Comparison and Evaluation of Various Techniques for Analysing Tear Proteins and Contact Lens Extracts

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Summary

With the increase in availability and use of contact lenses over the past few years, the number of cases of contact lens associated disease or discomfort has also risen. One such cause of these problems is thought to be an interference or alteration of the natural pattern of the tears in people who wear contact lenses. Contact lenses not only disrupt the film by their bulky physical presence; they may also absorb certain tear film constituents and can leak chemicals into the tears.

Complications in pinpointing changes in the tears occur due to the nature of the tear film. With a trilaminar appearance, the Precorneal Tear Film consists of an external lipid layer, a middle aqueous layer, and the third mucosal layer, which is in contact with the cornea itself. The problems observed with lens wear could result from an alteration in just one of these layers or from a complex combination of changes across the layers.

Due to the complications outlined above, various groups are working to study the separate components of the tears, using varied techniques. Within this study, the focus of work will be with regards to the protein component of the tears. Techniques chosen here are High Performance Liquid Chromatography (H.P.L.C.), one- and two-dimensional PolyAcrylamide Gel Electrophoresis (P.A.G.E.) and Western Blotting.

The hope is that, either in isolation or combination, these methods of analysis will provide some new insight into the pattern of tear proteins in contact lens wear. In addition the protein deposition with different lens types and wear regimes will be explored as will the 'normal' tear pattern. This will be achieved via the study of the tears of asymptomatic contact lens wearers and non-wearers as well as extracts derived from worn lenses.

Future work based on the comparison of normal tear patterns to those of contact lens associated disorder sufferers may yield important benefits in time with regard to diagnosis.

Keywords: Tear proteins, Western blotting, immunostaining, lens extracts, kininogen

Dedicated to my parents and my older brother Simon.

Special thanks also to all those people who I am lucky enough to be able to call 'friend'.

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Chapter 1

Introduction

1.1 Introduction

The proteins of the human tear film have been widely investigated by numerous research groups, each of which has had its own particular favoured technique or combination of techniques for the analysis and identification of these proteins. A detailed description of the research carried out by these various groups can be found in Section 1.7 'A Review of Previous Work' and so will not be discussed here. Whilst a lot of work has been done in the past using electrophoresis in general (and sodium dodecyl sulphate polyacrylamide gel electrophoresis in particular), the equipment used, the running conditions, type of gel, staining system, sample treatment method and many other variables change from group to group. Optimising these variables in order to obtain the best possible picture of the tear protein pattern would be of great benefit in analysing tear proteins and was the driving force behind the research presented here.

Of particular interest in regard to tear proteins is the amount and composition of those proteins that bind to and are taken up by a contact lens as part of the process of lens spoilation. This phenomenon is important not just due to the resulting possible impairment of the lens (both in terms of visibility and tolerance) but also because of the effect such a loss/change may have on the proper functioning of the tear film and in triggering an immune response in the ocular tissue. The tear film has many important functions which can be affected, and these will be explained later.

By analysing the tears of non contact lens wearers, asymptomatic contact lens wearers and disease sufferers, along with contact lens extracts from the worn lenses of people using different lens materials and modalities, we should be able to assess the effects of lens wear. In turn, this should allow us to start to link changes in tear protein composition with various disease states and other complications associated with lens wear.

In order to understand how important these proteins are, we must first understand their role in the tear film together with exactly what impact a change/modification of protein quantity or conformation in the tear film can have.

1.2 Structure of the Tear Film

The classical model for the tear film is that of a trilaminar (three layered) structure. These 'layers' are the mucin layer which lies on the epithelial surface of the cornea, the middle aqueous layer which contains a variety of components such as proteins and electrolytes and lastly the thin outer surface coating of lipids[1].

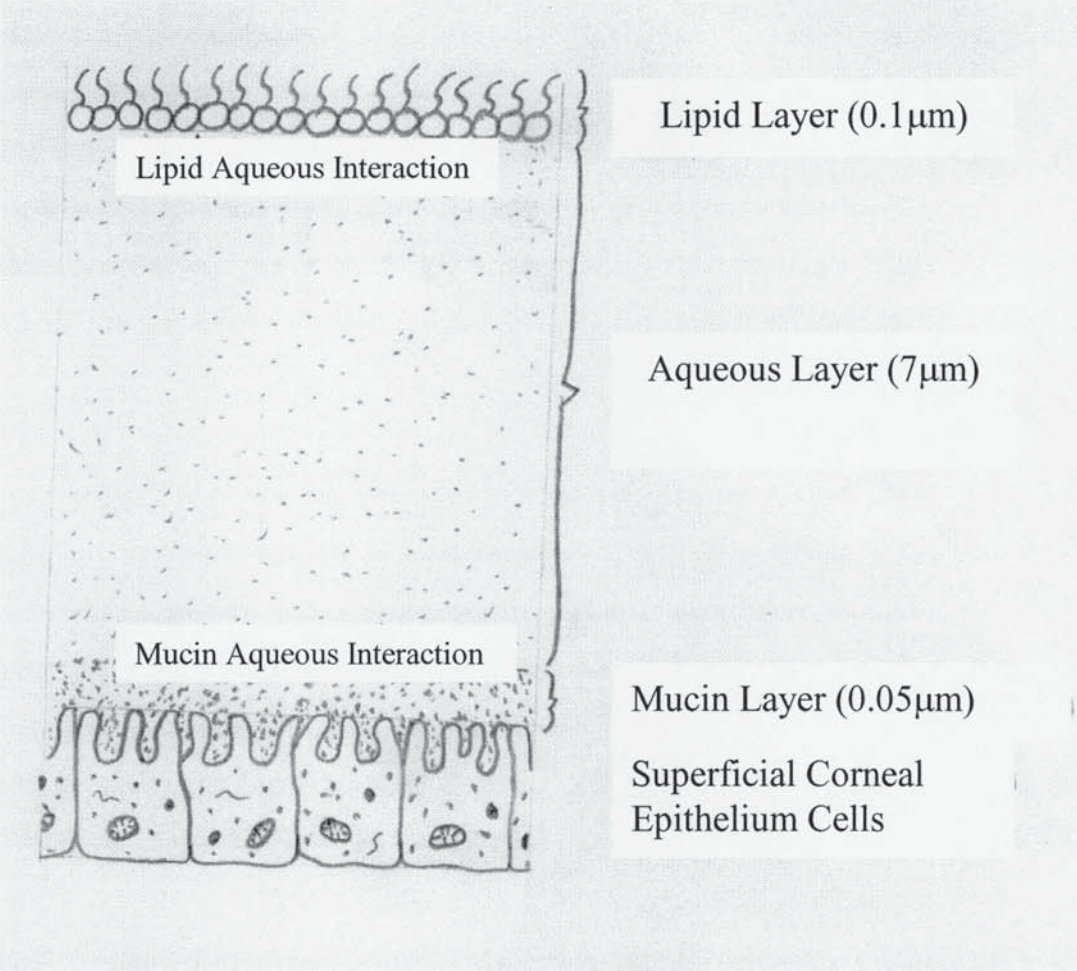


Figure 1.1. Structure of the tear film. The above diagram depicts the classical structure proposed by Wolff[2] in 1946 which remains widely used to this day.

This simplified idea of the tear film with enforced discrete boundaries allows us to easily identify its' main components. However, in reality each layer interacts with the adjacent layer to give an integrated system (e.g. certain components of the aqueous influence the lipid layer, the mucins can be found dissolved within the aqueous, etc.). In spite of this, each layer has its own unique composition which enables it to carry out a particular function(s) within the ocular environment.

The physical properties of the preocular tear film have been widely reported, with many different values being put forward as a result of different methods of analysis, collection protocol and patient groups. Values for thickness range from 7 to 45μm [2, 3], whilst a tear volume of around 7μl is most commonly accepted[4]. In the normal eye, the precorneal tear film accounts for 1μl of total tear volume, with the balance consisting of tears occupying the marginal strips and conjunctival sacs[5].

1.2.1 The Mucin Layer

The main source of mucins in tears is the goblet cells of the conjunctiva, but there are also mucus secreting cells that have been found in the lacrimal gland[6, 7]. Non-goblet epithelial cells that contain mucus secretory vesicles have also been described[8]. Mucins are composed of unique glycoproteins, free proteins and salts which form a viscous, sticky, thin layer that covers the surface of the cornea. The mucin layer is anchored in place by a structure called the glycocalyx. The glycocalyx is composed of oligosaccharide side chains of glycoproteins and glycolipids associated with the microvilli of the corneal and conjunctival epithelium[9].

Whilst traditional models considered the mucins to be found exclusively in this area in a layer 0.02 - 0.05μm in thickness, more recent studies have described a model wherein mucins exist in a more diffuse manner throughout the tear film[3, 10]. This structure is termed the 'mucus gel' model and is becoming accepted as the true structure of the tear film as it is based on data collected using very accurate modern methods/apparatus. The mucus gel tear film is much thicker than traditional tear models with a reported thickness of about 40μm as measured by laser interferometry[3] compared to traditional values of only 7μm. In addition it has been shown by treatment with mucolytic agents that the majority of this thickness is due to mucus. Tear layer thickness was reduced from 40μm to only 11μm upon treatment with a mucolytic agent[3]. The mucus gel model also describes a structure that would allow the mucins to interact with the other layers while still maintaining their main function.

The major role of mucins derives from their inherent hydrophilicity. This acts to mask the hydrophobic nature of the corneal surface and makes the cornea wettable by the aqueous tear[11]. Mucins are also thought to lower the tear surface tension, again enhancing corneal wettability as well as aiding the even spread of the tears during a blink[12, 13].

Other functions of the mucins have also been proposed and certain assumptions can be made because of the similarity of this mucous layer to those found elsewhere in the body. For example, the ability of the mucous layer found in the respiratory tract to trap harmful agents such as bacteria and irritants is well known. These are then cleared by sneezing or swallowing. The ocular mucus acts to immobilise infectious species which are then cleared by normal continuous tear turnover, by flushing via excess reflex lacrimation and blinking, or by nocturnal exudation[12, 14, 15]. In addition, the mucin layer is thought to provide a site for immune agents, such as IgA and lysozyme, to anchor thereby contributing to local immunity[16].

1.2.2 The Lipid Layer

The outermost layer of the tear film comprises oily secretions from the meibomian glands and the glands of Moll and Zeiss. These lipids are a mixture of sterol esters, wax esters, triacylglycerols, polar lipids and some free fatty acids[17, 18]. The lipid layer acts independently of the underlying aqueous, the lipids do not follow the normal flow of tears but are anchored at the orifices of the meibomian glands. The lipid layer is completely reformed with each blink. The glands secrete meibomian oil onto the lid margins and when the lids touch during the blink, the separate reservoirs of each lid merge. As the lids part, oil is drawn from the pooled reservoir into the preocular tear film to produce a continuous surface layer[19].

The lipid layer carries out several important functions. Foremost amongst these are the slowing or prevention of evaporation of the underlying aqueous layer from the ocular surface and a reduction of the tear surface tension leading to better spreading of the tear after the blink[20, 21].

Another role involves the prevention of skin lipids and applied cosmetics from contaminating the tear film from the lid margins[21, 22]. If skin lipids were allowed to pass onto the ocular surface they would disrupt the ability of the normal lipid layer to function correctly. This occurs because skin sebum has a higher spreading pressure than meibomian oil. In addition, it has been suggested that the lipids perform other tasks ranging from an antimicrobial action related to the presence of fatty acids, to a complexing action with mucin and even the production of sexually attractive chemicals called pheromones[19].

1.2.3 The Aqueous Layer

By far the largest of the layers at approximately 7μm and accounting for over 90% of the total thickness of the tear film, the aqueous layer is also the most diverse. Contained within the aqueous is a wide array of inorganic and organic species that are mainly produced by the main and accessory lacrimal glands, as shown in Figure 1.2. Other components are derived from the plasma and serum as a result of vascular leakage and/or active and passive transport across the blood-tear barrier. In some circumstances, such as eye closure and inflammation, the proportion of serum derived species is increased[23, 24]. The primary constituents of the tear film are a range of electrolytes and a large number of proteins.

The electrolytes of the tear film consist of both cations and anions. The main cations are sodium and potassium, but lower concentrations of magnesium and calcium are also present. The anions found in the aqueous are chloride and bicarbonate. These electrolytes act in two main ways; by contributing to the control of tear pH within a healthy range and by adding to tear osmotic pressure[25].

There are reportedly sixty different, identifiable proteins in the tears[26]. Whilst most of these are present in very small concentrations, they carry out very important functions. The main proteins of interest in the aqueous layer are lysozyme, lactoferrin, albumin, lipocalin (tear-specific prealbumin), the immunoglobulins (in particular IgA) and other mediators of inflammation/immunity (for example kininogen and the cytokines). As they encompass both native and serum derived proteins as well as possessing a

range of functions, these proteins and their actions will be discussed later. The source of these proteins (i.e. whether they are derived from the lacrimal gland or plasma) will be highlighted in these later sections. Also present in small amounts in the tears of normal individuals are species such as glucose, urea and free fatty acids which are classed as tear metabolites[6, 27].

