and alanine).

The amino acid composition of Peaks 3 and 4 is not at all what would be expected for any mucin. The amino acid composition is almost equal for each amino acid. Most of the amino acids consist of approximately 6% of the total protein. There are some that are noticeably less (Phe 3%, Pro 3.5% and His 4%) and lysine is higher at 11.5%.

When analysis of cysteine residues was attempted there was insufficient material to accurately quantify the results.

4.3.3.2 Monosaccharide and Sulfate Composition

The monosaccharide composition of the four peaks recovered from the high ionic strength anion exchange chromatography of bovine OHMG are listed in Table 4.8 and presented in Figure 4.8. Peaks 1 and 2 exhibited monosaccharide compositions that were slightly different to each other, however, both were comparable to a monosaccharide composition of a "typical" mucin species. Both species were slightly higher in GlcNAc than GalNAc (1.38:1 for Peak 1 and 1.44:1 for Peak 2) and considerably higher in Gal (4.54:1 for Peak 1 and 7.38:1 for Peak 2). Mannose was not observed in either Peak 1 or 2. The major difference between the monosaccharide composition of the two peaks was that Peak 2 had a higher sulfate content than Peak 1 (2.41 nmol sulfate per nmol amino acid in Peak 2 compared to only 1.93 for Peak 1). This would account for the later elution of Peak 2.

Table 4.8: *Monosaccharide composition of the species recovered from the high ionic strength ion exchange chromatography of bovine OHMG. Presented are both the total monosaccharide composition of the species [A] and the monosaccharides normalised to GalNAc [B]*

A

	Peak1		Peak2		Peak 3a		Peak4a		Bovine OHMG	
Mono.	**nmol/AA**	**SD**	**nmol/AA**	**SD**	**nmol/AA**	**SD**	**nmol/AA**	**SD**	**nmol/AA**	**SD**
Fucose	*	*	*	*	0.01	0.04	0.20	0.05	0.02	0.12
GalNAc	0.01	0.01	0.01	0.04	0.03	0.00	0.61	0.19	0.12	0.61
GlcNAc	0.02	0.00	0.02	0.02	*	*	0.55	0.26	0.14	0.28
Galactose	0.07	0.30	0.11	0.52	0.08	0.00	0.37	0.17	0.07	0.04
Mannose	*	*	*	0.03	0.22	0.00	0.05	0.15	0.05	0.16
NeuNAc	**	**	**	**	**	**	**	**	*	*
NeuGc	**	**	**	**	**	**	**	**	*	*
Sulfate	0.03	0.05	0.04	0.17	*	*	*	*	0.05	0.02

B

	Peak 1	Peak 2	Peak 3	Peak 4	Bovine OHMG
Mono.	Norm.	Norm.	Norm.	Norm.	Norm.
Fucose	*	*	0.33	0.32	0.17
GalNAc	1.00	1.00	1.00	1.00	1.00
GlcNAc	1.38	1.44	*	0.89	1.15
Galactose	4.54	7.38	2.41	0.60	0.62
Mannose	*	0.17	6.5	0.08	0.38
NAcSial	**	**	**	**	*
NGlySial	**	**	**	**	*
Sulfate	1.93	2.41	*	*	0.42

** indicates that the monosaccharide was below the detection limit of the procedure used*

*** Sialic acid was not examined in the IEC fractions as it was not detected in whole bovine OHMG*

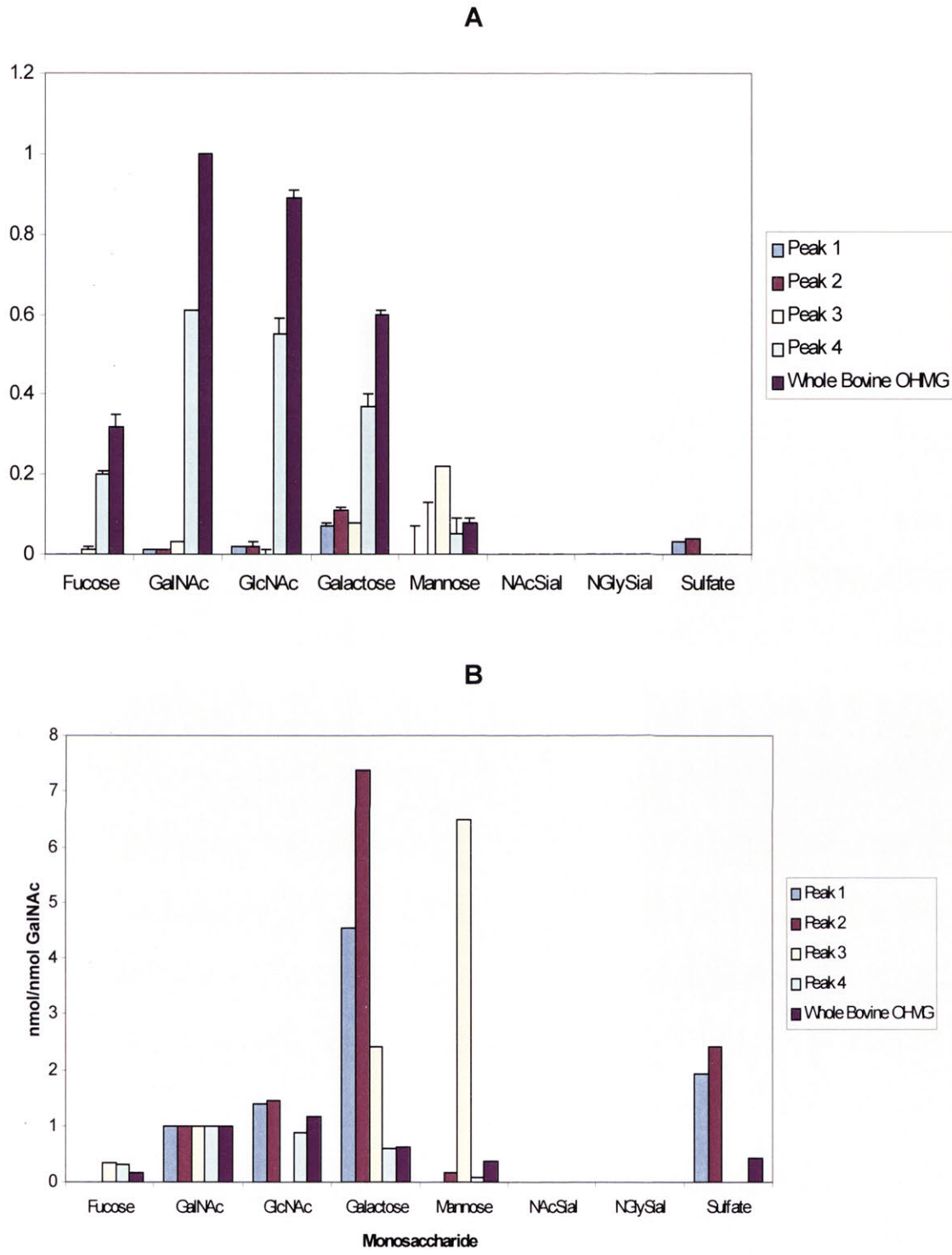

Figure 4.8: *Monosaccharide composition of the species recovered from the high ionic strength ion exchange chromatography of bovine OHMG. Presented are both the total monosaccharide composition of the species [**A**] and the monosaccharides normalised to GalNAc [**B**]. Note that though the amino acid compositions of Peaks 1 and 2 are similar, their monosaccharide composition is different. This is also the case for 3 and 4*

The monosaccharide content of Peaks 3 and 4 were significantly different to that of Peaks 1 and 2, as well as to each other. Peak 3 contained small amounts of fucose and GalNAc, with some Gal and a large amount of Man. The amount of mannose detected was unusually large, suggesting the possibility of contamination from glucose or co-elution with xylose (if mannose is present in large numbers then it would be expected that significant GlcNAc would also be present as they are associated in N-linked oligosaccharides). Peak 4 contained all of the monosaccharides commonly found in glycoproteins, with GalNAc and GlcNAc being the most prevalent. Galactose and fucose were also observed in significant amounts (0.6 nmol Gal per nmol GalNAc, 0.33 nmol Fuc per nmol GalNAc). Mannose was only observed in small quantities (0.08 nmol Man per nmol GalNAc). Sulfate was not detected in either Peak 3 or 4.

4.3.3.3 Overview of the Composition of Fractions of the High Molecular Weight Glycoconjugates of Bovine Ocular Mucus Separated by Ion Exchange Chromatography

Some of the calculated characteristics of the peaks recovered from high ionic strength anion exchange chromatography (HIAEC) of bovine OHMG are presented in Table 4.9. It is interesting to note that, although Peaks 1 and 2 have an amino acid and monosaccharide composition which is similar to mucins recovered from other tissues, they are only lightly glycosylated (Peak 1 13.2% carbohydrate by weight; Peak 2 17.9%). Conversely, Peaks 3 and 4, which are not enriched in the amino acids associated with oligosaccharide attachment (Ser, Thr, and Asx) and would, therefore, be expected to contain little carbohydrate, are in fact heavily glycosylated (32.3% and 74.7% carbohydrate by weight respectively).

Of note is the finding that the fractions recovered from the IEC column accounted for only 60.5% of the protein, 27.1% of the carbohydrate and 27.4% of the sulfate loaded, suggesting that a highly sulfated, highly glycosylated protein is being retained on the IEC column. This description would be satisfied by a proteoglycan-like glycoconjugate.

Table 4.9: *Some calculated characteristics of the species recovered from high ionic strength anion exchange chromatography of bovine high molecular weight ocular glycoconjugates. Note that the species recovered from the ion exchange column account for 60.4% of the amino acids loaded but only 27.1% of the carbohydrate and 27.4% of the sulfate, suggesting that a high carbohydrate/highly charged component of the whole OHMG was not recovered by the procedure*

	Peak 1	Peak 2	Peak 3	Peak 4	Bovine Whole OHMG Fraction
% by wt Carbohyd.	13.2	17.9	32.3	74.7	39.3
% Sulfate	1.7	2.0	0.0	0.0	1.9
% total Ser + Thr	32.5	30.2	10.9	11.9	13.6

4.3.4 Compositional Analysis of Human Tears

Two concerns with using the glycoconjugates extracted from bovine conjunctiva as a model for the human tear film are that there is some form of artefact being introduced by the excision of the conjunctival tissue (proteoglycans are a major component of the stroma) and/or the model is unrelated to human ocular mucus. As discussed in Chapter 2 (Section 2.2), when the conjunctiva was extracted by swabbing the surface of the eye rather than incubating the tissue overnight, similar amino acid compositions were observed. There is, however, still the possibility that the extraction buffer is efficient at removing proteoglycans from the ocular tissue. In order to further address these concerns, analysis of basal tears collected from humans was undertaken. Tears were collected by placing a glass microcapillary tube into the tear film reservoir at the lid margin, and purified and characterised as per procedures used for the characterisation of the bovine

conjunctival extract.. When amino acid analysis is performed on tears, however, the surface of the eye has not been extracted. It is unrealistic to suppose that the major component of the tear film mucus layer is a breakdown product from the stroma. Therefore, if a similar trend is observed between the amino acid analysis of tears and conjunctival extract, it is reasonable to conclude that the composition observed when the conjunctiva was extracted was not artefactual. Furthermore, as human tears are being analysed, if the composition is similar to that observed for bovine conjunctival extract, it can be surmised that bovine conjunctival mucus might serve as a good model for the human ocular tear film.

4.3.4.1 Amino Acid Composition

Amino acid composition of human tears (Table 4.10, Figure 4.9) indicates that a bovine model may be suitable as an indicator of the properties of the human tear film.

Table 4.10: *Comparison of the high molecular weight fraction recovered from human tears when separated by high salt SEC under reducing conditions to bovine OHMG*

	Human OHMG		Bovine OHMG	
A. Acid	% Comp.	SD	% Comp.	SD
Asx	14.5	0.64	22.6	3.18
Glx	7.5	0.12	10.8	0.29
Ser	6.8	0.10	8.1	0.45
Gly	15.3	0.30	9.3	0.61
His	0.5	0.00	1.3	0.15
Arg	3.0	0.06	5.2	0.29
Thr	5.7	0.12	5.5	0.26
Ala	8.5	0.12	6.7	0.40
Pro	4.8	0.17	3.7	0.12
Tyr	0.9	0.06	1.3	0.15
Val	1.4	0.29	5.7	0.20
Met	0.4	0.06	1.0	0.15
Ile	2.3	0.06	3.4	0.21
Leu	24.5	0.31	7.2	0.35
Phe	2.0	0.21	3.0	0.17
Lys	2.0	0.15	5.2	0.23

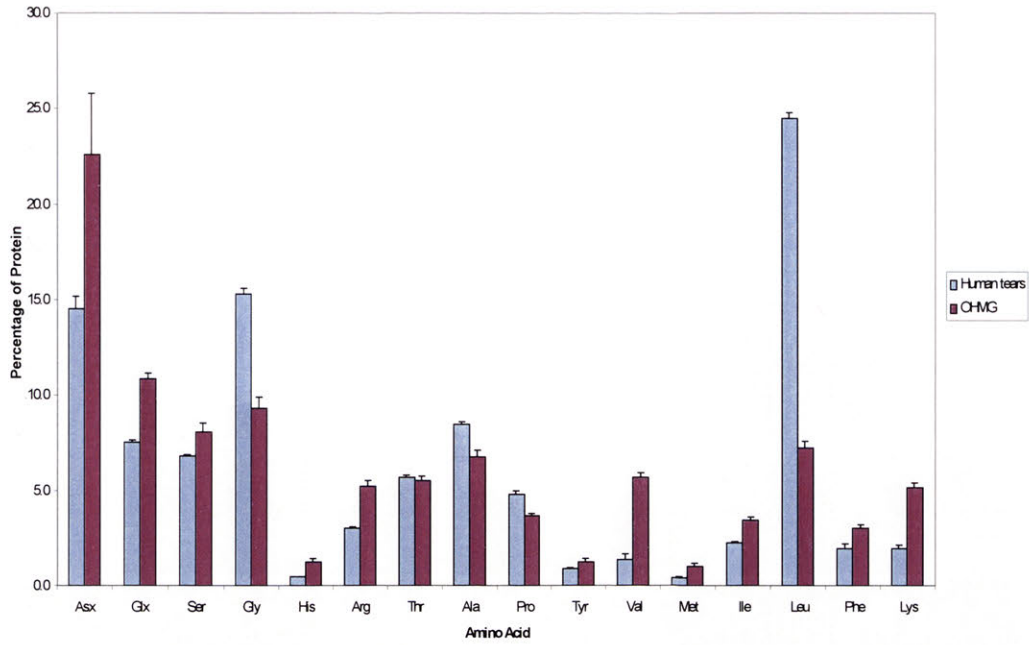

Figure 4.9: *Comparison of the high molecular weight fraction recovered from human tears when separated by high salt SEC under reducing conditions to bovine OHMG. With the exception of Asx and Leu, the general trends of the amino acids appear to be similar, suggesting that bovine OHMG may be a useful model for human tears*

It can be seen that while the total percentages were not exactly the same, the general amino acid compositions of the high molecular weight glycoconjugate fraction of bovine conjunctival mucus were similar in some instances to the amino acid composition of the high molecular weight glycoconjugate fraction of human tears. As in the bovine OHMG the levels of Ser and Thr in human OHMG are relatively low, and the total Ser is higher than the total Thr (Ser 7%, Thr 6% of the total amino acids of the fraction). Asx and Glx also comprise a major portion of the total amino acids in the human OHMG fraction (22% of the total amino acids). The level of Asx, although high in human OHMG, was, however, considerably lower than those of bovine OHMG (14.5% for human OHMG as compared to 22.5% for bovine OHMG). Some other noticeable differences are the levels of leucine (almost 25% in human OHMG as compared to 7% in bovine OHMG) and valine (1.4% in human OHMG, 6% in bovine OHMG). However,

these differences aside, it is obvious that, as in bovine OHMG, the glycoconjugate fraction of human ocular mucus includes a component that is not a mucin in the traditional sense.