Due to the number of components found in the aqueous layer, its' functions are also numerous[28]. These functions include:

- Supplying the corneal epithelium with essential nutrients.
- Facilitating the removal of foreign materials from the surface of the eye.
- Protecting the cornea from mechanical stresses via improved lubrication.
- Buffering tear pH.
- Localised immunity against invasive viruses and bacteria.

Figure 1.2. Position of the major glands of the eye that are involved in tear production[29].

1.3 Functions of the Tear Film

As indicated in the previous section, the pre-ocular tear film exists for a number of reasons. The many roles that are performed by the tears can be subdivided thus:

- Protection of the eye. Due to the antibacterial activity of several of the constituents of the tear film, it acts as a barrier to ocular infection. In addition, waste products produced by corneal metabolism are removed before they can rise to harmful levels and the cornea is prevented from drying out[16, 30].

- Maintenance of vision. The tears provide a smooth, regular optical surface by filling in any irregularities of the corneal surface[6, 30].

- Lubrication of the blink. Tears reduce the friction that results as the eyelids pass over the ocular surface preventing any damage. They also remove cellular debris and particulate matter that would otherwise cause irritation[31].

- Nourishment of the cornea. Due to the fact that the corneal surface is avascular (i.e. it has no direct blood supply) it largely relies on the tears for nutrition. Oxygen dissolves from the surrounding air into the tear film and is transferred to the corneal epithelium. Other essential nutrients are passed from the blood vessels of nearby areas such as the conjunctiva into the tear film and also delivered to where needed[6].

1.4 The Roles of the Tear Proteins

As mentioned earlier there are a large number of proteins to be found in tears which carry out an extensive portfolio of essential functions. Whilst it would be impossible to cover all of these in the space available, it is important to at least describe the core proteins of interest in our work. To this effect the following sections will cover the key ocular proteins and their functions. Particular attention will be paid to those proteins involved in the defence and protection of the eye.

1.4.1 Antibacterial Agents

The outer surface of the eye is under constant assault by a broad range of microorganisms including bacteria, viruses and certain parasites. To maintain the integrity of the ocular surface, which is essential to retain corneal transparency, the eye has developed a large number of defence mechanisms. These mechanisms range from mechanical means such as the blink to more specific antibacterial activities like those carried out by lysozyme and lactoferrin.

1.4.1.1 Lysozyme

Lysozyme is a low molecular weight protein of around 14.6kDa found in tears that is derived from the lacrimal gland. The average concentration of lysozyme in the tears has been reported as 1.85mg/ml but the value measured has varied widely due to a number of methods being employed[29]. Whatever the value, it remains one of the major protein constituents of the tear film, and this is an indication of its' importance. Lysozyme possesses both bacteriostatic (prevents bacteria from multiplying/growing but is not toxic) and bactericidal (kills bacteria) activity[32], and mostly acts to destroy certain classes of bacteria, in particular those that are gram positive[27].

The reason for this preference for gram positive species is that lysozyme enzymatically cleaves the glycosidic bond of muramic acid moieties found in the cell walls of these bacteria leading to cell lysis. Gram negative species are resistant to the

action of lysozyme due to an extra external lipopolysaccharide coating that stops lysozyme from attacking their cell walls. If, however, the outer layer becomes damaged or weakened in some way, the bacteria will become susceptible to lysozymes' lytic action[16]. Despite its' weakness when attacking gram negative species, lysozyme has been reported to enhance the antibacterial action of complement and T cells against these bacteria[33].

1.4.1.2 Lactoferrin

Just like lysozyme, lactoferrin is bacteriostatic and bactericidal in its' action. Also common with lysozyme are the source of this protein (the lacrimal gland) and the high concentration found in tears (around 1.5mg/ml)[34]. Their molecular weights are quite different, however, with lactoferrin being measured at around 82kDa[35, 36]. The method of antibacterial activity is also quite different.

Lactoferrin is an iron-carrying protein of the transferrin family whose strength of binding (reported to be 300 times that of transferrin)[37] led to assumptions that its' antibacterial activity derived from its ability to remove iron from the environment[27]. Iron is an essential metabolite for bacterial growth without which they cannot colonise successfully, hence removal prevents infection. More recently, it has been shown that lactoferrin has a more direct action on certain bacteria. It is thought that lactoferrin somehow alters the membrane of gram negative species thereby initiating subsequent lysozymal lysis[12,14].

In addition to these antibacterial activities, the iron binding action of lactoferrin has been linked with protection of the mucosal surface from harmful active oxygen species due to blocking the catalysis of the production of hydroxyl radicals. Lactoferrin has also been associated with control of inflammation via involvement in lymphocyte proliferation, cytokine production, activity of natural killer cells and regulation of complement activation[16].

1.4.1.3 β-Lysin

β-lysin is an enzyme which causes cell lysis. Unlike lysozyme which attacks the cell wall of bacteria, β-lysin attacks the cell membrane underneath[16]. This makes it particularly successful in countering infection by micrococci species, many of which are resistant to lysozyme. In fact, the two enzymes act synergistically with the destruction of the bacteria being initiated by lysozyme perforating the cell wall allowing β-lysin access to the cell membrane[12, 38]. β-lysin exists in many body fluids and its presence in tears is likely to be the result of transport from the blood. The level of β-lysin in tears is around 150μg/ml[38].

1.4.2 Transfer/Carrier Proteins

Other proteins in the tear film carry out important carrier protein functions, moving a number of essential metabolites and other species from well supplied vascular areas to those that are relatively isolated such as the avascular cornea. The most thoroughly understood of these is lipocalin, but other tear proteins such as albumin can be considered to perform some degree of transport due to the established presence of such a role for these proteins in plasma.

1.4.2.1 Lipocalin (Tear Specific Prealbumin)

Having possessed a number of names through the years (Proteins Moving Faster than Albumin (PMFA), Anodal Protein, Tear Specific Prealbumin) some of which were quite confusing, recent studies involving protein and gene sequencing methods have established that this protein is a member of the lipocalin family[39] and the name tear lipocalin has been widely adopted. In fact, there are a number of lipocalins present in tears and at least six isoforms can be easily determined[40], each of which could possess a unique structure and therefore a unique function. Lipocalins are a group of hydrophobic carrier proteins that bind a wide range of lipids with specificity being determined by molecular structure[41]. In tears these lipid ligands include cholesterol, fatty acids, fatty alcohols, glycolipids and phospholipids.

As a result of the range of lipids involved, the functions of tear lipocalins are also thought to be numerous. Proposed activities include the prevention of tear film disruption and the transport of ligands from the lacrimal gland. The first of these involves lipocalins binding hydrophobic molecules that would otherwise contaminate the mucous layer. The presence of such hydrophobic species on the hydrophilic ocular surface mucins would lead to dry/unwettable areas on the eye and prevent proper spread of the tear film. This in turn would lead to ocular desiccation and potential corneal erosions[42]. The latter function is based on the fact that the tear film is a midway point in the passage from the lacrimal gland to the nasolacrimal duct. Tear lipocalins produced in the lacrimal gland may carry an important ligand from the gland that is displaced by meibomian lipids and hence delivered into the tear film[41].

Recent studies have discovered a contribution of lipocalin to the non-immunological defence against infection in saliva[43] and this could possibly be another function of the tear lipocalins[44]. The antibacterial function of tear lipocalin is supported by the fact that it has recently been co-localised in the Von Ebners' glands with lysozyme[45]. Whatever their actual role, the tear lipocalins must be important to the tears as they constitute between 15 and 33% of total tear protein with a concentration around 1.7mg/ml[40].

1.4.2.2 Albumin

The concentration of albumin (molecular weight = 68kDa) in tears is around 1.3mg/ml which is considerably lower than the 50mg/ml found in the serum. Whilst its purpose in tears is not clearly understood, in plasma albumin functions as a transport protein specific for free fatty acids. Due to this well-defined role, it can be postulated that albumin carries out a similar job in the tears.

In addition, because the albumin in tears is serum derived, the presence/concentration of albumin in the tears is an indicator of permeability of the blood-tear barrier. Thus, higher concentrations of albumin can indicate inflammation, epithelial damage or vascular leakage[46].

1.4.3 Antibodies/Inflammatory Mediators

In addition to the antibacterial enzymes detailed earlier, the ocular environment also boasts several other protein species that act to protect the eye from infection, as well as those that control the inflammatory response and participate in wound healing. These proteins include the antibodies (immunoglobulins), cytokines, the members of the complement cascade and, of particular interest to our work, kininogen which is discussed in section 1.4.3.4.

1.4.3.1 Antibodies

Principal amongst the other proteins involved in defence of the eye are the immunoglobulins (Igs) or antibodies, glycoproteins that combine in a highly specific way with the substance (antigen) that triggered their formation. The immunoglobulins make up the humoral (cell based) aspect of the immune response and their general action can be described simply, as follows. The two 'arms' of the Y shaped antibody (see Figure 1.3) have antigen-binding regions which can link antigen presenting species together into large, easily phagocytosed lattice structures. This binding is enhanced by a flexible 'hinge' which allows the distance between binding sites to vary. The fate of the antigen once it is bound depends on the class of the immunoglobulin involved[9].

In the human body, five classes of antibody have been described- IgA, IgG, IgM, IgE and IgD. Whilst having high sequence homology and similar general molecular structure, consisting of two light chains and two heavy chains, each class has a unique heavy chain which gives it characteristic properties of its' own. This allows each class to undertake a particular role in defence of the host against potentially harmful or infectious bodies[47], as described later.

1.4.3.1.1 IgA

Unlike plasma, where IgG is the predominant immunoglobulin species, in the tears it is IgA that is most abundant. In fact, IgA is the principal class of antibody in a number of secretions including milk, saliva and respiratory and intestinal secretions[9]. IgA found in external secretions varies from its' serum counterpart both chemically and immunologically[48]. Whilst serum IgA exists as a 150 kDa monomer, secretory IgA (sIgA) takes the form of a dimer of molecular weight 400kDa. The additional weight arises from the addition of both a J(joining) chain and a secretory component (sometimes called SC). The function of the secretory component is to enable sIgA molecules to be transported across mucosal tissues into secretions.

Figure 1.3. Schematic of a typical antibody molecule comprising two identical heavy chains and two identical light chains[9].

The majority of IgA found in tears is produced by plasma cells found in the lacrimal glands[49, 50], rather than being plasma derived. Epithelial cells in these glands produce the secretory component which wraps around the dimer and protects it from degradation[49]. In cases of trauma, some IgA may pass from the plasma, but this will be relatively small when compared to concurrent movement of IgG. sIgA prevents bacterial adherence to the ocular surface[51], causes bacterial agglutination and can neutralise certain toxins and viruses[52]. Concentrations of IgA recorded in tears vary according to collection method and assay used, with values ranging from 0.04 to 0.85mg/ml and an average of around 0.3mg/ml being widely accepted[53, 54].

1.4.3.1.2 IgG

IgG is the most abundant immunoglobulin in the serum, constituting around 80% of the total antibody recorded in this fluid; it acts as a memory bank of previous infection. IgG is the second immunoglobulin to be produced after IgM upon novel antigenic challenge during primary infection. Whilst IgM specific for that particular antigen eventually disappears upon removal of the infection, IgG molecules directed against the invasive organism persist in the serum. Upon secondary exposure IgG reacts faster and more strongly than IgM due to this retained memory of an old enemy.

Not possessing the all-important secretory component associated with the ability of IgA to exist in external environments, the concentration of IgG in normal, unstimulated tears is relatively low at around 0.13mg/ml[29]. The source of this IgG is the plasma and this probably occurs via some sort of passive movement/seepage across the blood-tear barrier[28].

1.4.3.1.3 IgM

Always the first antibody produced in response to a new invasive antigen, IgM is also the major class of antibody released into the blood in the first stages of a primary antibody response. The secreted form of IgM is a pentamer, consisting of five separate IgM molecules linked by a polypeptide called a J chain. This means that secreted IgM possesses ten antigen-binding sites and leads to IgM being particularly successful at aggregating antigens[9].

Unfortunately, due to the size of its' secreted form, IgM cannot diffuse into the central stroma of the cornea, and it is restricted to the periphery of the ocular surface. This may provide the peripheral cornea with enhanced protection due to the aforementioned proficiency of IgM in dealing with antigens[16]. Recently more sensitive methods using enzyme linked immunosorbant assays have been used to measure the presence of IgM in tears[55]. These have allowed a concentration of 5.6μg/ml to be recorded in normal tears, a concentration so low as to preclude any significant function.

One situation where IgM does play an important role in the tears is in incidents of patients with serum IgA deficiency. In normal individuals, IgM is only recorded in trace amounts, if at all, in the tear fluid. Patients with serum IgA deficiency, however, possess detectable levels of IgM and it has been suggested that in such cases serum derived IgM is secreted in some way so as to compensate for the immune deficiency[56].

1.4.3.1.4 IgE

IgE is a unique immunoglobulin which participates in allergic responses. It acts by recruiting mast cells and basophils, causing these cells to release a number of biologically active amines including histamine. These amines trigger a number of responses associated with allergic reactions including dilation and increased permeability of blood vessels.

Whilst not always reported in tears due to its' extremely low concentration (around 200ng/ml), levels have been shown to increase in cases of allergy. IgE should be considered important when analysing tears of known hay fever sufferers and can be regarded as an indicator of such conditions[48, 57].

1.4.3.1.5 IgD

Like IgE, the concentration of IgD in the body is modest when compared to that of IgM, IgG an IgA. Unlike IgE, the actual role of IgD is poorly understood, and to date its' presence in the tears has not been established/recorded[48].

1.4.3.2 The Complement System

The complement system is an important aspect of the human immune system. Composed of around twenty serum and cell membrane proteins and taking the shape of an enzyme cascade which allows signal amplification, there are two pathways of complement activation (see Figure 1.4). The classical pathway follows a specific sequence of complement components in the order C1, C4, C2, C3, C5, C6, C7, C8, C9[58] and ends in the formation of the membrane attack complex (MAC).

The membrane attack complex is a collection of twelve C9 proteins that insert themselves into the lipid bilayer of the target cell and form a transmembrane channel. The formation of this channel, which is unique in nature, allows water and ions to enter the cell, causing it to swell and eventually burst[59]. The alternative pathway bypasses this sequence up to C3 which is activated by microbial polysaccharides called Factor B and Factor D. After this initial difference, the pathways are identical and the end result is the same in both pathways.

1.4.3.1.1 IgA

Unlike plasma, where IgG is the predominant immunoglobulin species, in the tears it is IgA that is most abundant. In fact, IgA is the principal class of antibody in a number of secretions including milk, saliva and respiratory and intestinal secretions[9]. IgA found in external secretions varies from its' serum counterpart both chemically and immunologically[48]. Whilst serum IgA exists as a 150 kDa monomer, secretory IgA (sIgA) takes the form of a dimer of molecular weight 400kDa. The additional weight arises from the addition of both a J(joining) chain and a secretory component (sometimes called SC). The function of the secretory component is to enable sIgA molecules to be transported across mucosal tissues into secretions.

Figure 1.3. Schematic of a typical antibody molecule comprising two identical heavy chains and two identical light chains[9].

Studies of the components of the complement system in the ocular environment have shown the presence of elements of both pathways in the tears, aqueous humor and cornea [58, 63, 64]. Equally important is the discovery of those proteins involved in the regulation of such a complicated system such as Decay Accelerating Factor (DAF;CD55), and Membrane Co-factor Protein (MCP), which act on C3, and membrane attack complex inhibiting protein (CD59) whose action is self explanatory[61]. Unregulated complement activity could lead to unwanted host 'self' tissue/cell destruction and/or excessive inflammation. The fact that regulatory proteins have been found in tears in addition to the complement proteins themselves shows that complement acts and is controlled in the ocular environment rather than just being found as a result of vascular leakage[65].

1.4.3.3 Cytokines

The presence of cytokines in the tears has recently been described both in cases of infection and in the basal uncompromised tear[66, 67, 68]. Cytokines are a family of mediators involved in immunological and inflammatory responses. They are also sometimes referred to as interleukins or lymphokines and can promote a number of biological responses including activation and proliferation of immunocompetent cells, chemotactic, cytotoxic and antiviral activity and production of red blood cells (haematopoiesis)[66]. As a result of these myriad influences, their importance in the ocular environment is obvious.

The most widely studied of the cytokines in tears is Epidermal Growth Factor (EGF), whose principal role is epithelial and endothelial wound healing[68]. The actual function of EGF varies according to the balance of other cytokines present and it is also reported to function in tissue restructuring[69]. Despite being present at only low concentrations of 5.3 ng/ml in basal tear[68], the ability of EGF to trigger an amplified cascade of other responses means that even such low concentrations can be significant.

This is further highlighted by the fact that decreased levels of EGF are recorded in cases of Sjögren's syndrome and in ocular rosacea, wherein the concentrations of inflammatory cytokines such as interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) are elevated[69, 70].

Inflammation is the normal response of the body to injury, resulting in increased blood flow to the damaged area and enhanced blood vessel permeability. This allows cells such as macrophages and lymphocytes to move in to the site of insult where they participate in defence, tissue remodelling and healing[71]. However, in some situations, inflammation can become widespread leading to harmful effects such as hypotension, shock, organ failure and even death[72]. The increase in inflammatory mediators and upregulation of other cytokines may therefore have both beneficial and detrimental effects, but the actual role of cytokines in the eye remains poorly understood. Further study is essential as there is huge potential in the use of applied cytokines in fighting ocular damage and disease.

1.4.3.4 Kininogen

Kinins are bioactive peptides produced by the action of kallikreins (specific proteases) on kininogens (inactive precursors). The action of kinins is tied up in the inflammatory response where they exert either a direct effect producing vasodilation, pain and increased vascular permeability or an indirect effect by triggering the release of prostaglandins and nitric oxide[73].

The kinin system is a cascade, similar to that seen with complement earlier, an outline of which can be seen in Figure 1.5. Kininogens are single chain glycoproteins found in two forms; High Molecular Weight Kininogen (HMWK) and Low Molecular Weight Kininogen (LMWK) whose molecular weights are 120 kDa and 68 kDa respectively[74]. They share a common heavy chain but have unique properties conferred upon them by a unique light chain, with LMWK having a much shorter light chain[75]. Upon cleavage, HMWK produces bradykinin and LMWK produces kallidin (lysyl-bradykinin) and these go on to trigger the inflammatory responses described earlier.

Figure 1.5. The generation of kinins by kallikriens[72]

In addition to their part in the kininogen-kallikrein-kinin system, kininogens have been designated specific roles outside that of precursor. Functions including activity in the blood coagulation cascade as a cofactor, inhibition of cysteine protease activity, and down regulation of thrombin-induced platelet activation have all been described[74].

To date the majority of work done has concentrated on kininogen and kallikrein in the blood plasma and saliva. Due to existing knowledge about the relationship between proteins found in plasma and saliva and those in tears, in addition to the importance of the kinin cascade and the other functions of kininogens discovered to date, it has been resolved that it is important for us to explore the concentration and activity of kininogen in tears.

1.5 Types of Tear

So far we have considered the tears to be relatively unchanging or constant. In reality, there exists more than one type of tear, each of which has a particular unique composition/content of the separate elements outlined earlier. There is also a variation between age groups and indeed from individual to individual and even between the eyes of one individual. As a result of these variations, care must be taken when collecting tear samples, the nature of the tears must be recorded, and conclusions must be considered with regard to inter-subject variability. In addition the method of tear collection must be carefully selected so as to obtain the class of tears required for the experiment[76, 77, 78].

In summary, the different types of tear can be divided into a number of groups.

1.5.1 Basal Tears

These are the tears observed when only minimal stimulation is being applied that maintains the 'normal' aspect and functions of the tears. No intense outside stimuli are having any influence/being applied (i.e. there is no reflex tearing). These tears most closely represent the normal tear protein pattern, consisting of a lacrimal gland secretion rich in lactoferrin, lysozyme and lipocalin[79, 80].

1.5.2 Reflex Tears

Under conditions of damage, inflammation or infection, reflex tears are produced. Tear flow rate increases up to 50 fold, the concentration of plasma associated proteins such as IgG, transferrin and serum albumin increases due to plasma leakage and the normal tear proteins become diluted[80,81]. Increases in tear flow help to flush away irritants and enhance the delivery of infection-fighting entities.

1.5.3 Closed Eye/Nocturnal Tears

Upon eye closure, the tear film becomes a stagnant entity with no contact to the external environment (air). Tear movement and refreshment is restricted due to the absence of the blink, oxygen levels are reduced, carbon dioxide levels increase and the result is corneal oedema, hypoxia and oxygen debt[82]. A parallel alteration of the tear proteins occurs which may be both triggered by and contribute to these symptoms. Levels of IgA increase noticeably, as do quantities of serum proteins such as albumin[24].

The closed eye exists in 'a sub-clinical state of inflammation'[23, 82]. Activation of the complement system has been recorded, as was the conversion of plasminogen to plasmin. A large number of polymorphonuclear leukocytes infiltrate the closed eye tears. Collecting closed eye tears is almost impossible, with most experimenters attempting to harvest tears immediately following eye opening, either after sleep or an extended period of eye closure. However, the eye has been shown to be devoid of an aqueous layer upon opening after sleep. What is in fact collected is a mixture of the closed eye tear diluted by an immediate release of fresh tears onto the eye[82]. Closed eye tears are of particular interest due to the possible similarity between these tears and those of dry eye sufferers. Dry eye is of interest to us because of the potential of the studies in helping to diagnose/prevent this disease.

1.5.4 Emotional (Psychogenic) Tears

The number of studies on such tearing are very low, most likely due to the fact that finding a particular stimulus which affected every individual in the study equally would be incredibly difficult and different levels of emotion would be impossible to quantify. In addition, the influence of society on men which means open displays of emotion are frowned upon must be taken into account. Whilst also resulting in an excess in tear volume that causes tears to overflow their normal environment and spill down the face, the composition of emotional tears is quite different. The protein concentration of emotional tears has been shown to exceed that of irritant induced (reflex) tears, but the protein pattern was not statistically different[83].

1.5.5 Age Variation

As an individual ages the tear volume falls, along with the levels of lysozyme and lactoferrin. The levels of IgG and ceruloplasmin, which are both associated with plasma, both increase hinting at some level of plasma leakage[84, 85,86]. The stability of the tear film has also been shown to decrease with ageing, although the cause of this has not been established[87].

1.6 Diseases of the Eye Associated with Contact Lens Wear

The introduction of a lens can have several effects on the tear film and indeed the ocular surface. In some instances this can be associated with severe symptoms including various contact lens associated disorders and diseases. Amongst these disorders are meibomian gland dysfunctions, dry eye, microbial infiltrative keratitis and tear film dysfunction.

1.6.1 Meibomian Gland Dysfunction

Meibomian gland dysfunction (MGD) is a non-inflammatory clinical condition which leads to a change in the appearance of the lipid component of tears from clear and free flowing to viscous and cloudy. This is associated with smeary vision, increased greasy deposits on lenses, dry eye symptoms and reduced tolerance to lens wear[88]. MGD is seen both in non-lens wearers and lens wearers, with the incidence being around 10% higher in people who wear lenses[89].

Contact lens-associated meibomian gland dysfunction (CL-MGD) has symptoms of blurred vision that can be linked to the adhesion of abnormal waxy lipids to the lens surface. The lens becomes more hydrophobic the more lipid that deposits and so is less capable of sustaining a normal, continuous tear film. This, along with the fact that the aberrant oil layer is less efficient at retarding evaporation, leads to the feelings of discomfort and dryness[90].

The cause of CL-MGD is open to some debate and in fact there are two opposing theories. One of these states that a person wearing a contact lens is less likely to rub their eyelids in case they damage or dislodge the lens. Normal rubbing of the eyelid is considered to be an essential mechanical stimulus so that the meibomian glands remain open and free flowing[91]. Removing the stimulus means glands become blocked and secretion falls.

In the other theory, CL-MGD is linked to papillary conjunctivitis. It is postulated that the itching caused by contact lens associated papillary conjunctivitis leads to excessive rubbing of the eye, which in turn means damage to the meibomian glands and consequent dysfunction[92]. To date it has not been determined which, if either, of these ideas explains the increased number of lens wearers who suffer from MGD.

1.6.2 Dry Eye Disorders

The problem of dry eye is one which affects many people who wear contact lenses. The overall causes are open for investigation and discussion, but dry eye itself has been defined as 'A disorder of the tear film due to tear deficiency or excessive tear evaporation, which causes damage to the interpalberal ocular surface and is associated with symptoms of ocular discomfort'[88].

Dry eye can thus be split into two groups; evaporative disorders and aqueous deficiencies. Evaporative disorders involve an increased rate of tear evaporation which can occur as a result of an abnormality in the tear lipid layer, an incomplete or irregular blinking pattern and/or some imperfection of the corneal surface. The most common cause of evaporative dry eye is meibomian gland dysfunction (as discussed earlier).

The prevalent form of aqueous deficiency is age-related non-Sjögren dry eye (NSDE) which is brought about by the destruction of acinar and ductal tissue in the lacrimal gland. This tissue breakdown is due to infiltration of the gland by lymphocytes. The next most common form is an autoimmune disease that also damages the lacrimal gland and is called Sjögrens' Syndrome Dry Eye (SSDE). Other sources of dry eye include sarcoidosis, HIV infection and graft versus host rejection[88].

Dry eye of all types is accompanied by conjunctivitis. This is caused by the release of a variety of inflammatory mediators such as prostaglandins which can be detected in the tears of dry eye sufferers. Also present are cytokines which are released into the tears by the lymphocytes that infiltrate the lacrimal gland. The cytokines are pro-inflammatory mediators and enhance the symptoms (see section 1.4.3.3) .