4.3.4.2 Monosaccharide and Sulfate Composition

Table 4.11 and Figure 4.10 show the monosaccharide content of human OHMG and compares it to that of bovine OHMG. Human OHMG has a substantially different composition to that of bovine OHMG, having less GalNAc and GlcNAc. Human OHMG contains 0.2 nmols of GalNAc and 0.02 nmols of GlcNAc per nmol of amino acids, compared to 0.12 GalNAc and 0.14 GlcNAc for bovine OHMG, but much higher galactose and sulfate (human OHMG has 0.36 nmol Gal and 0.23 nmol sulfate per nmol of amino acids, compared to 0.07 Gal and 0.05 sulfate for bovine OHMG). Human OHMG also had measurable N-acetyl and N-glycolyl neuraminic acid, whereas only trace amounts of these monosaccharides are present in bovine OHMG.

Table 4.11: *Monosaccharide analysis of human OHMG and comparison to bovine OHMG. Both the total nmol per nmols of amino acid (nmol/AA) and nmol normalised to GalNAc are presented (Norm)*

	Human OHMG			Bovine OHMG		
	nmol/AA	SD	Norm.	nmol/AA	SD	Norm.
Fucose	0.06	0.01	2.61	0.02	0.01	0.17
GalNAc	0.02	0.00	1.00	0.12	0.00	1.00
GlcNAc	0.02	0.02	0.91	0.14	0.01	1.15
Galactose	0.36	0.01	15.66	0.07	0.00	0.62
Mannose	0.03	0.17	1.21	0.05	0.00	0.38
NAcSial	0.05	0.25	2.08	0.00	0.00	0.00
NGlySial	0.02	0.02	0.80	0.00	0.00	0.00
Sulfate	0.23	0.00	9.79	0.05	0.00	0.42

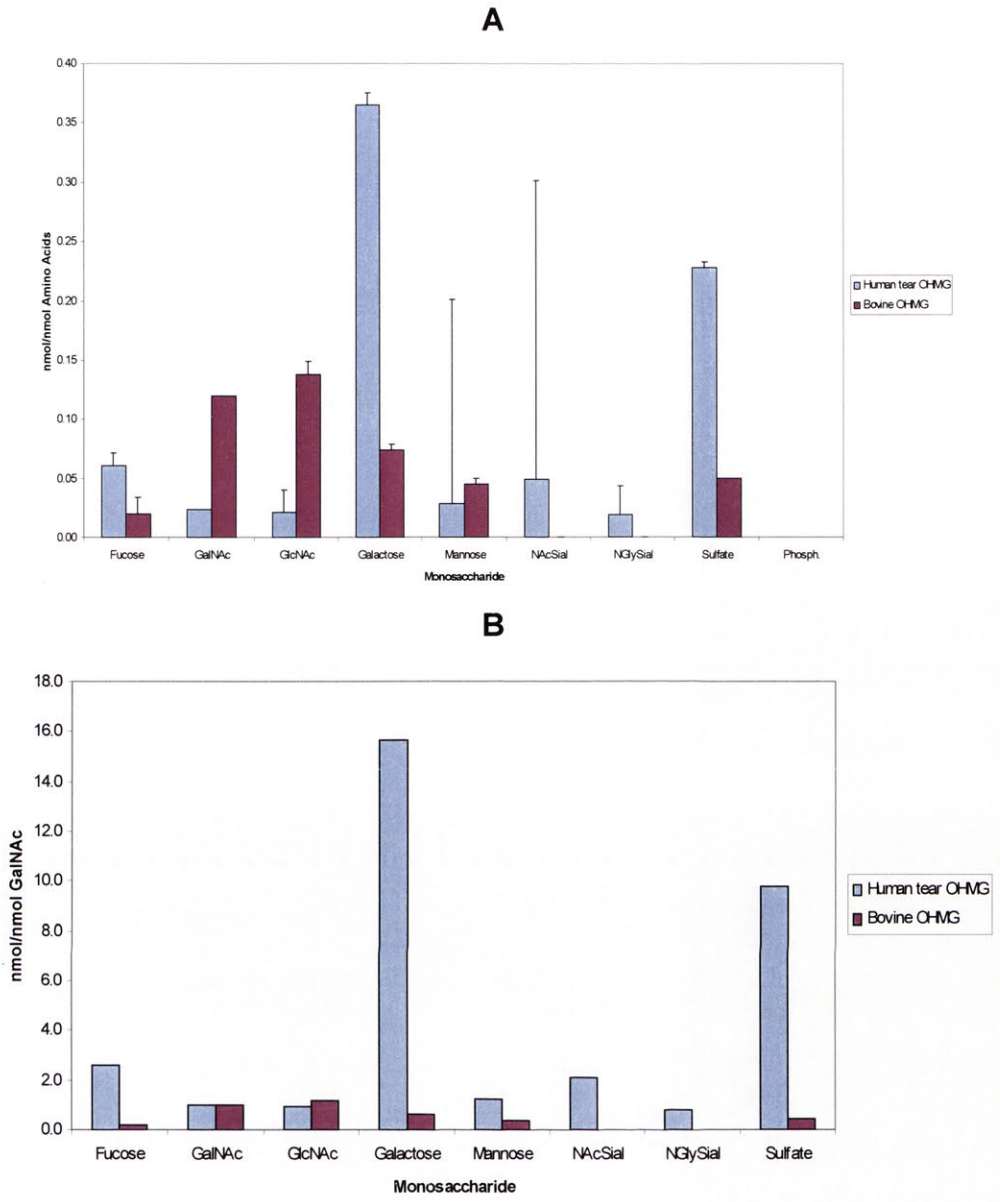

Figure 4.10: *Monosaccharide analysis of human OHMG and comparison to bovine OHMG. Both the total nmol [**A**] and nmol normalised to GalNAc [**B**]are presented. The monosaccharide content of human OHMG is significantly different to that of bovine OHMG. Most notably the lower levels of GalNAc and GlcNAc and the much higher levels of Gal and sulfate. The presence of N-glycolyl neuraminic acid is worthy of note as this monosaccharide is not usually associated with humans*

4.3.4.3 Overview of the Composition of Human OHMG and Comparison to Bovine OHMG

Table 4.12 lists some calculated characteristics of human and bovine OHMG. The two glycoconjugate fractions have a similar carbohydrate content, with human

OHMG being slightly higher. Human OHMG was determined to be 43.5% carbohydrate by weight compared to 38.7% for bovine OHMG. They also have a similar total Ser + Thr (12.5% for human OHMG and 13.6% for bovine OHMG). The GalNAc: Ser + Thr ratios are different however (1:6.25 for human tears compared to 1:9.42 for bovine OHMG) and human OHMG contains far more charge (having both Sialic acid and more sulfate).

Table 4.12: *Some calculated characteristics of human OHMG and bovine OHMG. Note that while the glycoconjugate fractions contain approximately the same carbohydrate by mass and total Ser + Thr, human OHMG is more highly charged – containing a higher proportion of sulfate per molecule as well as measurable sialic acid*

	HTM	OHMG
% by wt Carbohyd.	43.5	38.7
% Sialic.	8.4	*
% Sulfate	7.7	1.9
% total Ser + Thr	12.5	13.6

**denotes that the component was below the detection limit of the procedure used*

4.4 CONCLUSIONS / DISCUSSION

When the amino acid composition of bovine conjunctival extract is compared to the values reported in the literature for rabbit ocular mucin (Figure 4.11) (Tseng *et al.* 1987), it is clear that they share many of the same characteristics. The amino acid compositions of OHMG are different to those of mucins from other sources in that the total Ser in the sample is higher than Thr. Also, the total Ser + Thr content of OHMG and rabbit are low (approximately 14%) compared to the other mucins analysed (27-33%). Ocular mucins also contain a larger percentage of Asx and Glx compared to mucins that have so far been described (SWISS-PROT database, http://expasy.proteome.org.au/sprot/). This indicates

that there may be a component of bovine OHMG that is not mucin-like in the traditional sense.

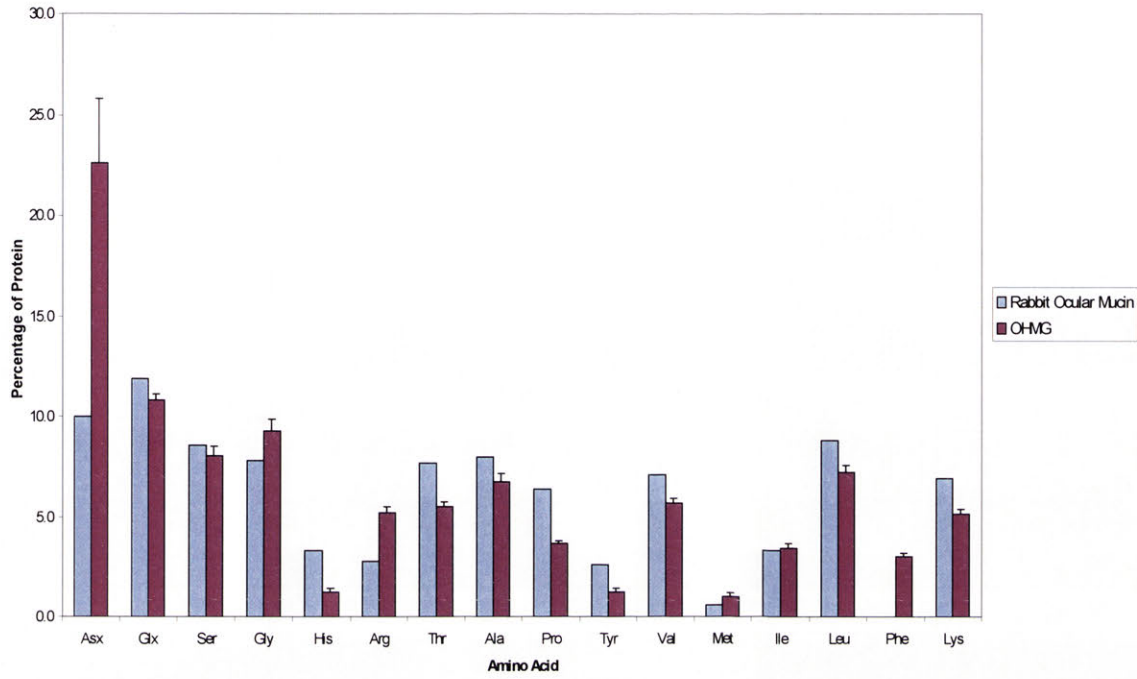

Figure 4.11: *Reported values of rabbit ocular mucin (Tseng et al. 1987) compared to those of bovine OHMG. Apart from the higher levels of Asx in bovine OHMG, the amino acid composition of the two is quite close. Of particular interest is the fact that Ser is higher than Thr in both cases. The total Ser + Thr is relatively low for a mucin species*

The monosaccharide composition of the bovine OHMG was also significantly different to that of the other mucins analysed. The bovine OHMG fraction contained significant mannose, suggesting that the oligosaccharides could have an N-linked structure, as mannose is present in the common core attached to asparagine. Many mucins have little or no N-linked oligosaccharides (Bertolini and Pigman 1969), and no significant mannose was found in either BSM or PGM. When levels of glucose contamination are high in a sample it is possible for it to spontaneously isomerise into mannose. In addition, xylose, a sugar not associated with mucin oligosaccharides, will elute at the same retention time as mannose using the separation conditions used in this thesis (Dionex technical note). When the amount of sample loaded onto the PA-10 column was increased two-fold, the

area of the mannose peak doubled for bovine OHMG, but not for either PGM or BSM (data not shown). This indicates that the mannose detected in OHMG was a real peak, not an artefact caused by glucose contamination. It does not distinguish between mannose and xylose however.

The presence of N-glycolyl neuraminic acid in the human tears is worthy of note as this monosaccharide is associated with cancer mutated mucins in humans (Devine *et al.* 1991). This is species-specific, however, as N-glycolyl neuraminic acid is normal on bovine and porcine glycoproteins (Mukuria *et al.* 1995; Jolles and Fiat 1979). When compositional analysis of PGM and BSM was performed, the resulting compositions were typical of mucin glycoproteins and consistent with the published data of the two species. Bovine OHMG, however, has been shown to be substantially different to both of these mucins. The high levels of Asx, Glx, and leucine, coupled with the low total Ser and Thr content of the amino acid backbone, suggest that the protein core of bovine OHMG is not typical for a mucin glycoprotein. In addition, the novel monosaccharide composition (high mannose and sulfate) suggest that the major high molecular weight glycoconjugate component of bovine ocular mucus is not a mucin at all.

The use of Rt41A to digest mucins, both heavily glycosylated from the colon or lightly glycosylated from ocular epithelium or sub-maxillary glands, has several advantages. While Rt41A appeared to be unable to penetrate into the glycopeptide regions of PGM, it was still more efficient in producing large glycopeptides than pronase. Only 1.2 ng (22 pmol) of Rt41A was required to digest 300 μg of mucin (a ratio of 2.5×10^{-5}:1 as opposed to 1:1 for pronase). It was possible to recover the larger glycopeptides from

PGM and the smaller glycosylated regions of BSM and OHMG by changing the time course and digestion conditions. As Rt41A is a non-specific serine protease, it cleaves the substrate at any accessible amino acid residue. When using this enzyme to digest the high molecular weight, heavily glycosylated mucins such as PGM, the enzyme can be allowed to cleave the glycosylated region indefinitely. This will effectively concentrate the glycopeptide to the form that is completely resistant to the protease, allowing for further analysis. If protein sequencing is required on the peptides recovered from the digestion of less densely glycosylated glycoconjugates (such as BSM or bovine OHMG) then further preparation would be needed (either by a second digestion with a specific protease or some form of affinity chromatography to enrich one of the peptides produced by the Rt41A digestion).

The digestion of PGM clearly shows two peaks, one at the void volume of Superose 12 (greater than 200-300 kDa) and one eluting just after the void (approximately 150-200 kDa). This is consistent with it being a large gel forming mucin such as MUC-6 (human gastric mucin) or MUC-2 (found in the intestines). MUC-2 has two distinct glycosylated regions: the large tandem repeat domain (labelled glycoprotein A) and a smaller less glycosylated region that contains only imperfect repeats (labelled Gly B) (Figure 2.13). As the peak corresponding to the smaller fragment was collected it is possible that this corresponds to a smaller glycosylated region similar to Gly B. Figure 4.12 compares the glycopeptide fraction recovered from the Rt41A digestion of PGM, to the whole fraction. The glycopeptide recovered from the digestion of PGM was found to be enriched in the amino acids which are most often found in tandem repeats, suggesting that the smaller glycopeptide in PGM may contain a repeat sequence. However it was relatively low in

sugar content (being only 76% carbohydrate by weight compared to 90% for the whole protein). The percentage of Ser and Thr was also low (Ser and Thr make up approximately 45% of the total amino acid content of the whole mucin, compared to only 26% for the glycopeptide recovered). The larger peak was not collected as it co-eluted with non-digested mucin.