Whilst lens wear does not actually cause dry eye in most cases, those individuals who have asymptomatic, marginal dry eye without lens wear often progress to clinical, diagnosable dry eye symptoms upon commencing lens use. This occurs due to the surface and physical properties of the lens, the nature of the materials used in making lenses and the effect lens wear has on blinking patterns and on the ocular surface tissues[93]. Thus, contact lens wear highlights an existing tear abnormality or deficiency rather than actually triggering the disease, but these cases are termed contact lens associated dry eye because without lens wear the sufferer would probably never notice they had the condition.

Although it may seem unlikely that a single protein abnormality might cause dry eye considering the complexity of the tear film, isolating a protein which is over or under expressed in dry eye sufferers compared to normal individuals could prove useful. Once these changes have been identified they could be used to highlight areas for the treatment of dry eye or indeed clarify the cause of the disorder in a particular patient.

1.6.3 Infiltrative Keratitis

Considered to be the most severe reaction to lens wear, contact lens-induced microbial infiltrative keratitis (CL-MIK) can lead to a wide range of effects from substantial pain and discomfort to complete loss of sight in its' most severe cases. Microbial keratitis is an infection of the corneal tissue by bacteria, virus, fungus or amoeba which causes inflammation and/or ulceration. In the vast majority of cases the infecting species is either *Pseudomonas*, a gram-negative bacterium, or *Acanthamoeba*, although many other species have also been isolated from infected corneas[94, 95].

The incidence of this disease has been reported as being 5.2 per 10,000 patients per year for daily soft lens wear, 18.2 per 10,000 patients per year for extended soft lens wear and 6.8 per 10,000 patients per year for daily wear of rigid gas permeable lenses[96]. Early signs of the disease include foreign-body sensation, pain, swollen lids, eye redness, discharge and photophobia[97]. A concurrent alteration in tear protein pattern may well be observable, although this is speculation.

Amongst the factors which lead to an increased number of lens wearers suffering from MIK are the inefficiency of cleaning solutions in removing invasive organisms, the fact that micro-organisms can adhere to the lens surface and thereby be introduced to the ocular surface[98], the effects of overnight lens wear (as described earlier) and patient non-compliance[99, 100].

Non-compliance is where the lens wearer does not follow the instructions of his optician; wearing the lenses for longer than they are supposed to, not cleaning lenses properly, not washing hands thoroughly prior to insertion, etc. Other factors outside the control of the lens wearer include mechanical trauma due to ill fitting lenses[101], diabetes[102] and even living in a warm climate[103] all of which have been shown to increases the chance of developing this disease. CL-MIK is a very good candidate for the exploration of the use of tear protein patterns in early diagnosis. This would be especially beneficial in the case of bacterial keratitis whose progression from early symptoms to severe corneal damage is extremely rapid[104].

1.6.4 Tear Film Dysfunction

Considering the complexity and relatively shallow nature of the tear film, it is no great surprise that the insertion of such a comparatively large invasive body as a contact lens can cause a great deal of disruption. Upon initial lens wear, increased reflex lacrimation occurs which dilutes the normal tear components that are not derived from the lacrimal gland and causes unwanted tear overflow[105]. After a period of adaptation wherein the eye becomes used to the presence of the lens this excess tearing ceases, but other effects may be seen. For example, reports exist of alterations in the levels of lysozyme and immunoglobulins with continued lens wear[106, 107, 108].

Another effect of lens wear is the division of the tear film into two distinct 'compartments'- the prelens and postlens tear films[109]. These differ both functionally and structurally from the unpartitioned tear film. As might be expected, the ability of the tear film to carry out its' normal functions may be compromised by this restructuring.

The prelens tear film, for example, is much thinner than the precorneal film. This leaves it prone to rupture before it can be renewed by the blink as a result of evaporation (i.e. the break up time of the film is too low)[93]. This can lead to desiccation of both the contact lens and the ocular surface beneath.

A major problem caused by lens wear is that of post-lens film stagnation. Normal removal of waste products and delivery of nutrients is impeded by the lens, producing a similar problem as seen in the closed eye. This is countered by the blink which acts to flush debris from under the lens, and the continuous turnover of the tears under the lens is called 'tear exchange'[110].

Other effects of lens wear include an increase in the permeability of the blood-tear barrier which leads to alterations in the levels of albumin and IgG measured in the tears[111]. This is most pronounced when the lens involved is poorly fitting, which can lead to damage of the ocular surface, and also varies with the type of lens involved and the pattern of lens wear. Rigid lenses tend to cause a heightened response, particularly if they are worn for extended periods. The least effect occurs with the use of daily disposable lenses which remove the problems associated with overnight wear.

1.7 A Review of Previous Work

As a result of the importance of proteins to the proper functioning of the tear film and the ongoing health of human beings, there has been a great deal of interest in exploring tear proteins using a variety of experimental methods. Amongst the most commonly used techniques are electrophoresis, high performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA).

ELISA has been used in the past to analyse tears for the concentration of lactoferrin[34, 112], cytokines[69, 77], immunoglobulins[55], components of the complement system[61], lysozyme and ceruloplasmin[113]. The flexibility of this technique is good and its' possible uses in analysing tear proteins are restricted only by the availability of antibodies that will react with the protein of interest. However, whilst ELISA is convenient for determining changes/differences in the concentration of known proteins found in the tears, it is not possible to investigate unidentified proteins or to investigate more than one protein in a particular run using ELISA. For example, an ELISA experiment might be able to pick up a fall in IgA concentration but would not be able to indicate if such a drop coincided with an increase in one of the other antibodies.

The use of HPLC has also been widespread with particular interest in purifying isoforms of tear proteins such as lipocalin[40], quantifying tear proteins[114] and in the analysis of the protein pattern produced in response to varying stimuli[80, 81]. Compared to ELISA, HPLC allows the complete pattern of the tear proteins to be visualised with each run, with a series of peaks representing the individual proteins. The relative concentration of each protein can also be determined by analysing the area of the peaks produced. Individual fractions can be collected at the end of each HPLC run and subjected to further analysis by further HPLC, electrophoresis or by ELISA. This makes HPLC particularly useful in tear studies.

A number of forms of electrophoresis exist such as immunoelectrophoresis[67, 115], cellulose acetate electrophoresis[111, 116, 117] and polyacrylamide gel electrophoresis techniques. In recent years polyacrylamide gel electrophoresis has become particularly popular due to its' flexibility and the fact that in the mini form it becomes unnecessary to use pooled or stimulated tears. This is because a sample of 2-5µl is sufficient when loading a gel and means that several individual samples can be compared at one time. The small volumes involved open up the possibility of contrasting tears taken at time intervals and of identifying a truer pattern than pooled or stimulated samples. In addition, the fact that several samples are run next to each other aids visualisation of any differences in the tears of 'normals' compared to disease sufferers or people who wear contact lenses.

The technique of polyacrylamide gel electrophoresis (P.A.G.E.) is still generally based around the work of Laemmli[118] and can be used in two ways. The first is as a simple one dimensional technique which separates the tear proteins according to their molecular weight and the second a two dimensional version where they are also separated due to their isoelectric point, or pI (see Materials and Methods section). These separations display a pattern of proteins that can be compared for any changes due to disease, lens wear, etc. Individual proteins can also be identified according to their relative mobility in the gel compared to molecular weight and pI markers. In addition, it is possible to transfer the proteins from the gel to membranes via Western blotting and they can subsequently be analysed by immunostaining or by amino acid sequencing. This allows proteins to be conclusively catalogued.

Some of the areas which electrophoresis has been used to explore include the determination of the number of proteins present in tears[26], the effect of lens wear on tear proteins[119], identification of markers of corneal damage[120], the effect of stimulus[83], the importance of tear proteins in tolerating lenses[121], the identification of individual proteins[122, 123, 124 125, 126, 127] and the relationship between tear protein patterns and a variety of diseases[56, 128, 129]. Two dimensional techniques have allowed the number of proteins present in tears to be measured at more than sixty of which a large number remain to be conclusively identified[26].

In P.A.G.E. a number of alternatives exist including several gel recipes giving different characteristics, the option to use stacking gels, a choice between one and two dimensions, the ability to produce gradient gels and a variety of staining techniques. To date although some researchers have compared the effect of such influences as collection technique[76], length of storage[130] and sample treatment[131] on the pattern seen with electrophoresis of tears and some work has contrasted normal and gradient electrophoresis[132], it appears that no-one has attempted to obtain the best possible visualisation of the tear proteins by adjusting the aforementioned variables. One aim of this thesis will be to address this point and to generate a reproducible process that represents the best reproducible portrayal of the tear proteins that can be generated by P.A.G.E.

1.8 Aims and Scope of Research

The aims of this work can be clearly indicated as a number of points.

- Evaluation of a number of techniques for analysis of the tear proteins. These include HPLC, two dimensional electrophoresis, one dimensional electrophoresis, Western blotting and immunostaining.

- To set up and optimise the technique of one dimensional electrophoresis and to determine the influence of factors such as use of stacking gels, various gel recipes, running conditions and staining methods on the pattern of tear proteins which could be produced.

- Comparison of the strengths and weaknesses of the data that can be generated using electrophoresis compared to that derived from Western blotting and immunostaining.

- To investigate the presence of kininogen in a number of tear samples and contact lens extracts and determination of differences between the results procured from each type of sample

- Formulation and investigation of a technique or techniques that utilise a lens as a probe for removal of the intact tear envelope.

In terms of work that was outside the scope of the period of this research there were many exciting areas of potential exploration. These included:

- Further use of the optimised electrophoresis technique in the assessment of a wider sample population. Of most interest would be a study which included clinical samples taken from sufferers of various ocular disorders and a comparison of these to a 'normal' protein pattern.

- Study of the immunoglobulin and cytokine protein families using Western blotting and immunostaining. Some problems of commercial availability of antibodies and cross reactivity of the immunoglobulins have been experienced but these may be overcome with time and extended work.

- Continuing appraisal and development of the tear envelope extraction methods and their application to a wider sample population. The centrifugation parameters need extended study to achieve optimal conditions.

- Ongoing evaluation of new techniques and apparatus for tear analysis in terms of their applicability to the study of the protein pattern

Chapter 2

Materials and Methods

2.1 Introduction

This chapter outlines the main techniques used for tear protein analysis and the various methods explored for their efficiency in this analysis. Further details are provided where necessary in the relevant results chapters.

2.2 Materials

H.P.L.C. grade water, Acetonitrile, Ethanol, Methanol, Perchloric acid and Glacial Acetic acid (Fisher Scientific International).

NaOH pellets, Glycerol (BDH Chemicals Ltd.).

Lysozyme, Immunoglobulins, Lactoferrin, Albumin, AnalaR Ammonium Persulphate, TEMED, Acrylamide, Dithiothreitol, Phosphoric Acid, Urea, Tergitol, Bromophenol Blue, Bis-acrylamide, Ampholytes, Sodium Lauryl Sulphate, Tris base, Agarose, NaCl, Glycine, 2-Mercaptoethanol, Coomassie Brilliant Blue R-250, PVDF (polyvinylidine difluoride) membrane, FAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets, 6-amino-n-hexanoic acid (SIGMA, U.K.). Hydrochloric acid (Aldrich, U.K).

Sodium Acetate, 5% Sodium thiosulphate, EDTA-Na2, Sodium carbonate, 2.5% Silver nitrate, 37% Formaldehyde, 25% Glutardialdehyde, RepelSilane (Pharmacia Biotech, Sweden).

Fixative Enhancer Concentrate, Silver Complex Solution, Reduction Moderator Solution, Image Development Reagent, Development Accelerator Reagent, High range molecular weight markers, Low range molecular weight markers, Broad range molecular weight markers (Bio-Rad, UK)

2.3 Tear Sample Collection

The number of methods available for collecting tears and the variation in results obtained when using a particular method has been widely reported. Following on from work done by others within the group[62] it was decided to use a method involving narrow bore glass microcapillaries obtained from The Binding Site, Birmingham. Due to a minimal amount of irritation to the corneal surface it can be seen that the tears collected using this method represent a 'truer tear' than those collected via Schirmer strips and other abrasive methods.

Collection is achieved by placing the tip of the capillary in the lateral canthus and/or the inferior marginal strip. Tears are then drawn into the capillary and these can then be wrapped in aluminium foil and frozen until needed. Any unusual response such as excessive lacrimation was noted and is reported in the results. In this work fresh tears were used whenever possible.

Samples were expressed using the plunger supplied with the capillaries into micro eppendorf tubes and treated in a variety of ways as described later. Most commonly tears had an equal volume of 2X treatment buffer added and were then boiled for 10 minutes at 95°C (see section 2.8.1 for recipe of 2X buffer). Treated samples were stored in a fridge at 4°C and used within seven days. Those samples not used within this time were frozen until needed. Tear samples were stored according to these parameters based on previous work carried out on the influence of storage conditions on the proteins in a tear sample[130].

Volunteers who had their tears collected showed no overt signs of ocular disease and reported having no feelings of dryness or ocular discomfort. All samples were collected during the period 10am to 11am to minimise any possible influence of diurnal variation. The use of contact lenses was noted and all results were interpreted with reference to lens wear. The continuing ocular health of the subjects was monitored by regular visits to their own optician. Tear samples were collected in line with the ethical guidelines of Aston University.

2.4 Preparation of Contact Lens Extracts

When a contact lens is worn proteins attach to the lens surface and penetrate into the matrix of the lens. This technique is designed to remove the proteins from the lenses to allow further analysis and to grossly quantify the amount of protein removed. The varying ability of different lens types and of lenses produced using different materials to take up proteins from the tear film was seen as a positive feature and as something that we would be able to explore and illustrate with the techniques available to us.

Initially a worn lens was studied using an on-lens UV spectrophotometric assay. This technique allows the concentration of total protein deposition to be calculated but the chemical nature of the spoiling material cannot be determined. A subsequent extraction method, where the lens is treated with a mixture of chemicals at high temperature, is used to remove as much protein as possible. The lens is then reassessed with the spectrophotometer and the remaining protein (and thus the amount of protein removed) can be measured.

In order to measure protein deposition, a worn lens was placed into a very clean cuvette filled with distilled water so that it would lie around the cuvette wall at 180° to the excitation beam. The cuvette was then placed into a Hitachi U2000 Fluorescence spectrophotometer which had been calibrated with a blank containing only distilled water. The lens was scanned at 280nm which is the excitation wavelength for proteins. After extraction the lens was scanned again and the percentage protein removed calculated using this equation:

$$\frac{\mathbf{i-x}}{\mathbf{i}} \quad X\ 100 = \%\ \text{protein extracted/removed}$$

where **i** is the absorbance recorded for the untreated lens and **x** is the absorbance recorded following extraction treatment.

A previous study was undertaken to assess the optimal extraction method based on the criteria of extraction efficiency, reproducibility, low extraction solution volume and removal of proteins of interest[62]. After extensive assessment it was decided that a method involving a solution containing 40% urea, 1% SDS, 1mM DTT and 100mM Tris solution (pH 8.0) fulfilled these criteria to the best degree.

Each lens to be extracted was heated at 90°C (on a heated stirrer) for 3 hours, in a 1ml eppendorf and allowed to cool. The solution was then pipetted off into fresh tubes and stored in a fridge until used. Lens extracts were analysed using polyacrylamide gel electrophoresis and Western blotting.

2.5 Reverse-phase H.P.L.C.

Reverse-phase High Performance Liquid Chromatography is so-called due to the fact that it behaves in the opposite way to adsorption (or Normal Phase) liquid-solid chromatography. The stationary phase is non-polar and hydrophobic and consists of silica with an alkylsilyl compound chemically bonded to it. Retention of proteins mainly occurs as a result of the complicated hydrophobic interactions between the stationary phase surface and the protein.

A polar mobile phase is used to elute the solutes. This is usually a mixture of water and a water-miscible organic solvent such as methanol or acetonitrile. In this method, a mixture of perchloric acid and acetonitrile was used. Solutes are eluted in order of increasing hydrophobicity (decreasing polarity) and as the aqueous (polar) component of the mobile phase is decreased solute retention decreases.

2.5.1 Method

The following protocol was adapted from that described by Moriyama et al[133]. A set of standard solutions was produced based on the published values for concentrations of tear proteins (see Table 1). The quantity of protein corresponding to ten times physiological concentration was dissolved in 1ml of H.P.L.C. grade water. The purpose of this experiment was to assess whether the technique could detect these higher concentrations before progressing to physiological values. Half of each solution was frozen, whilst the remaining 0.5ml samples were stored in a fridge at all times when not in use.

Initially, 10μl aliquots of undiluted solutions were analysed. 10μl was used due to the small size of the loop on the injection valve of this system. Later, 1/10 dilutions of these original solutions were produced because this system appeared to be very sensitive. These dilutions were termed A/10, B/10, C/10 and D/10.

The physiological concentrations reported in the following table are taken from an article by Bright and Tighe[29] who calculated these values by averaging the values reported in various different articles.

SOLUTION	Physiological Concentration[7]	Ten Times Physiological Conc.	Actual Conc. of Solution Made
Solution A LACTOFERRIN	211 mg/100ml	21.1 mg/ml	22.53 mg/ml
Solution B ALBUMIN	130 mg/100ml	13.0 mg/ml	12.87 mg/ml
Solution C IMMUNOGLOBULINS	13 mg/100ml	1.3 mg/ml	1.54 mg/ml
Solution D LYSOZYME	186 mg/100ml	18.6 mg/ml	19.71 mg/ml

Table 2.1 Standard protein solutions used for H.P.L.C. and the average recorded physiological concentrations of these proteins

For this process, a TosoHaas TSKgel SuperODS™ column was used (I.D. 4.6mm, Length 100mm). This column was selected because relatively short run times were needed for good separation.

The Moriyama protocol[123] requires a gradient of mobile phases, with a ten minute linear gradient of CH_3CN (acetonitrile) from 68% to 20%, the eluent being 13mM $HClO_4$ (perchloric acid). To enable this, it was necessary to use the Varian 9010 Solvent Delivery System. This was attached to a Varian 9065 Polychrom detector that monitors a range of wavelengths from 190nm to 370nm. Data was processed and analysed using Varian Star 4.0, with most interest being at 254nm (this being the closest wavelength selectable to that at which proteins are detected, namely 260 nm).

Ahead of running a sample, the Varian Star programme was initiated, and the detector turned on. The pump was readied by purging the tubes connecting the reservoirs containing the mobile phases to the pump in turn. The pump was then allowed to run for one hour at its initial composition (68% acetonitrile, 32% perchloric acid) to equilibrate the system.

To get a base line one or more injections of acetonitrile were used to flush the Rheodyne. This was done after every protein sample to clear the loop and obtain a base line. Once a satisfactory baseline was achieved, 10µl aliquots of the protein samples were injected and the resulting peaks saved into the programme. The syringe used was washed between samples with acetonitrile.

After initial observations of both undiluted and diluted versions of solutions A-D (above), various mixtures were analysed. These were as follows:

NAME OF MIXTURE	CONTENT OF MIXTURE
Solution E	100µl each of solutions A/10, B/10, C/10 and D/10
Solution F	100µl each of solutions A/10, B/10 and C/10 + 100µl of distilled water
Solution G	100µl each of solutions A/10, B/10 and D/10 + 100µl distilled water
Solution H	100µl each of A/10, C/10 and D/10 + 100µl distilled water
Solution I	100µl of solutions B/10, C/10, and D/10 + 100µl distilled water

Table 2.2 Composition of mixtures made for reverse-phase H.P.L.C.

These mixtures were run under identical conditions to the other solutions. In addition, some fresh tears were filtered with a Whatman Puradisc™ 25 AS filter disc (pore size 0.45 µm), then injected into the system and analysed in the same way.

2.6 Two Dimensional Polyacrylamide Gel Electrophoresis (2-D PAGE)

Two dimensional electrophoresis involves the separation of a mixture of proteins using two characteristics or 'dimensions'. In the first instance the proteins are divided according to their isoelectric point (or pI). The net charge of a protein varies with the pH of the surrounding medium, and the isoelectric point is that pH at which a protein possesses no net overall charge. Gels are made which contain chemicals known as ampholytes. When an electrical current is passed through the gel the ampholytes produce a pH gradient with different ampholytes giving different pH ranges. Proteins are treated with a lysis buffer which contains a nonionic detergent to solubilize, denature and dissociate the protein whilst leaving the charge unchanged. They are then loaded onto the gels and when current is reapplied the proteins migrate to the pH position along the gel where they have no charge (see Figure 2.1).

After the first dimension, the proteins are separated according to molecular weight (MW). This is achieved via SDS polyacrylamide gel electrophoresis. SDS is an ionic detergent which masks the charge on the protein with negative charge. The principles behind this technique are described in a later section.

The coupling of the two dimensions has the advantage over simple one dimensional separations in that proteins of similar/identical MW can be identified as a result of different pIs and those of similar pI can be visualised according to different MW.

The majority of the method described here was taken from the standard operating procedure supplied by Hoefer with the equipment[134].

2.6.1 First Dimension Tube Gel Electrophoresis (Isoelectric Focusing)

Figure 2.1 Separation of protein molecules by isoelectric focusing[9].

Six gel tubes (I.D. 1.5mm, Length 7.5cm) were placed into a casting cup. The tubes were then gathered into a neat vertical bundle and secured to the support column of the cup with an elastic band. A small gap was left between the bottom of the tubes and the bottom of the cup to allow acrylamide to be displaced into the tubes. 3.5ml of first dimension acrylamide stock was then prepared by mixing 0.175ml of Ampholytes (40%), 0.365ml of an acrylamide stock solution (15g acrylamide and 0.9g of bis-acrylamide dissolved in 50ml of distilled water), 1.13ml of distilled water and 0.35ml of 20% NP-40. Into this, 1.9g of urea was dissolved before 0.025ml of 10% A.P.S. and 0.025ml of TEMED were added.

The A.P.S. and TEMED were added after all the other ingredients had been mixed together because they initiate gel polymerisation. The mixture was stirred gently, and then immediately poured into the casting cup to allow time for the gel solution to enter the tubes before polymerisation occurred.

The casting cup was lowered into the casting chamber, and 23ml of distilled water was slowly added to exert a column pressure that displaced the acrylamide into the gel tubes. The water was poured carefully so as to minimise any mixing of acrylamide and water. At the point of equilibrium between the column pressure and atmospheric pressure, a gel 6-6.5cm in length was created, leaving a space of around 1cm at the top of the tube for sample loading.

The gel solution was left to polymerise for about 2 hours. The bundle of tubes was then taken from the casting cup, the rubber band removed and remaining acrylamide mass was trimmed from the end of the tubes using a spatula. Care was taken so as not to dislodge the gels, and the tubes were inserted into a tube gel adapter that had been thoroughly rinsed with distilled water. The adapter was installed into one side of a Hoefer Mighty Small II SE 250 Mini-Vertical Gel Electrophoresis Unit, with the tubes facing in toward the upper buffer chamber. A clean glass plate was clamped to the other side of the unit to seal this chamber.

At this point, the upper buffer chamber was filled with a cathode solution (20mM NaOH which had been degassed for 20 minutes). The top of each tube was checked to ensure it was submerged in the solution, and any air pockets were removed by injecting cathode solution into the tubes with a syringe. The lower buffer chamber was filled with the anode solution (0.085% Phosphoric acid), and the gels pre-focused at 250V for half an hour to establish the pH gradient created by the ampholytes.

Whilst the gels were pre-focusing, the samples to be run were prepared. 10µl of tears were centrifuged at 1,000g for five minutes to remove debris, and the resulting clear supernatant added to 170µl of lysis buffer to give a total volume of 180µl. The lysis buffer used comprised 5.7g of Urea, 1ml of 20% NP-40, 0.5ml of ampholytes (40%) and 0.45ml of 2-mercaptoethanol made up to 10ml with distilled water. 60µl of each sample was loaded onto one of three tube gels, with gels 1-3 having Left eye samples, and gels 4-6 being loaded with Right eye samples.

The lid was placed on the electrophoresis unit, and the gels run at 500V for 2 hours. After this time, the power supply was turned off, and the cathode solution carefully poured away. The adapter was then unclamped, the tubes were removed, and the bottom, acidic end was labelled with methyl violet dissolved in distilled water.

At this point the tube gels were extracted and frozen until they could be run in the second dimension. A 3ml syringe was filled with equilibration buffer (5ml glycerol, 65mg dithiothreitol, 1g Sodium Dodecyl Sulphate, 2mg bromophenol blue, 0.76g Tris base, 30ml distilled water, adjusted to pH 6.8 with 6M HCl) and attached to the extractor bolt. The tube gel was inserted into the bolt opening, and pressed in until it had passed the two o-rings of the extractor. The bolt was tightened ¼ turn to form a tight seal, and the plunger of the syringe was slowly depressed. The intact tube gels were extruded into jars that also contained equilibration buffer, and left to soak for 10 minutes. After soaking, the tube gels were moved into tissue culture dishes and frozen at - 60°C until required.

2.6.2 Second Dimension Separation (PolyAcrylamide Gel Electrophoresis)

The first step here was to cast a slab gel. Two gel sandwiches were constructed, each consisting of one notched alumina plate, two 1.5mm spacers, and one 10 x 8cm rectangular glass plate. To minimise leakage a light layer of Cello-seal was applied to the outside (glass side) of each spacer.

Once assembled and carefully aligned, the gel sandwiches were inserted into the casting clamps of a Hoefer Mighty Small SE 245 Dual Gel Caster. The alumina plates were placed so as to face the back block of the clamps, and the bottom of the sandwich was made to protrude very slightly below the block. Once arranged, the sandwiches were secured by tightening the screws of the clamps. Each clamp was now placed into the casting cradle, screw side out. Cams were inserted into each side of the casting cradle, with short ends pointing up. The sandwiches were then completely sealed by rotating the cams 180°, which pushed the protruding edge of the sandwich into the gaskets located in the base of the cradle.