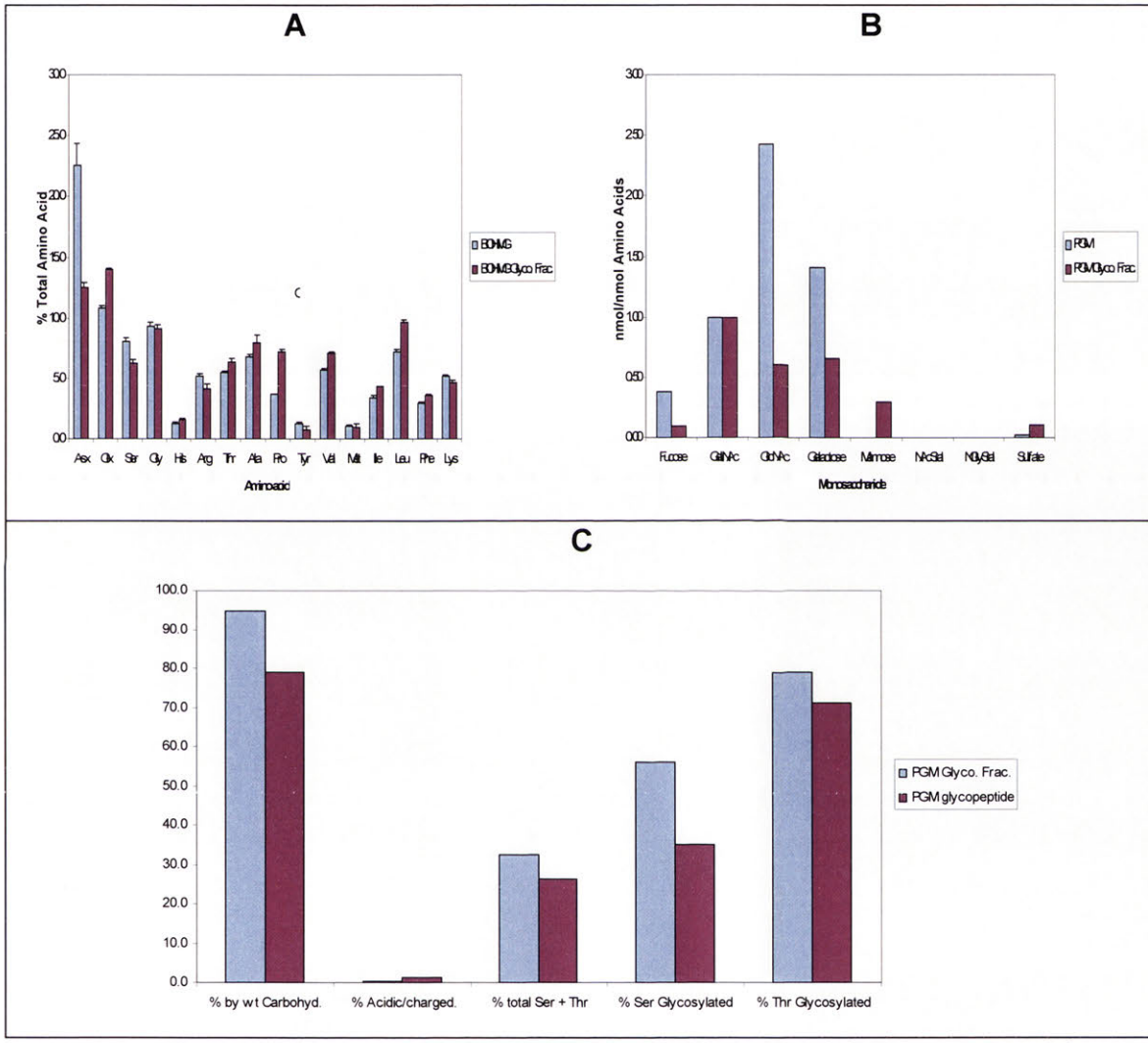

Figure 4.12: *Comparison of porcine gastric mucin to the glycosylated fraction recovered from its digestion with Rt41A. Presented is the amino acid composition [**A**], monosaccharide compositions normalised to GalNAc [**B**], and calculations derived from the compositional data [**C**]*

BSM was digested into a glycopeptides (approximately 75-120 kDa, as determined by molecular weight markers) when treated for only three hours. Further digestion resulted in the total destruction of the glycoprotein. Figure 4.13 compares BCM to this

glycopeptide fraction. Compositional analysis of the glycopeptide recovered revealed that it was essentially similar in characteristics to the whole mucin. The total amount of carbohydrate present was the same (approximately 71% in both cases) and, while there was a slight increase in the amount of Asx and Glx in the fragment of BSM, the amino acid composition was also close. Similarly the monosaccharide composition of the glycopeptide and the whole mucin was not significantly different. This suggests that the molecule of BSM is a molecule without a region that is highly glycosylated. Rather the oligosaccharides are spaced evenly along the majority of the molecule. Possibly the smaller material in the digest (after three hours) contains smaller glycoprotein fragments.

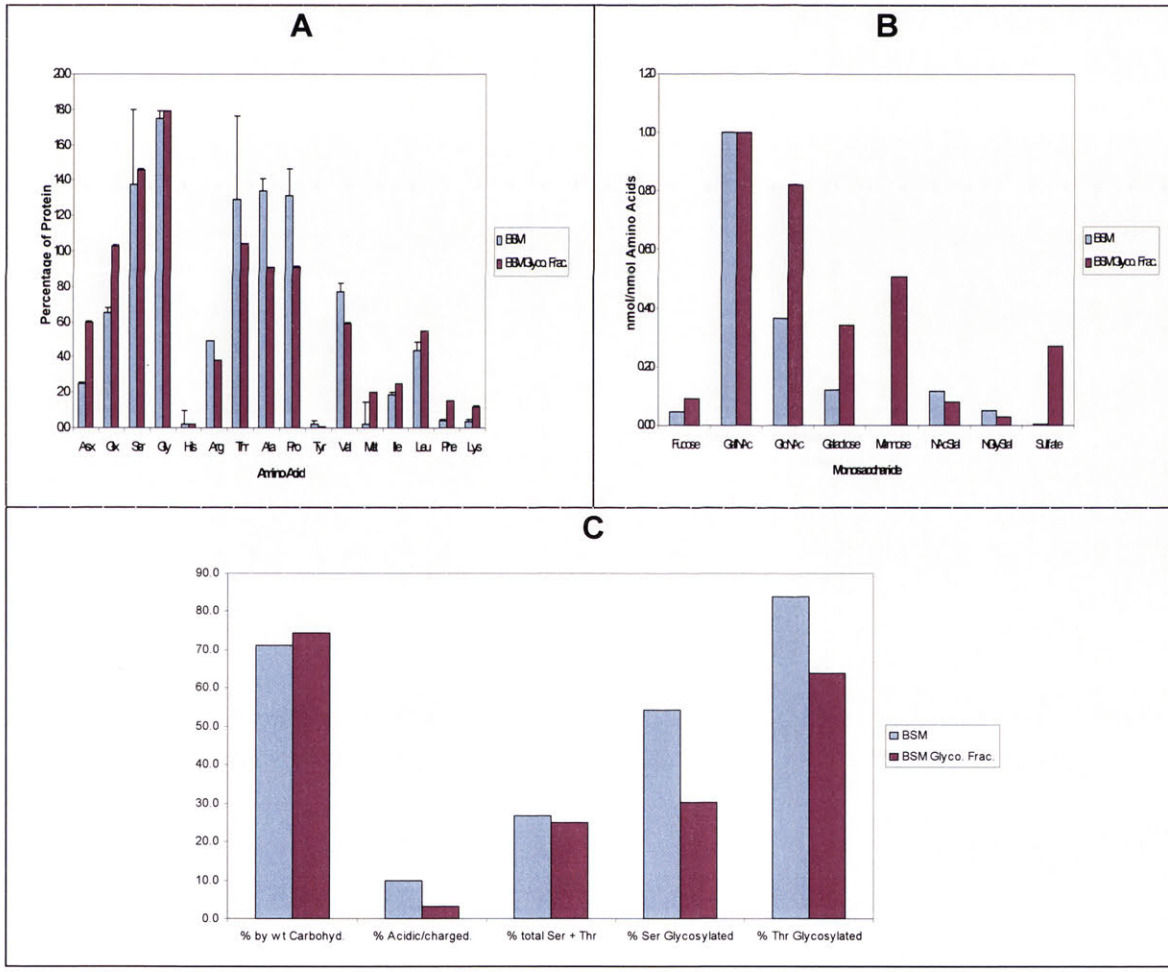

Figure 4.13: *Comparison of bovine sub-maxillary mucin to the glycosylated fraction recovered from its digestion with Rt41A. Presented is the amino acid composition [**A**], monosaccharide compositions normalised to GalNAc [**B**], and calculations derived from the compositional data [**C**]*

Figure 4.14 compares bovine OHMG to its glycopeptide fraction recovered from the RT41A digestion by high salt IEC. The glycosylated peptide fraction that resulted from the Rt41A digestion of bovine OHMG was different to the corresponding fractions from both PGM and BSM. The OHMG glycopeptides were still high in Asx, Glx and leucine suggesting that, if a repeat region is present in these glycoconjugates, it contains the above amino acids. The amino acids most commonly associated with tandem repeat sequences in mucins were not enriched in the glycopeptide fraction of bovine OHMG, indicating that a "mucin-like" repeat sequence is not present as a major component of ocular mucus. In addition, if a large gel forming mucin (such as MUC-5AC) was a major component of ocular mucus, it would be expected that a glycopeptide containing the large protease resistant "tandem repeat" region would be recovered after digestion. This was probably not the case.

The monosaccharide composition of the glycopeptide fraction recovered from the protease digestion of bovine OHMG further supports the deduction that mucin is not the major component of bovine ocular mucus. As with the whole OHMG fraction, the peptide fraction of bovine OHMG contained significant amounts of mannose/xylose. In addition, the total carbohydrate content was higher and total sulfate was enriched in the glycosylated fraction of the digest, suggesting that this fraction contained a heavily glycosylated, sulfate rich region of the glycoconjugates present in bovine OHMG. This glycosylated region is clearly not from any mucin that has been so far described. Rather the very high sulfate, possibly xylose or high mannose and amino acid composition of the glycopeptide fraction, is indicative of a typical proteoglycan-type glycoconjugate (Iozzo 1999).

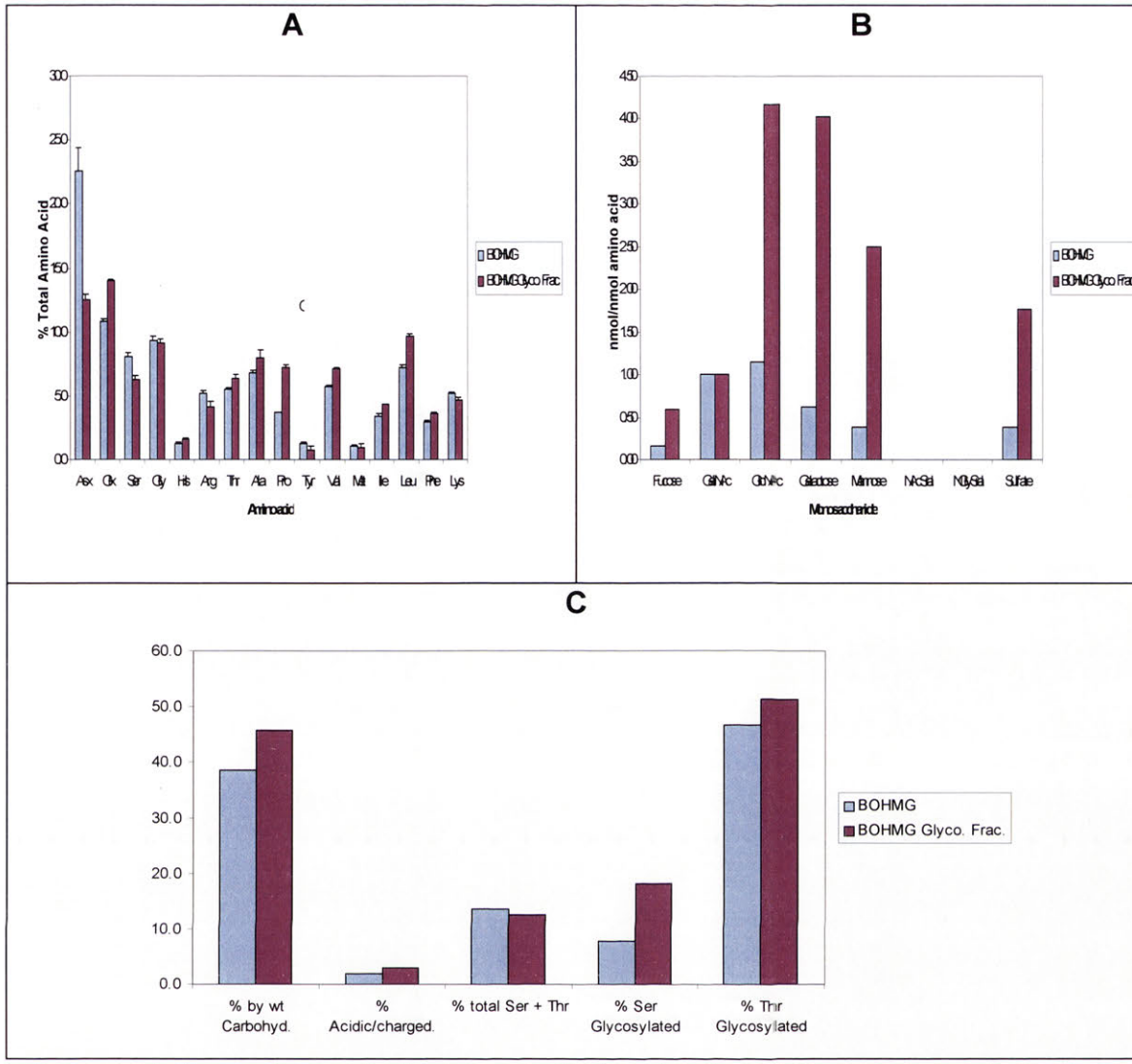

Figure 4.14: *Comparison of the bovine ocular high molecular weight glycoconjugate fraction to the glycosylated fraction recovered from its digestion with Rt41A. Presented is the amino acid composition [**A**], monosaccharide compositions normalised to GalNAc [**B**], and calculations derived from the compositional data [**C**]*

The species recovered from the high ionic strength anion exchange chromatography of whole bovine OHMG include a species that has a similar composition to that of a "traditional" mucin. Two major differences, however, are the higher total Ser to Thr content, and the low carbohydrate content of the species. When the amino acid composition was compared to the sequence of known proteins (in the SWISS-PROT database, http://expasy.proteome.org.au/sprot/) no significant match was achieved. The other species recovered had an amino acid and monosaccharide composition more typical

of a standard glycoprotein, however, a similar search also resulted in no significant match (many of the actin family of proteins were represented but the match was > 400). It is interesting to note, however, that there is no obvious reason for peak 3 and 4 to elute after peaks 1 and 2. They have no greater amount of negatively charged amino acids, nor do they contain more sialic acid or sulfate. This suggests the possibility of a component that isn't being observed by the analysis methods used (for instance the presence of uronic acids in glycosaminoglycan oligosaccharides), or an artefact of some kind in the procedure. This is worthy of further pursuit, as peaks 3 and 4 may be proteoglycans. Additionally the relative amounts of cysteine in the samples may provide insight into the differences between the species. Analysis of cysteine using the methods described here requires large amounts of material relative to other types of analysis, and when it was attempted the results could not be effectively interpreted. However as cysteine plays an important role in the formation of gel matrices, comparison of the cysteine content of the four peaks may prove useful. It is worth noting however that there appeared to be no discernable difference between the cysteine content of PGM BSM and the whole high molecular weight glycosylated fraction of bovine ocular mucus, so the information gained may in fact be minimal. Most interestingly the species eluted from the column accounted for approximately 60% of the protein but only 30% of the carbohydrate and sulfate that was loaded. While losses are incurred in any procedure, they should be consistent across the analysis. The fact that less sulfate and carbohydrate was recovered suggests that a heavily glycosylated/highly charged fraction has not yet been recovered and identified.

By comparing the amino acid content of the bovine conjunctival extract with that of basal tears collected from humans, it was possible to eliminate the possibility of artefacts

caused by the excision procedure. The composition of the human high molecular weight glycoconjugate fraction has enough homology with bovine OHMG to conclude that the compositions are similar. It is not reasonable to suggest that the dominant species in the healthy mucus layer of an eye would be a breakdown product from another tissue. Human ocular mucus appears to contain more highly charged glycoconjugates than that of bovine OHMG. Human OHMG has much larger amounts of sulfate and significant sialic acid content. Interestingly, human OHMG contains N-glycolyl neuraminic acid, a monosaccharide that is usually only associated with aberrations, such as cancer, in humans.

The compositional data suggests strongly that the major component of the mucus layer tear film is not, in fact, a mucin in the traditional sense. The most likely candidate is another class of high molecular weight glycoconjugates - proteoglycans.

When the composition of OHMG is compared to that of β-luminican (amino acid composition reported on the SWISS-PROT database) (a major proteoglycan found in the stroma of cow eyes) (Figure 4.15), an obvious similarity in composition was seen.

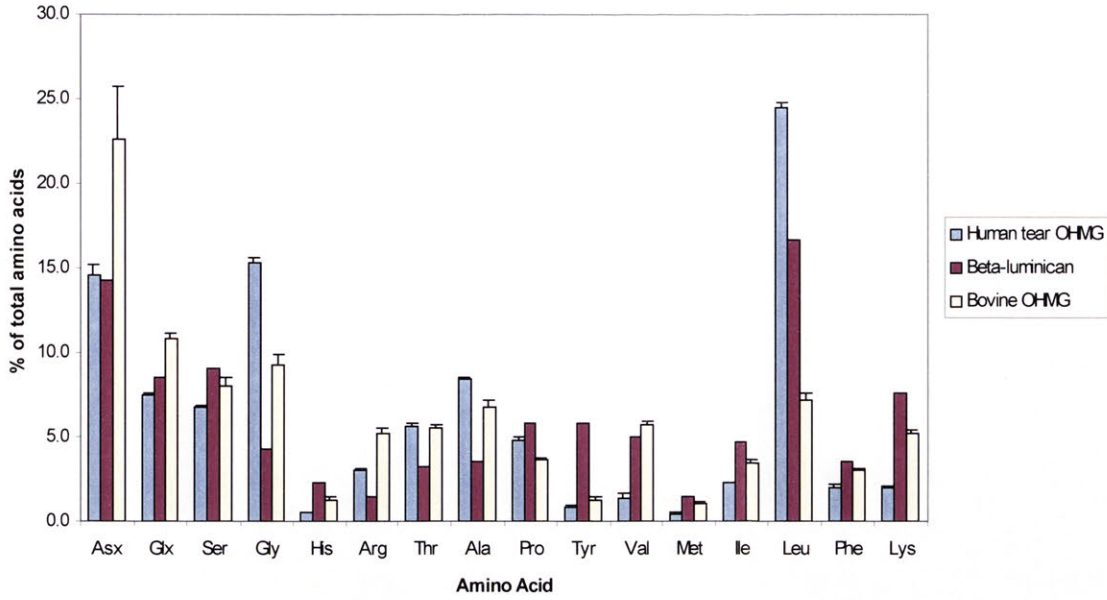

Figure 4.15: *Comparison of bovine and human OHMG with the reported amino acid composition of β-luminican – the major proteoglycan found in bovine cornea. Many similarities can be seen, such as the Ser and Thr content, as well as the relatively high Asx and Glx content. This suggests the possibility of the OHMG containing a proteoglycan-like component*

β-luminican is high in total Asx and Glx, low in total Ser and Thr relative to mucins, and the total Ser is greater than the total Thr. Leucine and lysine levels are also high in β-luminican. This is an indication that the major component of OHMG is a proteoglycan-like glycoconjugate rather than a mucin. Xylose is a monosaccharide component of many proteoglycan molecules. The elevated levels of supposed mannose detected in the bovine OHMG sample may be accounted for by xylose, if a proteoglycan-like molecule is a major component of the bovine ocular mucus layer. This possibility is further explored in the next chapter.

CHAPTER 5: DETERMINATION OF SPECIFIC COMPONENTS FOUND IN THE HIGH MOLECULAR WEIGHT FRACTION OF CONJUNCTIVAL MUCUS

5.1 INTRODUCTION

While compositional analysis is a powerful tool in the determination of what types of components are found in the high molecular weight glycoconjugate fraction of bovine conjunctival mucus, some information on specific components will help to create a more complete picture of what molecular species are present. There are many well-characterised methods that can be used for determining what species are present in a sample. Monoclonal antibodies are commonly used for the detection of specific epitopes on expressed proteins and mRNA hybridisation can be used to detect what the cell machinery is producing. In the past, the majority of work performed on mucin species detection in mucus has been carried out in these ways (see, for example, Porchett *et al.* 1991). These two techniques are very powerful but they have some significant drawbacks. Most importantly, they only show whether or not something already suspected to be present is actually in the sample i.e. the sequence of RNA must be known, or the epitope of the protein or sugar previously characterised. Anything that has not been found previously will not be detected. This approach could easily account for the discrepancies found between what has been previously reported for the tear film components and what is reported for the high molecular glycoconjugates in this project. In the simplest terms, major components of the tear film have not been found previously because they have not been looked for. Furthermore, analysis of mRNA will only provide information on the protein backbone that is produced. There is a large amount of

evidence suggesting that mucins with the same amino acid sequence can have many functions due to their post-translational modifications. MUC-1, for example, can be membrane bound or secreted and there is evidence to suggest that it can act as a receptor (Patton 1999; Mall *et al.* 1992). Monoclonal antibodies have, in the past, been shown to produce erroneous results when being used to identify mucins where cross reactivity with sugars has been responsible for false positive staining (Montagne *et al.* 2000). For these reasons these two methods of analysis have not been used for this project. Rather, specific removal of sugars and mass spectrometry were employed to gain specific knowledge of the oligosaccharide content of the glycoproteins found in ocular mucus.

In the previous chapters analysis of the total OHMG fraction indicated that the composition of this fraction was not consistent with mucin being the only, or indeed, major component of ocular mucus. In this chapter bovine OHMG was treated by chemical or enzymatic means to specifically remove sub-classes of oligosaccharides. Removal of traditional N-linked oligosaccharides was performed by incubation of the OHMG fraction with PNGase-F. This treatment will cleave any oligosaccharides that contain the ($GlcNAc_2$, Man_3) branched structure linked to asparagine. Chemical β-elimination, using alkaline conditions in a reducing environment, was used to remove any oligosaccharides that were O-linked to Ser or Thr. Finally, any specific glycosaminoglycan-like oligosaccharides were removed with keratanase. Keratanase cleaves GlcNAc-Gal linkages where GlcNAc is sulfated and Gal is not (Nakazawa *et al.* 1975). These released oligosaccharide fractions were then analysed for monosaccharide content and Electrospray-TOF or MALDI-TOF mass spectrometry was used to determine the mass fingerprint of the fractions.

Mass spectrometry has made significant developments over the past few years, enabling it to be used for the sequencing of both peptides and oligosaccharides (Tetaert *et al.* 1994). By treating a protein with a specific endoprotease such as trypsin, a peptide mass fingerprint can be formed. Each protein will produce an individual pattern of peptide sizes when treated with trypsin. By using a database of amino acid sequence, or of fragment size of known proteins, it is possible to match an unknown protein with any of the previously characterised gene sequences. Similarly, by removing oligosaccharides intact from the protein backbone, the mass can be used, with the aid of simple calculations, to predict the possible composition of monosaccharides present. There are, however, limitations to these procedures. When using a peptide fingerprint, the gene sequence must be known for a positive identification to occur. Peptide masses do not provide the sequence of unknown proteins. This can be overcome by using MS-MS fragmentation patterns. Similarly, the order of the monosaccharides in released oligosaccharides cannot be determined without MS-MS fragmentation. Even using MS-MS may prove difficult, as the branched structures of many oligosaccharides can make the fragmentation pattern difficult to interpret. Mass spectrometry also does not necessarily detect everything that is in the sample, as some species may not "fly" i.e. they may not ionise and reach the detector.

5.2 ANALYSIS OF OLIGOSACCHARIDES RELEASED FROM OHMG FRACTION

5.2.1 Materials and Methods

5.2.1.1 Release of Oligosaccharides

Three methods were used to remove specific oligosaccharide sub-classes from the amino acid backbone of the high molecular weight glycoconjugates.

1. O-linked oligosaccharides were removed from OHMG by β-elimination. To 1 volume of protein solution, 1 volume of H_2O, 1 volume 4 M $NaBH_4$ (made fresh) and 1 volume 0.4 M NaOH were added (in that order). The solution was mixed and incubated for 16 hours at 45°C. After incubation, the sample was chilled on ice and neutralised with 1 volume 0.4 M acetic acid added in 0.2 volume aliquots, mixing between additions. The pH of the sample was confirmed at between 6.4 and 7.0 with litmus paper.

2. N-linked oligosaccharides were removed from the protein with PNGase-F cloned from *Flavobacterium meningosepticum* and expressed in *E. coli* (Roche Diagnostics, Mannheim, Germany). Bovine OHMG was dissolved into 50 mM sodium phosphate buffer pH 7.5 and 2.5 µl 2% (w/v) SDS/1 M β-mercaptoethanol and the solution heated to 100°C for 10 minutes. After heating, the solution was cooled on ice and 5 mU PNGase-F and

2.5 µl 15% NP-40 was added. The sample was incubated for 16 hours at 37°C.

3. Proteoglycan-like oligosaccharides were detected using keratan sulfate endo-β-galactosidase from *Pseudomonas sp.* (Seikagaku Corporation, Tokyo, Japan). Bovine OHMG was dissolved into 50 mM sodium phosphate buffer pH 7.5 and 1 U keratanase added. The solution was incubated at 37°C for 16 hours.

5.2.1.2 Recovery of Oligosaccharides

Oligosaccharides that were removed from the glycoconjugates using the above methods were recovered from the solution using Alltech (Deerfield, Illinois, USA) Carbograph SPE Carbon columns, as described by Packer *et al.* (1998A). The columns were activated with one column volume (CV) of 80% acetonitrile in 25 mM ammonium formate buffer pH 9.0. The columns were then washed with 2 CV's of water before the sample was loaded. The columns were then washed with a further two CV's of water to remove salts. The oligosaccharides were eluted from the columns using two CV's of 50% MeCN in 25 mM ammonium formate buffer pH 9.0. The samples were then lyophilised and either hydrolysed, to their monosaccharide components, as per methods described in Chapter 4 (Section 4.2.3) or taken whole for MS analysis.

5.2.2 Monosaccharide Composition of Released Oligosaccharides

Monosaccharide analysis of the three released oligosaccharide fractions, compared to that of the whole OHMG, is listed in Table 5.1 and graphically represented in Figure 5.1.

Table 5.1: *Analysis of the monosaccharide composition of three types of oligosaccharides on bovine OHMG. Both total nmol recovered (nmol/AA) and nmol normalised to GalNAc (Norm) are presented*

	Whole OHMG			PNGase-F			β-eliminated			Keratanase		
	nmol/AA	SD	Norm.	nmol/AA	SD	Norm.	nmol/AA	SD	Norm.	nmol/AA	SD	Norm.
Fucose	0.73	0.03	1.82	0.06	0.01	0.53	0.05	0.01	0.34	0.20	0.00	7.81
GalNAc	0.40	0.00	1.00	0.11	0.06	1.00	0.14	0.00	1.00	0.03	0.10	1.00
GlcNAc	0.59	0.04	1.47	0.12	0.02	1.12	0.42	0.02	2.97	0.00	0.00	0.00
Galactose	0.12	0.00	0.30	0.03	0.00	0.25	0.07	0.01	0.53	0.02	0.00	0.63
Mannose	0.04	0.01	0.10	0.03	0.00	0.24	0.03	0.01	0.21	0.02	0.07	0.79
NAcSial	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NGlySial	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Sulfate	0.05	0.03	0.13	0.03	0.00	0.30	0.07	0.39	0.50	0.05	0.03	1.81

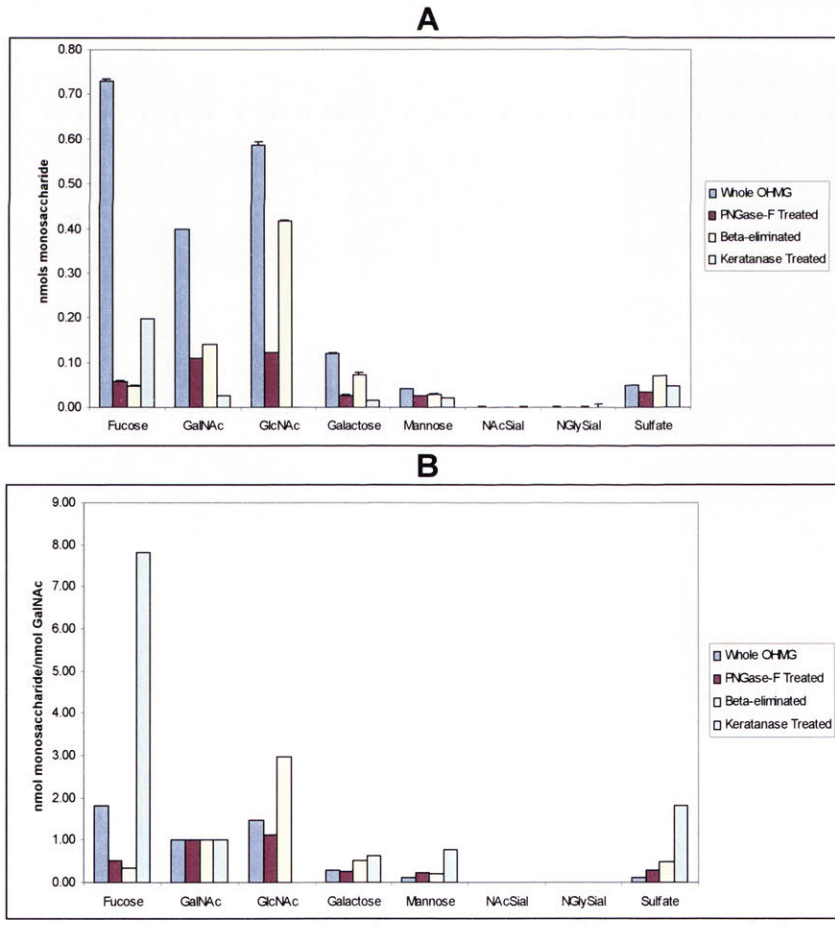

Figure 5.1: *Analysis of the monosaccharide composition of three types of oligosaccharides on bovine OHMG. Both total nmol recovered [**A**] and nmol normalised to GalNAc [**B**] are presented. 76.7% of the carbohydrate in the whole OHMG fraction can be accounted for by the three oligosaccharide removal techniques, suggesting incomplete removal of the oligosaccharides or losses in the analysis*

Most noteworthy is the composition of oligosaccharides released by treatment of OHMG with keratanase. This glycosidase is specific for the release of GaGs from keratan sulfate proteoglycans (Nakazawa *et al.* 1975). However, the monosaccharide composition of the released sugars is not consistent with that of keratan sulfate (keratan sulfate consists of polymers of sulfated N-acetyl glucosamine-galactose dimers). When bovine OHMG was treated with keratanase fucose, GalNAc, galactose, mannose and sulfate were released. This does not appear to be non-specific cleavage of mucin-like oligosaccharides, as no sugars were released by keratanase treatment of either BSM or PGM (data not shown). PNGase-F treatment of bovine OHMG released the sugars GlcNAc, mannose, galactose, fucose and some sulfate, which are common to N-linked oligosaccharides. Also present was a high content of N-acetyl galactosamine. This monosaccharide is usually only present as a small percentage of the monosaccharides in N-linked oligosaccharides. The monosaccharides typically associated with O-linked oligosaccharides in mucin sugars (GalNAc, GlcNAc, galactose, fucose and sulfate) were detected in the β-eliminated fraction of bovine OHMG. When O-linked oligosaccharides are β-eliminated from the protein backbone under these conditions, the reducing terminal GalNAc is converted to an alditol and does not elute with the rest of the GalNAc. As such, the reported levels of GalNAc for the β-eliminated fraction are lower than is present in the original sample. This can account for the missing proportion of GalNAc when the composition of all the fractions are totalled and compared to the whole high molecular weight fraction. There was an unusually high amount of fucose in the whole OHMG fraction, which is not totally recovered in the released oligosaccharides. As the carbon column was used to further purify each of the fractions, the contaminants which often occur early in the chromatographic separation, and often co-elute with fucose, may have been removed.