After it had been established that neither sandwich leaked using distilled water, the second dimension separating gel solution was made. This gel solution was different to the first dimension gel, and consisted of 5ml Second Dimension acrylamide Stock (30g high purity acrylamide, 0.8g bis-acrylamide made up to 100ml with distilled water), 2.5ml Separating Gel Buffer (36.3g Tris base, 150ml distilled water; adjusted to pH 6.8 with HCl, made up to 200ml with distilled water), 0.1ml 10% SDS and 2.33ml of distilled water. These ingredients were mixed thoroughly and then 50µl of 10 % A.P.S. and 5µl of TEMED were added. This gave 10ml of solution, which was swirled before being poured into a gel sandwich using a Pasteur pipette. 10ml was sufficient to give one 8 x 7cm-slab gel, and the gel solution was freshly mixed for each sandwich. After pouring, the gel solution was covered with water-saturated n-butanol and allowed to polymerise.

Once the gel had set, the water-saturated n-butanol was poured off, and defrosted tube gels were laid onto the top of the slab. Air pockets were removed via application of gentle pressure with a spatula until the gel/gel interface was smooth. The tube gel was sealed in place with a solution of 1% agarose melted in SDS electrophoresis buffer (145mg Tris base, 750mg glycine, 50mg SDS, 500mg agarose, plus distilled water to give 50ml). This agarose solution not only sealed the tube gel in place, but also ensured a continuous contact.

After the agarose solution had set, the gel sandwiches were clamped to either side of the electrophoresis unit, with the notched plate facing in. The upper and lower buffer chambers were both filled with a solution containing 1.74g Tris base, 9.0g glycine, 0.6g SDS dissolved in 600ml of distilled water. The upper chamber was filled first to ensure there were no leaks. This was done carefully so as not to dislodge the tube gel. The gels were run at 40mA (20mA per gel) constant current until the tracking dye (bromophenol blue from the first dimension equilibration buffer) reached within 1cm of the bottom of the gel.

The running buffer was poured away by inverting the unit, and the sandwiches then unclamped. The plates were separated carefully with a Hoefer Wonderwedge tool, and the gels gently lifted from whichever plate they had adhered to and then laid in trays containing Coomassie Brilliant Blue stain which forms covalent bonds with proteins. This stain comprised 0.5g Coomassie Brilliant Blue R, 800ml methanol and 140ml acetic acid which was made up to 2l with distilled water. The acetic acid was added a little at a time with constant swirling of the solution. Gels were stained for one hour, and then de-stained overnight in a weak solution of methanol:acetic acid. Once de-stained, gels were scanned into a computer then allowed to dry out before being discarded.

2.7 Silver Staining Method

Some gels were Silver Stained due to increased sensitivity compared to the Coomassie Blue method outlined earlier. A staining kit produced by Pharmacia Biotech was used which reportedly gives one hundred times greater sensitivity than the Coomassie Blue method previously mentioned. The method outlined here was supplied by the manufacturers of the staining kit and is based on the work of Heukeshoven and Dernick[135].

Gels run as above were placed in a stainless steel staining tray which is used because it has no effect on the staining solutions. This was left on a Janke and Kunkel KS 501 D flat bed rotary shaker throughout staining to provide necessary constant agitation. 250ml of Fixing solution (100ml ethanol, 25ml glacial acetic acid and 125ml distilled water) was added and left for 30 minutes. This solution was then poured off and 250ml of Sensitising solution (75ml ethanol, 1.25ml glutardialdehyde, 10ml sodium thiosulphate, 17g sodium acetate made up to 250ml with distilled water) was poured in. The glutardialdehyde was added to the mixture just prior to use.

After a further 30 minutes the gels were washed three times with 250ml distilled water before 250ml of a solution containing 25ml of silver nitrate and 0.1ml of formaldehyde was added and left for 20 minutes. At this point the gels were again washed with distilled water. This time it was two washes of 1 minute each. 250ml Developer (6.25g sodium carbonate, 0.05ml formaldehyde, made up to 250ml with distilled water) was subsequently added for four minutes before it was replaced with the Stop solution. The Stop solution contained 3.65g of EDTA-Na_2.$2H_2O$ dissolved in 250ml of distilled water. In both the instances of formaldehyde use, this was added just before the solution was utilised.

After a further three washes in distilled water of five minutes duration each, the gels were treated twice with a Preserving solution (75ml ethanol, 11.5ml glycerol, 163.5 ml distilled water) for thirty minutes each time. The gels were then placed upon glass plates that had been treated with RepelSilane and wrapped in Cellophane so as to dry them. Gels were subsequently scanned for permanent recording of the bands seen.

2.8 One dimensional Polyacrylamide Gel Electrophoresis (1-D PAGE)

This method is similar to the Second Dimension separation outlined previously and the theory behind it is described here. An illustration of the theory can be seen below in Figure 2.2.

Proteins usually have a net positive or negative charge that results from the mixture of charged amino acids they contain. When an electric current is passed through a solution containing protein, the individual proteins move at a rate that varies according to the net charge of the protein as well as its' size and shape. This is called electrophoresis[9].

SDS polyacrylamide-gel electrophoresis (SDS-PAGE) uses a highly cross-linked matrix of acrylamide as an inert matrix through which the proteins migrate. The pore size of this gel matrix can be altered by varying the total percentage acrylamide in the gel (i.e. the sum of the acrylamide monomer and the N,N'-methylene-bis-acrylamide crosslinker). This is called %T, and usually as the value of this increases the pore size decreases. The amount of crosslinker can also be varied so as to give different pore sizes. This is expressed as the percentage of crosslinker compared to the sum of monomer and crosslinker and is called %C. In most cases the %C used is arbitrarily kept at 2.6%[134].

The influence of charge and shape are overcome via treatment with a powerful negatively charged detergent (SDS) and a reducing agent such as mercaptoethanol. The SDS acts by binding to hydrophobic areas of the proteins, causing them to unfold and dissociate from other proteins or lipids. The negatively charged detergent molecules also act to cause all the proteins to become negatively charged, masking the natural charge of the protein. Now when an electric current is applied the proteins will all move toward the positive electrode.

The mercaptoethanol breaks any disulphide links in the proteins so that multi-subunit molecules are broken down into their constituent polypeptides. This means that these subunits can be analysed separately[9].

Figure 2.2 SDS polyacrylamide-gel electrophoresis (SDS-PAGE). Heating with SDS masks the native charge of the proteins and mercaptoethanol splits proteins into component subunits[9].

After treatment, the acrylamide gel will now act as a molecular 'sieve' with proteins being separated solely on the basis of their molecular weight. A complex mixture of proteins can thus be resolved into a series of distinct bands. Smaller proteins move faster through the gel whilst larger proteins are impeded, and distance travelled during a run is directly proportional to size.

2.8.1 Method

Construction of the casting cradle and gel sandwich is identical to that above. However, for 1-D P.A.G.E. a number of variables were explored. Gels were set with and without stacking gels, different gel percentages were used and two different gradients gels were explored.

In each case, samples were prepared by addition of an equal volume of 2X treatment buffer (2.5ml 0.5M Tris-HCl, 4.0ml 10% SDS, 2.0ml glycerol, 0.2 mg Bromophenol Blue, 0.2ml mercaptoethanol made up to 10ml with distilled water) followed by boiling for 10 minutes.

When a stacking gel was used, the chosen resolving gel solution was prepared as described above for second dimension gels and poured so as to reach a level around 3 cm from the top of the glass plate. Air bubbles were removed with the aid of a long spatula and the gel was left to polymerise. Water-saturated n-butanol was added to the top of the gel to prevent exposure of the gel to oxygen, and the gel was stored in a fridge at 5-10°C until needed.

Just prior to running samples, the overlay was poured off and a stacking gel mixture composed of 0.67ml acrylamide stock solution (30g acrylamide, 0.8g bis-acrylamide made up to 100ml with distilled water), 1.25ml Stacking Gel buffer (3g Tris base, 40ml distilled water; adjusted to pH 6.8 with HCl, made up to 50ml with distilled water), 0.05ml 10% SDS and 3ml distilled water was made. Polymerisation was initiated by addition of 25µl of 10 % A.P.S. and 2.5µl of TEMED just before pouring.

The stacking gel was then introduced on top of the resolving gel with a Pasteur pipette and a comb was inserted at an angle into the gel. This allowed sample wells to form in the stacking gel with a depth of around 1cm of stacking gel below the wells.

After the gel had set the comb was carefully removed and the gel was transferred to the electrophoresis unit. The upper and lower buffer chambers were filled with running buffer containing 1.74g Tris base, 9.0g glycine, 0.6g SDS dissolved in 600ml of distilled water. Samples were then underlaid into the wells using a syringe with a fine tip. Stacking gels were produced as close to electrophoresis being carried out as possible because this optimises resolution. Stacking gels are used to focus the samples into tight bands. Samples were run at 30 mA until the dye front ran off the bottom of the gel. At this time they were stained with Coomassie Blue or with the alternative silver staining method of Gottleib and Chavko[136] outlined later.

When no stacking gel was used 10ml of the selected resolving gel solution per gel sandwich was produced following recipes supplied by Hoefer with the equipment. By adjusting the amount of acrylamide in these solutions, gels of varying properties could be produced. High concentrations of acrylamide give gels with smaller pores and low concentrations give larger pores if the concentration of crosslinker is kept constant. Using the recipes supplied gels of 7.5%, 10%, 12.5%, 15% and 20 % acrylamide were possible.

Table 2.3 Gel Recipes as supplied by Hoefer (slightly adapted)[134].

The gel solution was poured to a level just below the top of the glass plate and a comb was introduced directly into this solution. Tapping vigorously on the top of the comb dislodged any air bubbles trapped under the teeth of the comb. The gels were allowed to set for at least two hours before the combs were removed. The surface of the gel was washed with distilled water and water-saturated n-butanol was then added. Gels were wrapped in cling film until needed. Before running, the water-saturated n-butanol was poured off, the gel was washed again with water and the gels were transferred to the electrophoresis unit where samples were loaded as above.

For gradient gels, two gel mixtures of differing composition were made. This was usually 10ml of 7.5% acrylamide solution with 10ml of either 15% or 20 % acrylamide solution. The higher percentage solution had 1.5g Sucrose added to increase its density. Gradients were produced by first pouring 5ml of high percentage solution and then adding 5ml of the lower percentage gel via a Pasteur pipette with some force to ensure mixing. The higher percentage solution also had half as much APS added to cause the gel to set from the top to the bottom.

Combs were again inserted to produce sample wells and air bubbles were removed as before . These gels were stored, loaded and stained as before except that in some cases gels were blotted using a semi dry blotter. Gloves were worn at all times.

2.9 Alternative Silver Stain for 1-D P.A.G.E.

This method involves a kit supplied by Bio-Rad and is based on the work of Gottlieb and Chavko[136]. Whilst not as sensitive as the previous silver staining system, this protocol has fewer steps, is more rapid (taking around an hour and a half) and is still reported to be fifty times more sensitive than normal Coomassie Blue R-250 stain.

One dimensional gels were run as previously described above. After the run the gels were placed in a glass container that had been thoroughly cleaned with nitric acid to remove laboratory detergent and then rinsed in distilled water. The glass container was placed on a Janke and Kunkel KS 501 D flat bed rotary shaker because all steps need gentle agitation of the solutions. 400ml of Fixative solution (200ml methanol, 40ml acetic acid, 40ml Fixative enhancer, 120ml distilled water) was added. This was left for 20 minutes before the fixative was poured off and replaced with 400ml of distilled water to rinse the gels. The gels were washed for two ten minute periods with fresh distilled water being used each time.

After washing, the gels were immersed in 100ml Staining/Developing solution (35ml distilled water, 5ml Silver complex solution, 5ml Reduction moderator solution, 5ml Image development reagent and 50ml Development accelerator solution). This solution was made within 5 minutes of use with constituents added in the order written above and with constant mixing. Image development reagent was added under a fume hood.

Gels were developed for about 20 minutes or until the bands could be distinguished without excess background staining. The process was stopped by the addition of 400ml of a 5% solution of acetic acid for 15 minutes. Gels were then washed in 400ml of distilled water for 5 minutes. Scans were made of the stained gels for permanent records. Gloves and safety glasses were worn at all stages of the staining procedure.

2.10 Semi-dry Western Blotting of Gels

Western blotting involves the transfer of proteins from gels to membranes under the influence of an electrical current. This allows proteins to be either visualised with antibody/ immunostaining or to be sequenced via Edman degradation reactions. Most often the membrane used is nitrocellulose, but this is prone to tear under stress. An alternative is PVDF (polvinylidene difluoride) which is mechanically stronger whilst still performing the same job.

The following blotting technique employs a Kem-En-Tec horizontal Semi Dry Blotter 2. This method is adapted from that supplied with the blotter and employs the discontinuous buffer system described by Khyse-Andersen[137].

The plates of the blotter were wetted with distilled water. Twelve Whatman Filter paper disks of 125mm diameter were soaked slowly in Anode solution 1 (36.3g Tris, 200ml methanol made up to 1000ml with distilled water) to avoid air bubbles. These were placed on the lower electrode with a rolling motion to minimise air bubbles between the papers and the electrode. A further three filter papers were soaked in Anode solution 2 (3.03g Tris, 200ml methanol made to 1000ml with distilled water) and these were added to the stack with the same action. Any bubbles were removed by rolling a pasteur pipette over the surface.

A piece of PVDF membrane cut to approximately the same size as the gel was wetted with methanol and placed on top of the filter papers. The slab gel to be blotted was then rolled onto the membrane and air bubbles were extruded by wetting the surface of the gel with a few drops of buffer and manipulating with gloved fingers.

Fifteen filter papers were now soaked in Cathode solution (5.2g 6-amino-n-hexanoic acid, 200ml methanol made to 1000ml with distilled water) and stacked on top of the gel. The upper part of the apparatus was used to cover the stack and pressure was applied to squeeze everything together. The power was then turned on and the apparatus left for 2 to 3 hours

After blotting was complete the gel was removed and Coomassie stained to check how successful the transfer had been. At the same time the membrane was removed and placed in a petri dish. The membrane was subsequently blocked overnight in a blocking solution that contained 40g MARVEL powdered milk in 1000ml Tris Buffered Saline (12.1g Tris, 29.22g NaCl, 0.75g Glycine dissolved in 800ml distilled water whose pH was adjusted to 8.0 before the solution was made up to 1000ml) before being immunostained.

2.11 Immunostaining of Western Blots

For this very sensitive system of detection, antibodies specifically directed towards a protein are used. First, a primary antibody that will bind to the protein of interest is applied to the membrane the proteins have been transferred to. For example this may be goat anti-human albumin. This antibody will bind only to human albumin and its breakdown products. Unbound antibody is washed away.

Next a secondary antibody is added. This antibody will be raised to bind to the primary antibody (for example it could be rabbit anti-goat) and also has some method of 'reporting' or identifying its own presence. Examples of these conjugated indicators include Alkaline Phosphatase (AP), Biotin, Horseradish Peroxidase (HRP) and even gold. When the membrane is subsequently washed, any unbound secondary antibody is washed away. A simplified depiction of this process can be seen in Figure 2.3.

Visualisation is achieved by reacting the reporter group in some way to give a colour change. For example, in our method of choice APS reacts with BCIP (5-bromo-4-chloro-3-indolyl phosphate) to give BCI and free phosphate. BCI subsequently reacts with NBT (nitro blue tetrazolium) to give an insoluble blue colour and so the protein can be seen.

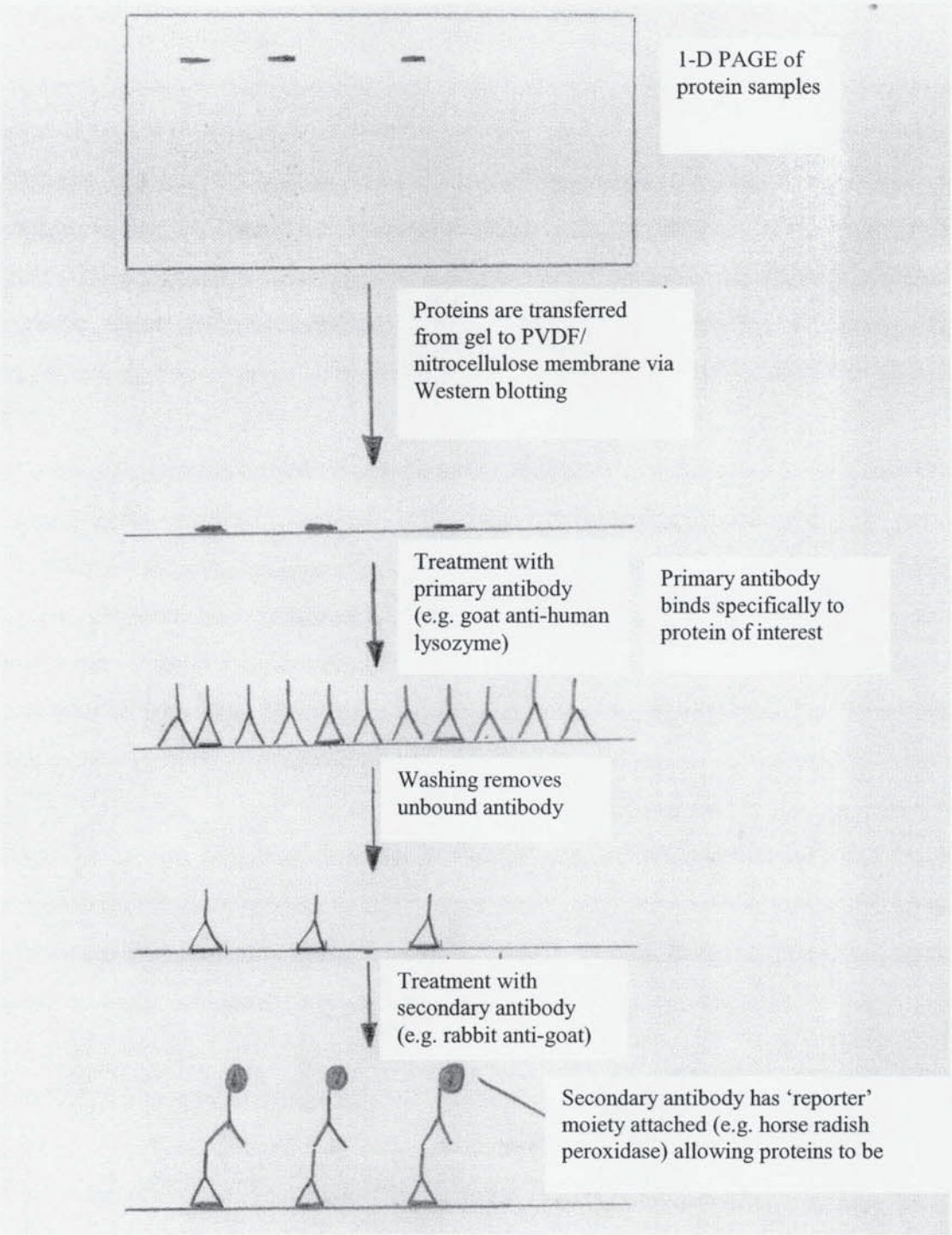


Figure 2.3 Immunostaining of proteins

2.11.1 Method

PVDF membrane that had been previously blocked overnight in MARVEL was rinsed with distilled water. 20ml of primary antibody solution was prepared by mixing 20µl of antibody in 20ml of 4% MARVEL in TBS (Tris buffered saline) and this was poured onto the membrane. After incubation in the primary antibody for 1 hour this solution was decanted and the membrane was washed five times in fresh TBS. Each wash lasted for 5 minutes.

During the washing the secondary antibody solution was made by adding 20µl of secondary antibody in 20ml of 4% MARVEL in TBS. This antibody was conjugated to alkaline phosphatase in order to elicit a colour reaction with the subsequent stain. The secondary incubation was carried out for one hour and this was followed by washing as above.

While the membrane was being washed 20ml of the staining solution was produced by dissolving two SIGMA FAST BCIP/NBT tablets that had been brought to room temperature in 20ml of distilled water. The solution was vortexed using a Whirlimixer to speed up the dissolving process. This was done no longer than one hour before use so as to obtain the best possible results. The substrate solution was poured into a petri dish and the membrane was laid into it. After around five to ten minutes, bands had developed on the membrane and staining was stopped by rinsing the membrane with distilled water.

The membrane was allowed to dry before being scanned. It was then stored away from light for future reference. The BCIP/NBT solution was poured into a storage container until it could be disposed of correctly.

Chapter 3

Assessment of a Reverse-Phase High Performance Liquid Chromatography System for Analysis of the Main Tear Proteins

3.1 Introduction

As mentioned in Section 1.8, one of the main aims of this thesis was to appraise a number of techniques for the analysis of tear proteins. The first technique to be evaluated for its' ability to provide good quality data regarding the tear proteins was reverse-phase High Performance Liquid Chromatography (HPLC). The particular system assessed involved a TSKgel Super-ODS column and a combination of mobile phases described earlier in Materials and Methods (Section 2.5). The reasons that this particular method was chosen from amongst the many available included:

- relatively short run times which allow a large number of samples to be analysed in a comparatively short period
- the high sensitivity of the method
- previously reported analysis of proteins including lysozyme and albumin in standard solutions at concentrations similar to those reported in tears[133].

Previous work using HPLC techniques for studying tear proteins have concentrated on using columns to fractionate samples via size-exclusion followed by a second step, such as ELISA or electrophoresis, for identification and quantification[40, 41]. One problem related in these reports was that of separating lactoferrin and albumin which can cause inaccurate results[40, 114]. The molecular weights of lactoferrin and albumin are relatively close (82kDA and 68kDa respectively) and this means that with certain methods the peaks that represent these proteins are too close together to separate. In addition the need for a second step makes the technique both more complicated and time consuming.

The aim of this study was to determine the methods' ability to provide a reproducible pattern of peaks that represented all the main proteins found in tears (addressing the problem highlighted above) and to allow a comparison of the patterns between 'normals', lens wearers and those individuals suffering from eye/tear film disorders. Production of such a pattern would be invaluable in diagnosis of lens wear associated problems and could allow early diagnosis or enable the cause of the problem to be determined.

3.2 Method (see section 2.5)

Standard protein solutions were produced which represented concentrations of ten times the reported physiological values of the proteins as found in tears. These solutions were lactoferrin (19.60mg/ml), lysozyme (18.15mg/ml), albumin (14.87mg/ml) and IgG (1.54mg/ml). Ten times physiological concentration solutions were used to assess the sensitivity of the apparatus (i.e. if the equipment showed no traces with ten times physiological, it would clearly not be able to analyse physiological concentrations). Following on from this, 1/10 dilutions of each solution were made and mixtures using the four diluted standards were produced. Each mixture had one protein missing and their use in combination was designed to identify the peaks observed in each trace by a process of elimination.

3.3 Results

Solution	Concentration of Solution (ten times physiological)	Average Retention Time		Standard Deviation	
		Peak 1	Peak 2	Peak 1	Peak 2
A	Lactoferrin 19.60 mg/ml	0.85 min.	1.12 min.	$\pm$0.01	$\pm$0.00
B	Albumin 14.87 mg/ml	0.85 min.	1.52 min.	$\pm$0.00	$\pm$0.00
C	IgG 1.54 mg/ml	0.85 min.	5.61 min.	$\pm$0.00	$\pm$0.04
D	Lysozyme 18.15 mg/ml	0.86 min.	1.97 min.	$\pm$0.01	$\pm$0.02
E	100µl A +100µl B +100µl C +100µl D				

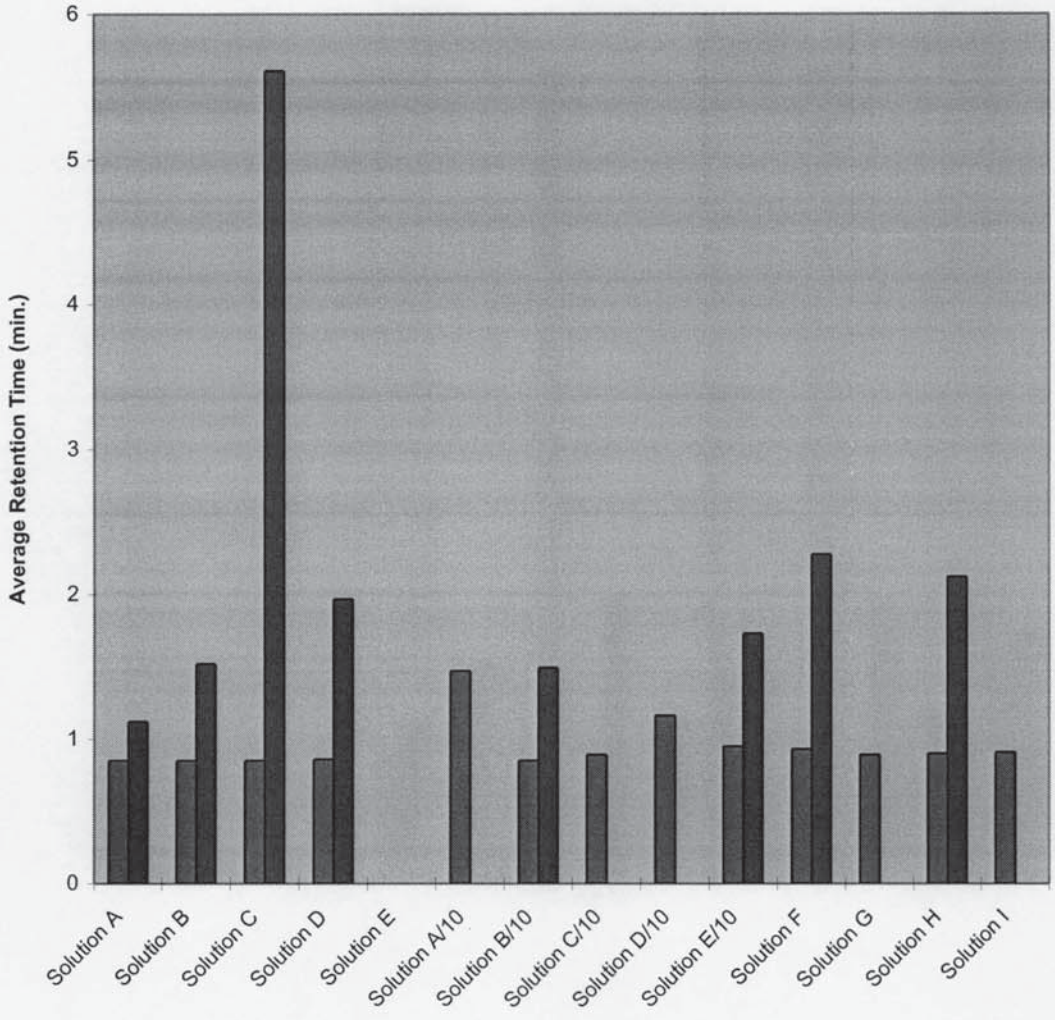
Table 3.1. Average retention times for reverse-phase HPLC of protein standards. It is clear that the peak at 0.85 minutes must be a result of either the mobile phase or the liquid the protein solutions were made with as it is seen in every result. It would appear that it is only the peaks called 'Peak 2' that are related to the individual proteins.