Alternatively, if the fucose is present in the whole OHMG fraction as a single residue linked to Ser or Thr, then it will not be retained by the carbon column. Mannose composition can be artefactual as it isomerises from glucose (a common contaminant due to the presence of cellulose dust). This could account for the presence of mannose in the β-eliminated fraction (mannose is typically only present in O-linked sugars in neural and muscle tissue, where it is associated with signalling pathways) (Endo 1999; Sasaki *et al.* 1998) and the keratanase fraction. Alternatively, the sugar xylose (which can be found as the O-linked monosaccharide in some glycosaminoglycan oligosaccharides), co-elutes with mannose under these conditions (Dionex technical notes, Dionex, USA). Contamination may, thus, account for the anomalies in the amounts of mannose and fucose. However, the relative amounts of GalNAc, GlcNAc and Gal for each of the fractions add up to the amounts of these sugars seen in the whole OHMG analysis. Sulfate recovered from each of the fractions is consistent with what would be expected for each of the oligosaccharide groups recovered. Each of the fractions contained roughly the same amount of sulfate per nmol amino acids as the whole OHMG fraction (whole OHMG contained 0.05 nmol compared to 0.03 for PNGase-F, 0.07 for β-eliminated and 0.05 for keratanase treated fractions). However, when these results are normalised to GalNAc, the ratios of the PNGase-F and β-eliminated fractions raise slightly (0.3 and 0.5 respectively, compared to 0.13 for the whole OHMG) whereas the keratanase fraction is almost 14 times higher (1.81:1 for the keratanase treated fraction). The total sulfate recovered from the fractions, however, only accounted for approximately 70% of the total sulfate loaded, suggesting that a highly sulfated species was not recovered.

5.3 MASS SPECTROMETRY OF OLIGOSACCHARIDES

5.3.1 Materials and Methods

Oligosaccharides were removed and recovered from OHMG as described above (i.e. PNGase-F, β-elimination and keratanase hydrolysis). Lyophilised sugars were redissolved into Milli-Q water at an equivalent concentration of 1 mg OHMG per 100 µl (i.e. 1 mg of protein was treated, desalted and lyophilised and the resultant oligosaccharides were then dissolved into 100 µl water).

Electrospray mass spectrometry was performed on a Micromass LCT-TOF system. The sample was loaded onto an HyperCarb 10 mm x 4 mm i.d. in-line graphitised carbon column (Alltech, Pennsylvania, USA) in 25 mM ammonium formate buffer at pH 9.0. The column was equilibrated for five minutes, then the oligosaccharides were eluted by raising the concentration of acetonitrile from 0% to 90% (in formate buffer) over 15 minutes. Negative ion mode was used to collect data in the 700-3000 m/z range. The possible monosaccharide compositions were predicted from the experimental masses using the GlycoMod informatic tool found at the ExPASy web site (http://expasy.proteome.org.au/tools/glycomod/).

MALDI-TOF mass spectrometry was performed using a Bruker Biflex III system. The sample was diluted 2:1 with 10 mg/ml matrix (2,5 dihydroxybenzoic acid) in 50% acetonitrile/water and spotted onto a target. The spot was air dried to crystal formation and analysed in negative ion mode for oligosaccharides.

5.3.2 Possible Glycan Structures Detected in Bovine OHMG

Mass spectrometry analysis of oligosaccharides released from bovine OHMG by β-elimination is presented in Figure 5.2, and the oligosaccharides released by PNGase-F treatment in Figure 5.3. Insufficient material was recovered in the keratanase-released fraction of OHMG to enable this type of analysis. The O-linked oligosaccharide compositions detected are indicative of those found in mucin-like oligosaccharides. The F-H_2-HN-Hnol species described most likely represents the Lewis-X structure (Zeng *et al.* 1999), with other masses representative of the core structures of mucins (see Chapter 1, Table 1.2). Interestingly none of the sugars detected contained sulfate. The largest oligosaccharide detected by this method contained six monosaccharide residues, which is quite a large O-linked structure. The detection of NeuGc in some of the structures observed indicates that, though this monosaccharide was observed in only trace amounts in the whole bovine OHMG fraction (see Section 4.3.1.2), it is still an important part of the O-linked oligosaccharide composition of ocular mucus.

M/z	Chg	F	H	HN	HNol	S	NeuAc	NeuGc	Delta
530.19	1	1	1		1				-0.018
733.27	1	1	1	1	1				-0.018
837.27	1	1	1		1			1	-0.028
895.26	1	1	2	1	1				-0.08
1040.23	1	1	1	1	1			1	-0.0148
1098.38	1	1	2	2	1				-0.04
1155.36	1		2	3	1				-0.081

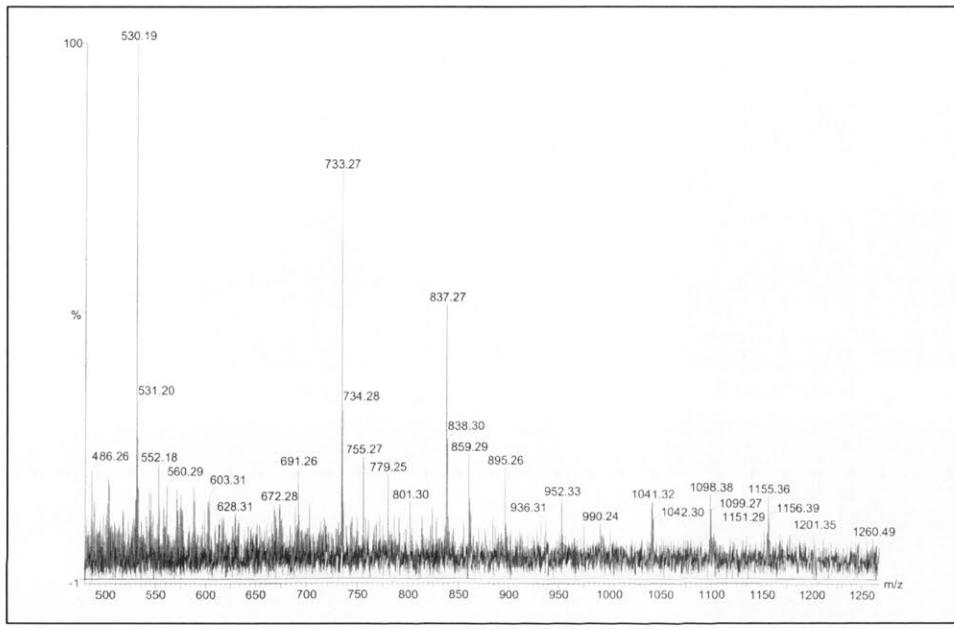

Figure 5.2: *Mass spectrometry analysis of β-eliminated oligosaccharides from OHMG. The trace depicts the mass spectra of the sample and the table lists the most likely monosaccharide composition of the masses observed (MS analysis performed by N. Karlsson, Proteome Systems Ltd):*

M/z = *lists the masses observed in negative charged ion mode*
Chg = *charge state*
F = *fucose residues*
H = *hexose (i.e. galactose glucose or mannose)*
HN = *amino hexose (i.e. GalNAc or GlcNAc)*
HNol = *reducing terminus (i.e. a GalNAc residue that has been reduced in the β-elimination process)*
S = *sulfate*
NeuAc = *N-Acetyl neuraminic acid*
NeuGc = *N-Glycolyl neuraminic acid.*
Delta = *error to proposed sugar structure from observed mass*

If glycosaminoglycan oligosaccharides are present as O-linked sugars, then β-elimination should remove them also. To test this theory the masses that were observed in the mass spectrometry analysis of the β-eliminated oligosaccharides were re-analysed using the GlycoMod infomatic database with expanded parameters (allowing the inclusion of xylose and hexuronic acids). Two of the masses returned reasonable potential structures:

- Mass 1040 returned the possibility of $(Hex)_1$ $(HexNAc)_1$ $(Deoxyhexose)_1$ $(Sulph)_2$ $(HexA)_2$ with a delta error of 0.059;

- Mass 1098 returned the possibility of $(HexNAc)_1$ $(NeuAc)_1$ $(Pent)_1$ $(Sulph)_2$ $(HexA)_2$ with a delta error of 0.162.

Both of these structures are possibly GaG structures (as determined by the CarbBank database) further supporting the possibility that proteoglycans are present in the mucus fraction of bovine conjunctival mucus.

M	Delta	Proposed structure
1914.697	0.108	$(Hex)_2$ $(HexNAc)_2$ $(Fuc)_2$ + $(Man)_3(GlcNAc)_2$
1930.692	0.073	$(Hex)_3$ $(HexNAc)_2$ $(Fuc)_1$ + $(Man)_3(GlcNAc)_2$
2092.745	-0.039	$(Hex)_4$ $(HexNAc)_2$ $(Fuc)_1$ + $(Man)_3(GlcNAc)_2$
2204.772	0.033	$(Hex)_2$ $(HexNAc)_2$ $(NeuAc)_2$ + $(Man)_3(GlcNAc)_2$
2220.767	0.018	$(Hex)_2$ $(HexNAc)_2$ $(NeuAc)_1$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
2236.762	0.183	$(Hex)_2$ $(HexNAc)_2$ $(NeuGc)_2$ + $(Man)_3(GlcNAc)_2$
2350.707	0.298	$(Hex)_3$ $(HexNAc)_4$ $(Sulf)_2$ + $(Man)_3(GlcNAc)_2$
2350.83	0.175	$(Hex)_2$ $(HexNAc)_2$ $(Fuc)_1$ $(NeuAc)_2$ + $(Man)_3(GlcNAc)_2$
2495.868	-0.202	$(Hex)_2$ $(HexNAc)_2$ $(NeuAc)_3$ + $(Man)_3(GlcNAc)_2$
2511.863	0.082	$(Hex)_2$ $(HexNAc)_2$ $(NeuAc)_2$ $(NeuGc)_1$ + $(Man)_3(GlcNAc)_2$
2576.764	0.361	$(Hex)_6$ $(HexNAc)_2$ $(Fuc)_1$ $(Sulf)_2$ + $(Man)_3(GlcNAc)_2$
2576.845	0.28	$(Hex)_2$ $(HexNAc)_2$ $(Fuc)_2$ $(NeuAc)_2$ $(Sulf)_1$ + $(Man)_3(GlcNAc)_2$

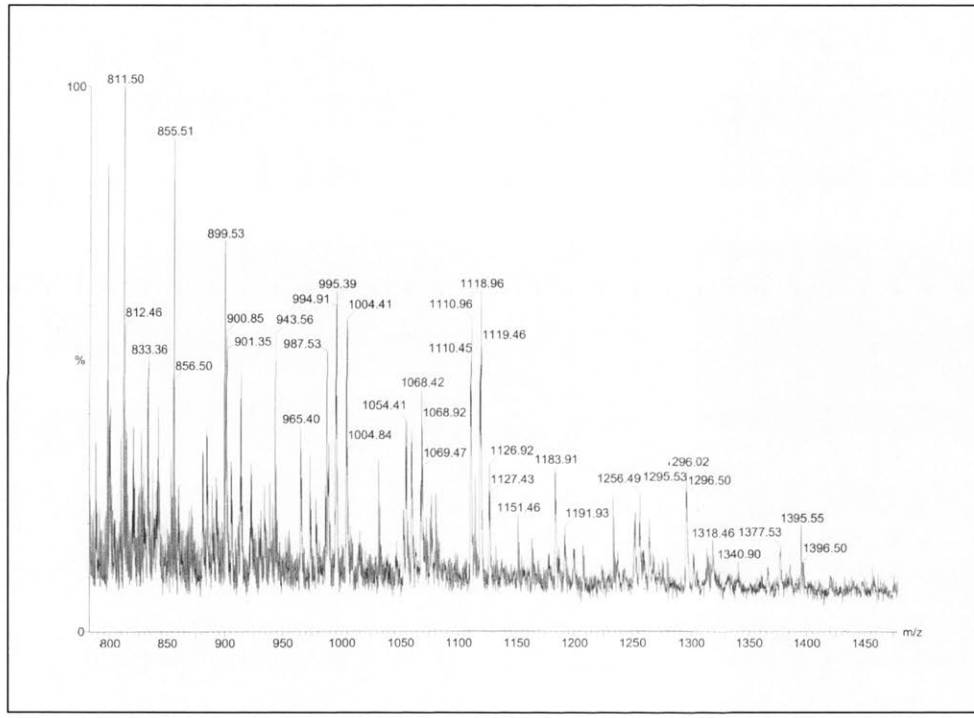

Figure 5.3: *Mass spectrometry analysis of N-linked oligosaccharides released from bovine OHMG by PNGase-F digestion. The spectra shows the masses detected and the most likely monosaccharide compositions of those peaks determined to be oligosaccharides, are listed in the corresponding table. Note that as N-linked oligosaccharides are doubly negatively charged in this mass spectrometry procedure, the masses listed have 1 added and then doubled from that of the peaks observed (MS analysis performed by N. Karlsson, Proteome Systems Ltd):*

M = *Mass of oligosaccharide calculated from the observed mass*
Delta = *error to proposed sugar structure from observed mass*
Hex = *Glucose mannose, galactose, etc.*
HexNAc = *GlcNAc or GlcNAc*

Possible N-linked oligosaccharide masses detected included a bi-antennary structure capped with N-acetyl neuraminic acid. Masses which could be accounted for by one or both of these terminal sugars replaced by N-glycolyl neuraminic acid were also detected. Masses of oligosaccharides with one or two fucose groups attached were also present. Several other peaks that had characteristics of oligosaccharides (i.e. they exhibited the appropriate peak pattern and the corresponding loss of water) were detected (e.g. masses 1990.827, 2135.868 and 2119.908). However, when these masses were computed by matching the masses in the GlycoMod infomatic database, no common monosaccharide combinations were found.

5.4 CONCLUSIONS

Structural analysis of bovine high molecular weight glycoconjugates has shown that there is indeed mucin-like species present. The O-linked component of the glycosylation of bovine OHMG is the major contributor to the oligosaccharides, being responsible for just over 40% of the total sugar present in the sample. The monosaccharide analysis of the O-linked sugars, coupled with mass spectrometry of the sugars themselves, is indicative of what would be expected of a typical mucin (indeed it matches the reports of O-linked oligosaccharide structures of other mucins such as MUC-1) (Baeckstrom *et al.* 1994). Mass spectrometry of the O-linked oligosaccharides indicated that there were several structures that are common to mucins, including Lewis-X and sialyl Lewis-X. However, it is also clear that other glycoconjugate species were present. The N-linked component of OHMG is significant, accounting for approximately 20% of all sugar (determined by

comparing the mass of sugar present in the whole sample with the PNGase-F fraction). Also the monosaccharide composition of this fraction contains GalNAc, not normally associated in such high amounts with N-linked oligosaccharides. Two possibilities are that there is a mucin-like protein with a significant N-linked component or that there is a completely separate protein that is predominantly N-linked. Mass spectrometry of the released N-linked sugars suggested the presence of standard oligosaccharide structures such as $GlcNAc_2$, Man_3 $(HexNAc, Hex, NeuNAc)_2$ and their variants. The presence of masses that did not correspond to familiar N-linked structures suggests the possibility of novel oligosaccharides. Further analysis by exoglycosidase digestion and MS-MS techniques would possibly identify these structures.

Interestingly, there are clearly proteoglycan-like sugars present in OHMG. Although keratanase is specific for sulfated glycosaminoglycan-like sugars (it digests polysulfated lactosamine into sulfated lactosamine units), fucose, GalNAc, Gal, Man, and sulfate were released from bovine OHMG by this enzyme. There are some reports that this enzyme can remove sugars from mucin molecules (Davies *et al.* 1991). However, when the enzyme was used to treat BSM, PGM and bovine fetuin, there was no evidence of sugars being released (data not shown). The presence of proteoglycan-like molecules is further supported by the amino acid and monosaccharide analysis of OHMG. Proteoglycans are typically high in asparagine, aspartic acid, glutamine and glutamic acid, as well as leucine and lysine, but have a relatively low Ser and Thr component, which is consistent with the composition of bovine OHMG. The fact that type monosaccharides are present in the keratanase released fraction indicates that there is either a proteoglycan in the mixture or that there is a "proteoglycan-like" mucin present. The proteoglycan present is not a

keratan sulfate, however, as the GalNAc is not indicative of polylactosamine. Unfortunately the method of monosaccharide composition analysis used is unable to effectively separate iduronic acids (all GaGs other than keratan sulfate consist of repeats of a HexNAc-Iduronic acid disaccharides). It is, therefore, not possible to determine which of the glycosaminoglycan sugar structures are present with this method.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTIONS OF OCULAR GLYCOCONJUGATE RESEARCH

The human tear film represents a unique environment in which the epithelium must survive. As is the case for all epithelial surfaces, the mucus layer must protect the cells in the epithelium from bacterial invasion and physical debris. However all of these properties must be achieved under the constraints of providing a clear pathway for light to reach the retina. It would stand to reason then that the protein composition of the mucus layer of the tear film might have a different composition to that of other epithelial layers. The eye is constantly exposed to a hostile environment without having the benefits of intricate filter systems (such as that provided for the respiratory and digestive tracts). In addition, the tear film is only microns thick (reported as being 7-50 μm [Anderton and Tragoulias 1998; Prydal *et al.* 1993]) as opposed to 200-4000 μm for the respiratory and digestive mucus layers (Turner *et. al.* 1999). In the past, it has been shown by Western blotting and mRNA hybridisation that many of the mucins found in other mucus layers are also present in the tear film and on the surface of the eye. It was reasonable from this data to then draw the conclusion that the mucus layer of the tear film had a comparable composition to that of the mucus layers found on other epithelial surfaces.

Indeed, the little compositional data that has been reported (primarily by Carrington *et al.* 1998) has shown that the chemical composition of the canine tear film is similar to the composition of the mucus layer of the respiratory tract. However the purification protocol used (CsCl gradient centrifugation followed by SEC) may be specifically enriching the mucin components of the tear film at the expense of any other. This possibility was explored more fully in Chapter 2. The reported values for other ocular mucins such as

rabbit (Tseng *et al.* 1987) have described amino acid and monosaccharide compositions similar to those reported in this thesis. In both cases purification by CsCl gradient was not used, rather the high molecular weight material was purified by SEC. As CsCl gradient centrifugation separates the mucin from other components on the basis of density rather than size, the procedure will separate the mucin glycoproteins from any other high molecular weight glycoconjugates such as proteoglycans. Detailed studies of the effects of purification methods on species enrichment of mucus high molecular weight glycoconjugate components have not yet been performed. Analysis performed in this project, by using a purification protocol that selects for total high molecular weight material (the fraction we have designated as bovine OHMG) has indicated a new ocular mucus composition.

The physical properties of bovine OHMG have similarities to both high molecular weight gel forming mucins, such as PGM, and non-gel forming mucins, such as BSM. OHMG forms a mucus gel layer in the eye, and has been shown to form gels *in vitro*, which is similar to the gel forming characteristics of PGM in porcine stomachs. However, OHMGs are easily digested by proteases, similar to BSM. Electrophoresis of OHMG demonstrated that it had three dominant species, which was similar to both PGM and BSM. In addition, several minor species were observed in OHMG, suggesting that there were more high molecular weight species present in OHMG than in either PGM or BSM. Separation by anion exchange chromatography supported this finding and indicated that a positively (or at least not highly negatively) charged species was present in OHMG (as determined by the fact that a species does not bind to an anion exchange column when applied in pH 7.0 buffer). Neither PGM nor BSM were found to have a component that

did not bind in this manner. Chemical compositional analysis of the high molecular weight glycoconjugates showed that there must be other high molecular weight species present than those that have been previously described. It also suggested some potential candidates for what these species might be.

Analysis of the total high molecular weight fraction indicated that the OHMG was high in both Asx and Glx, accounting for a third of the total amino acids present. Total Ser and Thr content was low - only half of what it would be if mucin was the only high molecular weight glycoconjugate present in the mucus. Additionally, the total content of Ser was higher than that of Thr. None of the mucin species that have so far been reported as present in ocular mucus (MUC-1, 2, 4, 5AC, 5B, 7), nor any combination of them, is able to produce an amino acid composition similar to that reported here as all of the mucins reported in the tear film are higher in total Thr than Ser. Similarly, the monosaccharide composition of OHMG was not consistent with that reported to date for mucins. Bovine OHMG had a significant amount of mannose, suggesting a more N-linked dominated oligosaccharide composition. The mucin species that have been described as present in the tear film have only minor N-linked components (Bhavanandan *et al.* 1998; O'Hara *et al.* 1994; Reddy *et al.* 1993; Maorett *et al.* 1989), as such mannose would not account for a significant amount of the total monosaccharide content. Furthermore, bovine OHMG contained an order of magnitude more sulfate than was found in either of the mucins analysed.

Comparison of the amino acid and monosaccharide composition of the high molecular weight glycoconjugate fraction of human tears indicated human OHMG's have a similar

composition to that of bovine conjunctival extract, i.e. they are high in Asx, Glx, Leu, Lys and Gly but low in Ser and Thr and the other amino acids most often associated with tandem repeat sequences in gel forming mucins. The values for human OHMG was consistent with the data reported for rabbits by Tseng (1987) and canines suffering from dry eye conditions (Hicks *et al.* 1998). This suggests that it was an ocular, rather than species, phenomenon.

Analyses of glycopeptides recovered from OHMG after digestion with Rt41A and purification by high salt SEC demonstrates that they are also high in Asx and Glx, suggesting that, if there is a repeat sequence in the molecules, it then contains these residues. The monosaccharide composition of the glycopeptide was also unusual, containing significant mannose and high sulfate. The possibility that the mannose detected was in fact xylose further supports the theory that the major component of bovine ocular mucus is a proteoglycan-like species. In any case, mannose is a significant component in N-linked proteoglycan oligosaccharides. The monosaccharide composition of the glycopeptide fraction recovered from the Rt41A digestion of whole bovine OHMG does not match that of a mucin. Therefore, if mucin molecules are present, then they must be in low quantities, not significantly impacting on the monosaccharide composition. Alternatively they were not recovered from the separation. The glycopeptides from PGM were significantly larger than those recovered from either OHMG or BSM (Figures 2.10, 2.11 and 2.12 respectively). If this was the case, the mucin component was not sufficient to cause a significant rise in absorption during the separation.

Analysis of the individual species recovered by high ionic strength ion exchange chromatography provided some potential candidates for the glycoconjugate that was responsible for the anomalous amino acid and monosaccharide compositions. Two species were found with similar amino acid compositions to a classical mucin containing high levels of Ser and Thr, as well as enrichment in the amino acids (such as valine and proline) that are associated with tandem repeat sequences in mucin glycoproteins. However, the Ser and Thr ratios in these molecules were the reverse of a gel forming mucin such as PGM. Monosaccharide compositions of these species were slightly different but also shared the same properties of a gel forming mucin, having similar ratios of GalNAc to the other monosaccharides. They were both low in total carbohydrate, however. Carbohydrate accounted for less than 20% of the molecular weight of the species.

Two other species, also with similar amino acid compositions but with different monosaccharide content, were re covered. They had compositions similar to a non-glycosylated or lightly glycosylated protein, i.e. they were not significantly enriched in the amino acids that are associated with oligosaccharide modification. These proteins had little enrichment in any of the amino acids. They must be glycosylated, however, as they were visualised after the ion exchange separation using a general glycosylation stain (the Bio-Rad Immuno-Blot kit). Analysis of the monosaccharide content of these species produced interesting results. The monosaccharide composition of Peak 3 was not typical for N or O-linked sugars. It may be a proteoglycan-like species. However, the methods used did not detect iduronic acids. Peak 4 had a more typical monosaccharide

composition of a glycoprotein, although the total carbohydrate content of this species was unusually high (being over 70% by weight carbohydrate).

All the species recovered from the ion exchange chromatography of the whole high molecular weight glycoconjugate fraction of bovine OHMG did not include a glycoconjugate species that could account for the high amounts of Asx and/or Glx found in the whole OHMG fraction. Furthermore, the majority of sulfate and carbohydrate of the whole fraction were also not recovered. As the starting material was tested before application to the IEC column, this was not due to any previous treatment of the fraction. This suggests that there were one or more species high in Asx, Glx, total carbohydrate and sulfate that were not eluted from the IEC column with the protocol used. Negative results are difficult to use as an indicator due to the possibility of unaccounted artefacts, but this result suggests one or more proteoglycan-like species are present in the tear film. This species would have to have a high negative charge to account for the inability of the buffer system to elute the molecule(s) from the column. In order to determine if this molecule does exist, a different system of separation must be used. The use of a weaker anion exchange column, such as DEAE Sepharose, may overcome this problem.

Analysis of the high molecular weight glycoconjugate fraction of human tears determined that human mucus contained a similar amino acid composition to that of bovine OHMG and agreed with what has been reported for other species in the literature (Tseng *et al.* 1987; Hicks *et al.* 1998). This suggests that the proteoglycan-like composition of bovine mucus is not artefactual. The oligosaccharides on human OHMG were much more highly

charged that those of bovine OHMG, containing a higher percent total sulfate and significant sialic acid content.

The trace amounts of sialic acid that were detected on bovine OHMG included both N-acetyl and N-glycolyl neuraminic acid. This was thought to be a species-specific phenomenon, as N-glycolyl neuraminic acid is not usually associated with healthy human tissues. Analysis of human OHMG, however, has also detected N-glycolyl neuraminic acid (previously thought to only occur in cancer in humans) (Devine *et al.* 1991). In the past it has been reported (Corfield *et al.* 1997) that ocular mucus contains oligosaccharides that are considered cancer markers (such as Tn attachments) in other tissues.

As recovering the individual species present in the OHMG fraction by ion exchange chromatography could not provide conclusive proof of proteoglycan-like species, specific deglycosylation protocols were used to recover the types of oligosaccharides present. The whole OHMG fraction was treated with PNGase-F to release N-linked oligosaccharides of the $GlcNAc_2,Man_3$ configuration and β-elimination was used to release any oligosaccharides O-linked through Ser or Thr. As the amino acid composition of the whole OHMG suggested the possibility of a proteoglycan, similar to one of the small leucine-rich proteoglycan family, the whole OHMG was also treated with keratanase to remove glycosaminoglycan type oligosaccharides.

β-elimination of the whole high molecular weight glycoconjugate fraction of bovine conjunctiva released oligosaccharides that are consistent with those which would be

released from mucins. The levels of GlcNAc are highest with some GalNAc, Gal and Fuc. When compared to the values recovered for PGM (Figure 6.1) it was clear that the components are similar. However there was some mannose detected in the O-linked released sugars. Mannose has not been reported as being present in O-linked sugars for mucins. As discussed previously, this could be accounted for by the presence of xylose in the sample. β-elimination of the oligosaccharides in the whole OHMG fraction would also remove any O-linked glycosaminoglycan oligosaccharides, thus, they could account for the mannose. The apparent presence of mannose may also be an artefact caused by the isomerisation of glucose. The β-eliminated fraction also contained significant amount of sulfate. One could speculate from this data that the high sulfate species may be glycosaminoglycans O-linked through Ser and Thr via xylose. Glycosaminoglycan oligosaccharides share many of the same monosaccharides as those of mucins. It is, therefore, possible that they are present in this fraction.

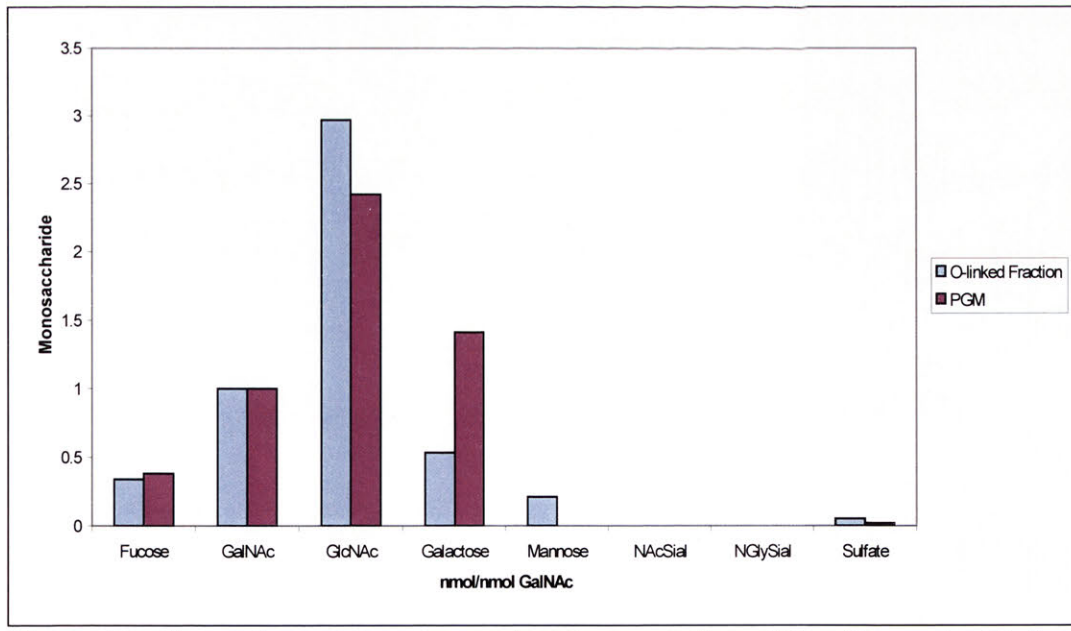

Figure 6.1: *Comparison of the monosaccharide composition of the oligosaccharide fraction of bovine OHMG released by β-elimination to the monosaccharide composition of PGM. Note that though OHMG has mannose (or possibly xylose) present in the O-linked fraction and galactose was somewhat lower, the general composition of this class of oligosaccharide was similar to that observed in a typical mucin (PGM)*

Mass spectrometry of the O-linked oligosaccharides released by β-elimination are similar to those found in the mucins that have been previously reported to occur in the eye, such as Sialyl Lewis-X (Karlsson *et al.* 1997A; Sandrin *et al.* 1997; DiIulio and Bhavanandan 1995). There were, however, several masses that did not have a mass similar to previously reported O-linked oligosaccharides (as determined by the GlycoMod tool). These masses could correspond to glycosaminoglycan structures.

The monosaccharide composition of the PNGase-F released fraction of the oligosaccharides present in the whole OHMG indicated the presence of GalNAc, in addition to the GlcNAc, Gal, Fuc and Man that would be expected in N-linked structures. It is, therefore, possible that these structures have been further modified with the addition of this monosaccharide. The addition of GalNAc to N-linked sugars usually results in it being only a minor component (e.g. the formation of the LacdiNAc dimer in the antennae of the sugars) (Nitz and Bundle 2000). In OHMG, however, GalNAc accounted for 30% of the total monosaccharides. This suggests either an N-linked component that is not of the standard "antennae" structure, or that the N-linked oligosaccharides of the classic type have been extensively modified. Some glycosaminoglycans are N-linked to asparagine (such as the keratan sulfates). However it is unlikely that PNGase-F would remove these in any large amounts as it is specific for $GlcNAc_2$ structures (Fan and Lee 1997). Some sulfate was detected in the PNGase-F released oligosaccharides, however, it was not significant as compared to the content of the other monosaccharides. The whole bovine OHMG fraction was almost 2% by mass sulfate. This suggests, therefore, that the N-linked oligosaccharides do not account for the sulfate.

Several species of oligosaccharides were identified from the PNGase-F digestion of bovine OHMG by mass spectrometry. A basic, non-sialylated, bi-antennary structure (the simplest of the complex N-linked structures), as well as masses accounted for by N-acetyl and N-glycolyl neuraminic acid substitutions, were detected. Also present were these species with fucosyl additions. It was not possible to determine if the desylylation was due to fragmentation in the mass spectrometer, however, the low levels of total neuraminic acid (determined by monosaccharide analysis) suggest that this was not be the case. Mass spectrometry analysis is unable to distinguish between hexoses (Glc, Gal and Man) and HexNAcs (GlcNAc and GalNAc). Therefore, it was also not possible to determine if these structures are substituted with GalNAc (as the monosaccharide composition would suggest). Also present were masses that had the characteristics corresponding to an oligosaccharide, such as the isotopic distribution pattern, but did not correspond to the mass of any typical oligosaccharide so far reported (as determined by the GlycoMod tool). As with the O-linked oligosaccharides that were recovered, this may correspond to species that are from glycosaminoglycans. The glycosaminoglycan oligosaccharides are not readily accounted for using this method, as their structures follow no defined pattern other than the inclusion of a polydisaccharide (Schwartz 2000). MS-MS analysis coupled with exoglycosidase digestion may result in the identification of some or all of these peaks.

Sugars were also released from the whole OHMG fraction by keratanase, a glycosidase specific for digestion of keratan sulfate glycosaminoglycans. This enzyme is specific for the digestion of polylactosamine (the backbone of keratan sulfate glycosaminoglycans) into lactosamine monomers (Nakazawa *et al.* 1975). As the lactosamine moiety is present

in mucins, both in O-linked and N-linked oligosaccharides, there are some references to keratanase removing sugars from mucins (Davies *et al.* 1991), however, when tested on PGM and BSM, there was no evidence of oligosaccharides being digested. The monosaccharide composition of the released oligosaccharides was not consistent with lactosamine. Rather GalNAc and Gal were recovered with some Fuc and Man (again the Man may in fact be xylose or an artefact caused by glucose contamination). Also present in the sample was a significant amount of sulfate. Sulfate comprised 16% of the total monosaccharide composition. This is consistent with what has been reported for glycosaminoglycans (Schwartz 2000; Iozzo 1998). The amino acid analysis suggests the possibility of a proteoglycan that is part of the small leucine rich proteoglycan family (Figure 4.12 compares bovine OHMG to β-luminican, a member of this family of proteoglycans). To date this family has been reported exclusively as keratan sulfate containing proteoglycans (Schwartz 2000; Iozzo 1998). This again indicates the presence of a proteoglycan-like species that has been as yet unreported.

Saso *et al.* (1999B) reported that glycosaminoglycans specifically interact with ocular mucins, suggesting that they can be used as therapeutic delivery systems. It is possible that he has unwittingly described the interactions that are occurring naturally in the tear film mucus. A possible scenario is that the reported mucins are present in small amounts, perhaps acting as the frame work for the mucus gel, and that other components are present in larger amounts (accounting for the chemical composition when the whole OHMG fraction was analysed). These other components may be either a secreted form of a known proteoglycan or an as yet uncharacterised proteoglycan-like glycoconjugate. Hassel *et al.* (1992) have indicated the importance of sulfation in keeping the corneal

stroma transparent. They have shown that when the proteoglycans in the stroma are desulfated, opaque areas occur. It is possible that the highly sulfated proteoglycan-like component is required to make ocular mucus transparent also.

To fully understand the tear film, several approaches can be followed in the future. Firstly, the DNA coding for these unknown proteins could be determined using some of the well documented methods of molecular biology (shot-gunning the genome, for example). Slam freezing (a process where tissue is placed against a polished copper mirror cooled by liquid nitrogen) has been successfully used in the past to recover an intact tear film on the surface of a cornea (Chen *et al.* 1997). This process, coupled with *in situ* hybridisation techniques and differential staining, could provide useful information into the structural aspects of the interactions between elements in the tear film. Further analysis of the species, separated by ion exchange chromatography from the high molecular weight fraction, could be performed to identify the oligosaccharide composition of each of the species present. Peptide mass fingerprinting of the amino acid backbone may provide some information as to the identity of these proteins. Many of the mucins that have been reported in the eye are partially or fully characterised (MUC-1, MUC-2, MUC-4, MUC-5AC and MUC-7) and all have reported sequences for at least their tandem repeat (SWISS-PROT database, http://expasy.proteome.org.au/sprot/).

It is important to try to identify the role these mucins play in the formation of a stable tear film. *In situ* hybridisation and mAb staining of frozen sections of the tear film and ocular epithelium may help to indicate the structural relationship of the known mucins. *In vitro* binding studies could also be used to identify chemical bonding of the mucins to each

other and to other tear film components. Coupled with further understanding of the as yet unidentified component(s), a three dimensional picture of the tear film may start to form. Attempting to recover the minor bands observed when the high molecular weight fraction is separated by PEG-SDS-PAGE may provide some useful information on the ratios of the species present. Just as important would be identifying whether or not proteoglycans play an important role in other mucus layers. It is possible that these glycoconjugates have been missed in the past because they have been separated from the mucin fraction in the purification process.

Proteoglycans have a different apparent density when separated by CsCl gradient centrifugation (proteoglycan species are recovered in the density range of 1.30-1.38 g/ml rather than 1.40-1.50 g/ml for mucin species) (Ledbetter *et al.* 1987; Bhaskar *et al.* 1986). It is conceivable that those groups that use the CsCl method of purification have overlooked proteoglycans as they were focussing on the mucin fraction. Finally, to properly understand the tear film it is necessary to study the interactions that the mucus layer has with the other components. The mechanism by which three seemingly incompatible components (hydrophobic lipids, hydrophilic aqueous proteins and gel forming mucus) can create such a stable layer is still not understood. In the past these components have been regarded as separate layers with little interaction (see Figure 1.2). Common sense dictates that this cannot be the case, as biology does not usually involve the rigid demarcations found in chemistry and indeed current thinking regards the tear film as more of a continuum, with the epithelium and glycocalyx at one end and the lipid/air interface at the other. The components that act as intermediaries in this continuum may shed some light on the gel layer itself.

When a biomaterial (such as a contact lens) is placed into a system that has a defined structure made up of diverse components from several sources (such as the tear film), it is going to be difficult to make sure that it does not disrupt that system. Indeed, there is no current contact lens on the market that has overcome this problem. In order to achieve this, science must do something never before accomplished. In the past the goal for biocompatible materials has been to convince the body to ignore the presence of the prosthetic device. This will not suffice for a contact lens, however, as its mere presence disrupts this highly ordered system. Rather we must convince the eye that the contact lens is in fact a part of the continuum that is the tear film. In order to achieve this, the specific components responsible for recognition must be identified and a suitable mimic synthesised so that it may be attached to the surface of a lens. Initial studies carried out by Carney (1997) have indicated that the mucus layer may be the place to start looking for these components. Continuation of the work carried out in this project may well lead to the discovery of those components.

This thesis has identified one or more components of the high molecular weight glycoconjugate fraction of the tear film that have not been so far reported. The compositional analysis of the whole fraction and the separation of the fraction into individual species has suggested that this is a major component of the conjunctival mucus. Previously proteoglycan-like compositions have been reported in the mucus layers of the gut (Gupta 1989), bladder (Buckley *et al.* 1996), eye (Carreras and Porcel 1995) and lung (Bhaskar *et al.* 1991). Glycosaminoglycan components (such as xylose) have been reported in the human tear film (Gipson *et al.* 1995). However, in all cases these were reported as minor components. As the focus of mucus glycoproteins has

always been on mucins, this tendency has translated to ocular research, the majority of which has been done using antibody staining and mRNA hybridisation, processes which indicate mucins are present but do not test for other components. It is suggested here that in the bovine conjunctiva, at least, mucins are not the major component of the mucus layer of the tear film, rather it is one or more proteoglycan molecules substituted with glycosaminoglycan oligosaccharides. As the bovine conjunctival mucus layer and human tears have a similar total amino acid and monosaccharide composition, this may also be true of human ocular mucus.

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APPENDIX A: TERMINOLOGY AND ABBREVIATIONS

A1: AMINO ACIDS

Amino Acid	3 Letter Code	1 Letter Code	Molecular Weight	Molecular Weight (attached)
Alanine	Ala	A	89.1	71.1
Arginine	Arg	R	174.2	156.2
Asparagine	Asn	N	132.1	114.1
Aspartic Acid	Asp	D	133.1	115.1
Cysteine	Cys	C	121.2	103.2
Glutamic Acid	Glu	E	147.1	129.1
Glutamine	Gln	Q	146.0	128.0
Glycine	Gly	G	75.1	57.1
Histidine	His	H	155.2	137.2
Isoleucine	Ile	I	131.2	113.2
Leucine	Leu	L	131.2	113.2
Lysine	Lys	K	146.2	128.2
Methionine	Met	M	149.2	131.2
Phenylalanine	Phe	F	165.2	147.2
Proline	Pro	P	115.1	97.1
Serine	Ser	S	105.1	87.1
Threonine	Thr	T	119.1	101.1
Tryptophan	Trp	W	204.2	186.2
Tyrosine	Tyr	Y	181.2	163.2
Valine	Val	V	117.1	99.1

A2: MONOSACCHARIDES

Monosaccharide	Abbreviation	Average Molecular Weight
Fucose	Fuc	146.1
2-deoxyglucose	DeoxGlc	146.1
N-acetyl galactosamine	GalNAc	203.2
N-acetyl glucosamine	GlcNAc	203.2
Galactose	Gal	162.1
Glucose	Glc	162.1
Mannose	Man	162.1
N-acetyl neuraminic acid	NeuAc	250.2
N-glycolyl neuraminic acid	NeuGc	307.3
Xylose	Xyl	132.1

A3: COMMON PROTEIN AND OLIGOSACCHARIDE ABBREVIATIONS

A3.1: Glycoconjugates

BCM	=	Bovine conjunctival mucin
BSM	=	Bovine sub-maxillary mucin
BOHMG	=	Bovine ocular high molecular weight glycoconjugates
Gly A	=	Large tandem repeat glycosylated region of MUC 2
Gly B	=	Smaller glycosylated region of MUC 2 that does not contain a tandem repeat
HOHMG	=	Human ocular high molecular weight glycoconjugates
MUC	=	The genes and their products that code for the mucin glycoprotein molecules in the human body. To date 13 are known
OHMG	=	Ocular high molecular weight glycoconjugates
OSM	=	Ovine Sub-maxillary mucin
PGM	=	Porcine Gastric Mucin
PSM	=	Porcine sub-maxillary mucin

A3.2: Oligosaccharides

- ***GaG:***
  Glycosaminoglycan, an oligosaccharide attached to either asparagine, serine or threonine, containing one of the poly glycosamino glycan dimers (poly-lactosamine, or poly HexNAc-iduronic acid).

- ***N-linked:***
  Oligosaccharide attached to asparagine through the amino group of the amino acid and the reducing terminus of N-acetyl glucosamine. Always contains the core structure $GlcNAc_2$, Man_3. May be high mannose, hybrid or complex types depending on the organism that produces them, and protein to which they are attached.

- ***O-linked:***
  Oligosaccharide attached to serine or threonine through the hydroxyl group of the amino acid and the reducing terminus of the oligosaccharide. May contains one of many core structures, usually linked to a N-acetyl galactosamine residue in mucins.

A4: REAGENTS / BIOLOGICAL

DIG	=	Digoxigenin
DTT	=	Dithiothreitol
ECM	=	Extra cellular matrix
EDTA	=	Ethylamine diamine tetra acetic acid
EtOH	=	Ethanol
F-moc	=	9-fluorenylmethyl chloroformate
MeCN	=	Acetonitrile
MeOH	=	Methanol
PBS	=	Phosphate buffered saline
PEG	=	Polyethylene glycol
PMSF	=	Phenylmethylsulfonyl fluoride
PVDF	=	Polyvinyliden difluoride
SDS	=	Sodium dodecyl sulfate
TEA	=	Triethethylamine
TFA	=	Trifluoro acetic acid

A5: PROCEDURES

IEC	=	Ion exchange chromatography
HIAEC	=	High ionic strength anion exchange chromatography High pH anion exchange chromatography
HPLC	=	High pressure (performance) liquid chromatography
MALDI-TOF	=	Matrix assisted LASER desorption ionisation time of flight mass spectrometry
PM-SDS-PAGE	=	Polyethylene glycol modified sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	=	Size exclusion chromatography

APPENDIX B: GENERAL METHODOLOGY

B1: SAMPLE PREPARATION

B1.1: Mucin Sources

B1.1.1 Bovine Ocular High Molecular Weight Glycoconjugates

Bovine eyes were obtained fresh from the abattoir and the conjunctiva excised. Excess muscular and adipose tissue was removed before the conjunctiva were incubated in one of two extraction buffers (two conjunctiva per 15 ml containing either 4 M urea, 1 mM DTT, 0.1% SDS, or 6 M guanidinium HCl 1 mM DTT, both containing 5 mM EDTA, 5 mM N-ethylmaleimide, 100 mM aminohexanoic acid, 5 mM benzamidine, 1 mM PMSF) at 4°C for 16 hours. The tissue was removed and the extract centrifuged (10000 g, 30 minutes) to remove precipitated debris. The supernatant was stored at 4°C until used in purification methods. After analysing both extraction techniques only the urea extraction was continued as it was determined to solubilise more of the mucin fraction.

B1.1.2 Other Mucins / Glycopeptides

Bovine sub-maxillary mucin was purchased from Sigma Aldrich (MO, USA), and purified by size exclusion chromatography (see Section 2.3.2.2) to remove contaminants/fragments.

Porcine gastric mucin was purchased from Sigma Aldrich (MO, USA) and purified by size exclusion chromatography (see Section 2.3.2.2) to remove contaminants/fragments.

Glycopeptide A (Gly A) was produced by the digestion of murine MUC-2 analog and consists of the large tandem repeat sequence. Gly A was prepared as per Carlstedt *et al.* (1993) and was provided by Ingmar Carlstedt. Glycopeptide B (Gly B) corresponds to the small tandem repeat region of murine MUC-2 and was prepared in a similar manner (also provided by Ingmar Carlstedt).

B1.2: Reduction and Alkylation Techniques

B1.2.1 AEDANS / Iodacetamide

Mucin was dissolved in 0.6 M guanidinium hydrochloride and dithiothreitol (DTT) added to a concentration of 20 mM and the tube sealed under O_2 free N_2. The solution was incubated at room temperature in the dark for 16 hours. Excess reducing agent (either 5-iodo-acetamido-fluorescene or AEDANS was used as the alkylation agent) was added to the tube. The tubes were resealed under O_2 free N_2 and incubated in the dark for a further four hours. The sample was dialysed against water until no fluorescence was detectable in the dialysate (approximately 48 hours with six changes of water).

B1.2.2 Coumarin Maleimide

Lyophilised mucin (2 mg) was dissolved into non-buffered Tris (pH 10.4) at a concentration of 50 mM containing 6 M urea. The solution had DTT added to a concentration of 20 mM before O_2 free N_2 was bubbled through it and the tube sealed. The sample was then incubated at room temperature for at least 16 hours in the dark. 1 mg of coumarin maleimide (7-diethylamino-3-[4'-maleimidylphenyl]-4-methylcoumarin [CPM]) was then added and the solution vortexed until dissolved. The oxygen was again removed from the solution with O_2 free N_2 and allowed to incubate for at least six hours at room temperature in the dark. The solution was centrifuged (10,000 g 30 minutes) to remove any particulates and desalted using a Pharmacia Fast desalting column (Method 2.2.4).

B1.2.3 Acrylamide

Adapted from Yan *et al.* 1998.

To 1 vol of protein solution (1 mg/ml) add 1 vol 1 M N-ethylmorphiline acetate (ml), 1 wt guanidinium hydrochloride (g) and 4 wt DTT (mg). Seal tube under O_2 free N_2. Incubate at room temperature for four hours in the dark. Add acrylamide to a 2 M excess of DTT present in the tube. Reseal the tube under O_2 free N_2 and incubate at room temperature for 16 hours in the dark. Dialyse against water or desalt as per Section 2.2.4.

B1.3: Concentration Techniques

On occasion the mucin extract, or partially purified mucin, was concentrated using Amicon Centriprep 100 concentrators. The solution was centrifuged at 500 g for several hours until the volume was reduced to a useable level (typically the partially purified mucin was concentrated 2.5-10 x).

Purified mucins were lyophilised using either a Savant Speed-Vac (New York, USA) for small volumes or were freeze dried for preparative volumes. The samples were dried to powder in all cases.

B2: CHROMATOGRAPHIC SEPARATION

B2.1: High Ionic Strength Anion Exchange Chromatography

Taken from Thornton *et al.* 1994.

Anion exchange chromatography was performed by a Bio-Rad (California, USA) BioLogic preparative LC system using a Pharmacia (Uppsala, Sweden) Mono Q (5 mm x 50 mm) column.

Buffer A = 10 mM piperazine buffered to either pH 5.0 or 7.0 with HCl, containing 6 M urea and 0.02% (w/v) CHAPS.

Buffer B = 10 mM piperazine buffered to either pH 5.0 or 7.0 with HCl, containing 6M urea, 0.02% (w/v) CHAPS and 0.5M lithium perchlorate.

Program: Flow rate was 0.5 ml / min µl / min

Time (min)	Concentration B (%)
0	0
5	0
50	80
55	80
60	0
65	0

B2.2: Size Exclusion (SEC)

Analytical scale SEC was carried out on a Pharmacia (Uppsala, Sweden) SMART system using a Superose 12 (4 x 300 mm) column at a flow rate of 40 µl/min of 150 mM sodium phosphate buffer pH 7.1 containing 1 M NaCl and 1 mM DTT (PHS buffer).

Preparative scale SEC was performed by either a Pharmacia (Uppsala, Sweden) FPLC system or with a Bio-Rad Biologic intermediate pressure LC system. Columns used on these systems were the same being either a self-poured column of Pharmacia Superose 12 media (I.D. 15 mm, length 600 mM) (flow rate 0.8 ml/min run time 180 minutes), a Pharmacia HR 10/30 pre-poured Superose 12 (flow rate 0.5 ml/min run time 90 minutes) or a Pharmacia HR 10/30 pre-poured Superose 6 column (flow rate 0.5 ml/min run time 90 minutes).

B2.3: Desalting

Analytical scale desalting was performed using a Pharmacia (Uppsala, Sweden) SMART system and a Fast Desalting column. Desalting was into water at a flow rate of 100 µl/min. Typically 100 µl sample was desalted into 300 µl water.

Preparative scale desalting was performed by either a Pharmacia (Uppsala, Sweden) FPLC system or with a Bio-Rad Biologic intermediate pressure LC system using a pre-poured HR 10/10 Fast Desalting column at a flow rate of 1 ml/min into water. Typically 1 ml of sample was desalted into 2 ml water.

B3: ELECTROPHORETIC TECHNIQUES

B3.1: Pre-cast SDS-PAGE

All pre-cast SDS-PAGE was performed using either a Novex (Invitrogen, California, USA) gel apparatus with Novex 4-20% precast gels, or a Bio-Rad Mini Protean II system with Bio-Rad precast 4-20% mini-gels. In both cases a Bio-Rad (California, USA) PowerPac 1000 power supply was used to run the gels at 100 V for 10 minutes (to run sample into the gel) followed by 150 V constant voltage until the buffer front remained just on the gel (approximately one hour). Gradipore (New South Wales, Australia) pre-made sample buffer was used with either 5 mM DTT or 5% 2-mercaptoethanol.

B3.2: Self-Poured Gels

Self-made gels were poured to the following recipe:

To make 10 ml gel solution (enough for 2 mini gels) 2.6 ml 1.5 M Tris-HCl pH 8.8 containing 0.1% SDS, 2.5 ml Gradipore (New South Wales, Australia) Liqui-gel 29.1 mix (a pre-mixed stock solution containing 40% acrylamide with 3.3% bis-acrylamide), and 4.85 ml H_2O were added to a 50 ml Erleymeyer flask and mixed under O_2 free N_2. 50 µl 10% ammonium persulfate and 10 µl TEMED were added and the solution mixed well. The solution was then poured into Bio-Rad mini gel plates, filling the plates to about 2/3 full. The mixture was allowed to set at room temperature for one hour. After the separating gel had polymerised a stacking gel was added. 5 ml of stacking gel solution was made by mixing 1.3 ml 0.5 M Tris-HCl, pH 6.8 containing 0.4% SDS with 1.3 ml Gradipore (New South Wales, Australia) Liqui-gel and 3.2 ml H_2O. 100 µl of a saturated solution of bromo-phenol blue was also added to the solution to aid in sample addition (by making the wells visible). The solution was mixed, 35 µl 10% ammonium persulfate and 7.5 µl TEMED were added, and the solution was mixed again. The stacking gel was poured on top of the separation gel and 10 well combs added. The gel was allowed to set at room temperature for one hour.

Gels were run in a Bio-Rad Mini Protean II system using a Bio-Rad Power-Pak 3000 set at 100 V constant voltage until the sample reached the separation gel (approximately 15 minutes) and then at 150 V constant voltage until the buffer front was at the end of the gel (approximately one hour).

B3.3: Blotting Procedures

Polyacrylamide/agarose composite gels or agarose slab gels were blotted using capillary action. A raised platform was placed into a dish and a strip of filter paper placed across it so the ends were in the dish. 4 x SSC (0.6 M NaCl in 0.06 M Na Citrate buffer, pH 7.0) was used as the transfer buffer. The gel was soaked in 4 x SSC for 10 minutes then placed onto the blotting paper on the stand. One or two nitrocellulose membranes were wet with 4 x SSC and placed on top of the gel ensuring that all air bubbles are removed. Several layers of blotting paper cut to the size of the gel were then placed on top of the membranes and finally several cm thickness of paper towels were added. A weight was placed on the paper towel and supported to avoid toppling when the paper was damp and 4 x SSC was placed into the tray. The gel was allowed to blot overnight, before fixing the membrane in 1% KOH.

Alternatively, the mini gels (both Novex and Bio-Rad) were blotted using a Gradipore (New South Wales, Australia) semi-dry blotting device. The gel and membrane (either nitrocellulose or PVDF that had been pre-wet with methanol) were washed for 10 minutes in 25 mM Tris HCl pH 10.1 containing 20% methanol. Onto the positive electrode were placed six pieces of 3 MM blotting paper cut to the size of the gel and soaked in 300 mM Tris HCl pH 10.7 containing 20% methanol. A further three pieces of blotting paper, soaked in the same buffer as the gel, were added before the membrane and then gel were placed on the stack. Finally nine pieces of blotting paper soaked in 25 mM Tris HCl, 40 mM ε-Amino-n-caproic acid, pH 9.38, containing 20% methonol were placed onto the gel and the negative electrode onto the apparatus. The gels were blotted at 12 V for one hour, the membrane removed and fixed in 1 % KOH for five minutes.

B4: DETECTION TECHNIQUES

B4.1: Silver Stain

The silver stain technique employed for the detection of proteins separated by SDS-PAGE was taken essentially from Rabilloud *et al.* (1994):

- Solution 1 = 40 % (v/v) ethanol 10% (v/v) acetic acid.
- Solution 2 = 30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.52% (v/v) glutaraldehyde, 0.2% (w/v) sodium thiosulfate.
- Solution 3 = 0.2% (w/v) silver nitrate.
- Solution 4 = 3.7% (w/v) sodium carbonate containing 0.05% (v/v) 37% formaldehyde.
- Solution 5 = 1.5% (v/v) acetic acid.

Gels were fixed in Solution 1 for one hour at room temperature. The gels were then incubated in Solution 2 for 16 hours. The gel was washed three times of 15 minutes each in Milli-Q water. Solution 3 was added for a minimum of 30 minutes, then the gel was washed in Milli-Q water for five minutes. Gels were rinsed in Solution 4 twice then incubated until bands developed. Colour development was arrested with Solution 5. Finally the gel was washed in Milli-Q water for at least 10 minutes before storage.

B4.2: Glycoprotein Detection

B4.2.1 Glycoprotein Detection Kits

Glycoproteins were stained using the DIG Glycan detection kit developed and marketed by Roche Diagnostics (Mannheim, Germany), or the Immuno-Blot Glycan Detection kit marketed by Bio-Rad (California, USA)

B4.2.2 Periodic Acid Schiff's Reagent

This thesis used the periodic acid - Schiffs reagent staining method developed by Thornton *et al.* (2000):

- Solution A = 1.0% (v/v) periodic acid (50% solution) purchased from BDH in 3% acetic acid.
- Solution B = 0.1% (w/v) sodium metabisulphite in 10 mM HCl.
- Schiffs reagent = A commercial reagent from Sigma Aldrich (MO, USA) was used.
- Solution C = 50% (v/v) ethanol.
- Solution D = 0.5% (w/v) sodium metabisulphite in 10 mM HCl.
- Solution E = 7.5% (v/v) acetic acid 5% (v/v) methanol in distilled water.

Solution A, B and D should be made up fresh, but if a large number of stains are being done in a relatively short time, then make up the acid solutions and add the metabisulphite and periodic acid as needed.

1. Soak the gel in Solution C for 30 minutes.
2. Wash in distilled water for 10 minutes.
3. Incubate in Solution A for 30 minutes.
4. Wash in distilled water for at least 6 x 5 minutes.
5. Wash in Solution B for 2 x 10 minutes.

6. Incubate in Schiffs reagent for one hour in the dark.
7. Incubate in Solution B for one hour in the dark.
8. Wash several times in Solution D for a total of at least two hours.
9. Store the gel in Solution E.

B5: AMINO ACID ANALYSIS

Taken essentially from Yan *et al.* 1998

Two separate analyses were performed. The first was a strong acid hydrolysis of the mucin. Glycoprotein samples prepared as described above were lyophilised into pre-fired glass hydrolysis tubes (approximately 100 picomoles of sample). The tubes were places into a hydrolysis vessel and 250 µl of constant boiling (6 M) HCl added. A crystal of phenol was placed onto the wall of the vessel and a vacuum applied. The vessel was flooded with O_2 free N_2. This process was repeated twice more and a final vacuum applied to the chamber. The samples were hydrolysed at 110° C for 22 hours and any HCl removed by drying. 30 µl of 2:2:1 ethanol:triethylamine:water was added to each tube and the samples re-dried. Each sample was dissolved into 10 µl of 250 mM Borate buffer containing 64 pmol of hydroxy proline as an internal standard and treated by automated F-moc analysis. The derivatised amino acids were then separated on a GBC Aminomate system using a Keystone Scientific 150 x 4.6 ODS Hypersil reversed phase column. This analysis gave a precise determination of the majority of the amino acids. However strong acid hydrolysis removes the amine groups of some amino acids, converting Gln to Glu and Asn to Asp, they are, therefore, combined in the results as Glx and Asx. Also cysteine and tryptophan are labile in strong acid conditions and these amino acids are, therefore, destroyed by the hydrolysis process. Cysteine residues may be protected from hydrolysis if the thiol group is protected by an alkylating group. A second analysis was performed on mucins reduced with DTT and alkylated by iodoacetamide (as described in Section 2.1.2.3). This was done to determine the percentage composition of Cys in each of the mucins.

β-elimination

To determine the extent of O-linked glycosylation present in a sample β-elimination was used. Amino acid analysis was performed before and after β-elimination. Protein samples were treated with 0.1 M NaOH for 16 hours at 45°C. This treatment removed the oligosaccharide from the amino acid backbone, in the process converting serine to dehydroalanine, and threonine to 2-amino butanoic acid. These converted residues do not have the same retention times as the original amino acids and, as such, a drop in peak area will be observed. By taking the protein and performing amino acid analysis (as described above), it was possible to determine how many Ser and Thr residues were still present in the protein (i.e. how many were not glycosylated). This can then be compared to the sample that has not been β-eliminated to calculate the number of sites that had oligosaccharide attachment.

All analysis was performed at least three times.

The program used for analysis of the amino acids was:

- Solution A = 30 mM ammonium phosphate buffer pH 6.5 in 85% MeOH in Milli-Q water.
- Solution B = 85% MeOH in Milli-Q water.
- Solution C = 90% MeCN in Milli-Q water.

Time	Concentration Solution A (%)	Concentration Solution B (%)	Concentration Solution C (%)
0.00	18.0	66.0	16.0
1.00	18.0	66.0	16.0
31.00	11.0	43.0	46.0
31.05	0.0	0.0	100.0
34.00	0.0	0.0	100.0
34.05	20.0	64.0	16.0
35.05	18.0	66.0	16.0
45.00	15.0	68.0	17.0
45.10	15.0	68.0	17.0
55.00	0.0	80.0	20.0
56.00	0.0	100.0	0.0

B6: MONOSACCHARIDE ANALYSIS

Taken from Packer *et al.* 1998B.

Glycoprotein samples were treated three ways to liberate monosaccharides.

1. Hydrolysed with 0.1 M TFA at 80° C for 40 minutes to release N-Acetyl and N-Glycolyl neuraminic acid.

2. 2 M TFA heated to 100°C for four hours to release neutral sugars (galactose, glucose, fucose and mannose).

3. 4 M HCl heated to 100°C for four hours to release amino sugars (N-acetyl-galactosamine, N-acetyl-glucosamine).

All of the hydrolysates were lyophilised using a Savant Speed Vac system (New York, USA), redissolved in 100 µl water containing 4 nmols of internal standard (for neutral and amino sugars the internal standard was 2-deoxy glucose while the internal standard for sialic acid analysis was lactobionic acid). The samples were then centrifuged at 10,000 g for 30 minutes to remove protein debris. The supernatant was transferred to a fresh tube and analysed by high pH anion exchange chromatography using a Dionex LC 20 system with a GP40 pump and an ED40 pulsed amperometric detector. Analysis of neutral and amino sugars was performed using a Dionex CarboPac PA-10 column with a constant hydroxide concentration of 15 mM over 20 minutes. A CarboPac PA1 column was used to separate sialic acid monosaccharides using the following gradient.

- Solution A = H_2O
- Solution B = 0.4 M NaOH
- Solution C = 0.1 M NaOH
- Solution D = 0.1 M NaOH, 1 M NaAc

Time (min)	Concentration A (%)	Concentration B (%)	Concentration C (%)	Concentration D (%)
0	0	50	50	0
2	0	50	50	0
30	0	50	10	40
31	0	100	0	0
40	0	100	0	0
41	0	50	50	0
45	0	50	50	0

B7: SULFATE ANALYSIS

Sulfate/phosphate analysis was performed essentially by the method described by Harrison and Packer (2000).

Samples taken for sulfate/phosphate analysis were hydrolysed in 4 M HCl at 100°C for four hours. The HCl was removed by drying the samples in a Savant Speed Vac (New York, USA) system. Samples were then redissolved into 100 µl H_2O. 25 µl of sample was injected onto a Dionex LC 20 system with a GP40 pump and a ED40 conductivity detector. Separation was performed by a Dionex IonPac AS11 Anion exchange column using the following gradient:

- Solution A = H_2O
- Solution B = 0.4 M NaOH
- Solution C = 0.1 M NaOH
- Solution D= 0.1 M NaOH, 1 M NaAc

Time (min)	Concentration A (%)	Concentration B (%)	Concentration C (%)	Concentration D (%)
0	95	0	5	0
2	95	0	5	0
12	70	0	30	0
15	70	0	30	0
20	95	0	5	0

The hydroxide ions were neutralised using a Dionex Anion Micromembrane suppressor (AMMS-1)(50 mM H_2SO_4 as the neutraliser) and the SO_4 /PO_4 ions detected using a conductivity detector.