Solution E was only ever run as a 1/10 dilution. It is reported in the above table solely to indicate its composition. The results of running the dilution of solution E can be seen in Table 3.2.

SOLUTION	Content of Solution	Average Retention Time		Standard Deviation	
		Peak 1	Peak 2	Peak 1	Peak 2
A/10	1/10 dilution of solution A	1.47 min.		±0.60	
B/10	1/10 dilution of solution B	0.85 min.	1.49 min *	±0.85	± 1.49
C/10	1/10 dilution of solution C	0.89 min.		±0.89	
D/10	1/10 dilution of solution D	1.16 min.		±0.03	
E/10	1/10 dilution of solution E	0.93 min.	1.73 min.	±0.25	±0.53
F	100µl A/10 + 100µl B/10 + 100µl C/10 + 100µl distilled water	0.93 min.	2.28min.	±0.87	±0.41
G	100µl A/10 + 100µl B/10 + 100µl D/10 + 100µl distilled water	0.89 min.	2.13 min.	±0.06	±0.57
H	100µl A/10 + 100µl C/10 + 100µl D/10 + 100µl distilled water	0.90 min.		±0.04	
I	100µl B/10 + 100µl C/10 + 100µl D/10 + 100µl distilled water	0.91 min.		±0.08	

In the table * = a peak only seen once in the five traces produced for the Albumin sample.

Table 3.2 Average retention times for reverse-phase HPLC of diluted protein standards and mixtures of these diluted solutions. The large degree of deviation in the results along with the appearance of some peaks in only one out of five runs means that no confidence can be placed in these results.



Blue = Peak 1 Dark Red = Peak 2

Figure 3.1 Graph showing average retention times for various protein solutions with reverse-phase HPLC. The position of peak one at around 0.9 minutes in the majority of samples again hints at an artefact of the mobile phase or liquid used to produce the solutions. No error bars or standard deviations are shown as they would often cover the entirety of each bar on the chart. It is evident that no faith can be placed in these results and that is why the decision was made not to continue with this technique.

3.4 Discussion

The above results clearly show that the technique is unsuitable for its proposed role. Initial use of stock solutions containing ten times the physiological concentration of the proteins of interest appeared to demonstrate a degree of reproducibility. However, one peak was seen at around 0.85 minutes in every trace no matter the identity of the protein analysed (Table 3.1). This was considered most likely to be a solvent peak or an artifact of the HPLC grade water that the proteins were dissolved in. The problem with such a peak is that it might mask other traces that actually represented the proteins being studied. In addition to this, it took several washes to clean the column between samples and establish a flat baseline. This suggested that the column was being overloaded and extended the time taken to analyse a sample by several degrees.

At this point it was decided to dilute the samples down to physiological levels to answer the overloading dilemma and hopefully to obtain a clearer picture of whether the apparatus was sensitive enough to analyse simple, unpooled tear samples. After dilution it was observed that two of the proteins (albumin and lactoferrin) seemed to have very similar retention times, as had been reported in previous studies, and the overall lack of reproducibility appeared untenable (Table 3.2).

Mixtures of these diluted samples were also made, with each mixture having one particular protein omitted and replaced with HPLC grade water. This was done to establish if the apparatus could indicate which protein had been left out of a particular mixture, an essential feature if the technique was to succeed in identifying tear protein abnormalities. Results obtained from this study showed even less reproducibility than the simple dilutions of the protein standards. The majority of traces only had one peak, rather than the desired and expected three, and the same peak observed as an artifact with the earlier, undiluted solutions was also present (Table 3.2). The data that was gathered indicated that the technique was not suitable for the proposed application as it could not provide reproducible data regarding mixtures of these important proteins.

Having determined that the system should be sensitive enough to detect physiological concentrations of protein, it was decided that it would be useful to analyse a tear sample in spite of the poor results obtained with the standard solutions. Fresh tears were filtered through a 0.45μm Whatman™ filter disk to remove debris before being loaded onto the column. The result of loading the tear sample was that the column became blocked and was unable to process the tears. This may have been due to residual debris in the tears or due to the physical properties of the tears themselves. Whatever the reason, it was determined that the technique was not worthy of further time investment and the focus of the work shifted to the use of electrophoretic techniques.

Chapter 4

Analysis of Tear Protein Patterns Using Two Dimensional Polyacrylamide Gel Electrophoresis

4.1 Introduction

This chapter outlines the results of an appraisal of the technique of two dimensional polyacrylamide gel electrophoresis (2D-PAGE) for tear analysis. Using the mini system developed by Hoeffer, which comprises the use of tube gels as a basis for separation in the first dimension and 10cm x 8cm slab gels in the second, it was hoped that a clearer picture of the tear protein profile could be established. Advantages of the system included the small volume of samples required (less than 5µl) and the ability to separate proteins according to both their molecular weight and their isoelectric point. The use of a second dimension eliminates problems that arise due to proteins having similar molecular weights which are common to other methods such as one dimensional PAGE and size-exclusion HPLC.

Previous work done using the technique of 2-D PAGE has often involved the pooling of tear samples[26] or the collection of stimulated tears in order to obtain sufficient sample volume for the system used[126, 127]. More recently the development of mini gel systems has allowed researchers to use smaller volumes of unstimulated tears, collected from individual patients meaning that direct comparison between individuals has become possible[121]. Applications of this technique in screening of tear samples of people suffering from various disorders have been discussed[121], but to date such studies remain unpublished. The potential of the technique in studying contact lens wearers for any change in tear protein profile remains unexplored.

The aim of this study was to establish a two dimensional map of the tear proteins of healthy individuals and to assess the possibility of applying the technique (and the 'normal' map) to the analysis of the tears of contact lens wearers and patients suffering from a variety of disorders such as dry eye, keratitis and meibomian gland dysfunction. In addition, a comparison between the tube gels with added ampholytes employed in this study (Materials and Methods) with the results reported for immobilised pH gradient (IPG) strips used in the majority of other studies was made.

Although this technique ultimately gave no results of value, this chapter has been included to show that this particular method has not been overlooked and was indeed investigated as to its applicability to our research.

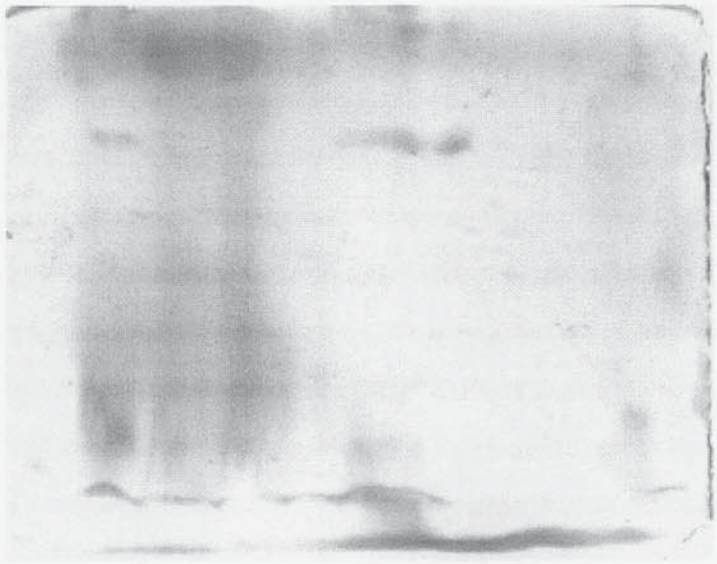
4.2 Method

(see Section 2.6)

During this study two different gel recipes were assessed for molecular weight separation, one with 10% cross-linker and the other with 15%. This was done to see which provided the best possible end result. In addition a number of carbamylate standards were used. These are designed to give a row of spots of identical molecular weight in a straight line across the gel, indicating the pH range. A range of carbamylates were used to see which one interfered the least with the proteins of interest. The type of gel and the particular standard used are indicated in the title of each gel scan. In each case the acidic end of the gel is to the right.

4.3 Coomassie Blue Staining of 2-D Gels

Figure 4.1 Scan of a typical 15% gel loaded with tears collected from the left eye of Dr Val Franklin and creatine phosphokinase carbamylate. The dark line roughly one third of the way down the gel represents the carbamylate standard. The standards should be seen as a series of spots, but they have not fully resolved. No reproducible spots that could be conclusively associated with tear proteins are present.

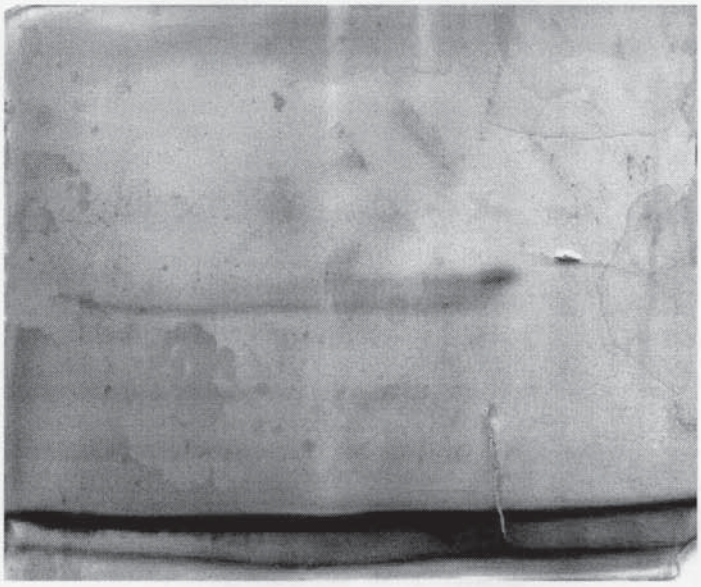


9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5

pH range

Figure 4.2 A typical 15% gel loaded with tears collected from the right eye of Val Franklin and creatine phosphokinase carbamylate. The standard carbamylate sample has again not fully separated and is seen as a line around one third of the way down the gel. The reason for the lack of separation is not known. There are no tear sample protein spots visible.

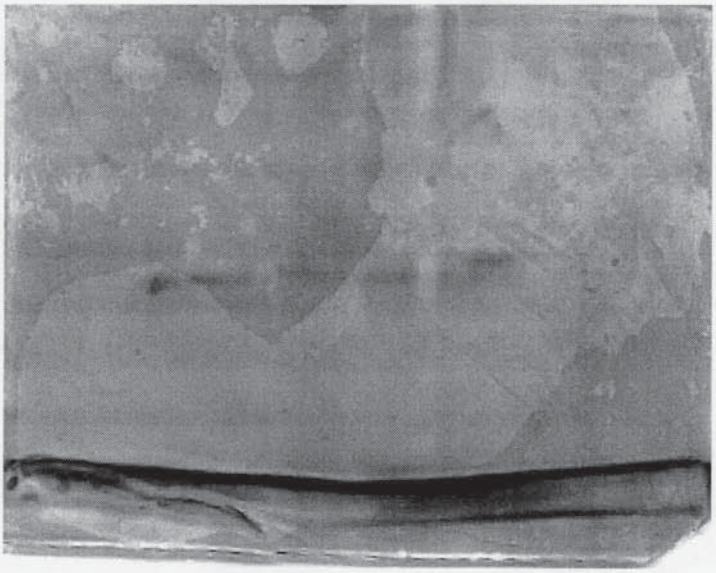
The above gels were loaded with fresh tears obtained from a subject using a glass microcapillary that were then treated with appropriate buffers (see sections 2.3 and 2.5). In addition they were loaded with creatine phosphokinase standards which possess a molecular weight of 40 kDa and should separate over a pH range of 4.9-7.1 to give around thirty individual spots. As can be seen, full separation of these spots did not occur, but a straight line occurred across the gels which can be assumed to represent the carbamylate standards. Whilst some indistinct spots can be seen, these were not reproducible and were considered to be artefacts due either to the staining system or possibly to failures in cleaning components of the apparatus.



9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5

pH range

Figure 4.3 Typical scan of a 10% gel loaded with left eye tears of Val and creatine phosphokinase carbamylate. The carbamylate standard is now around half way down the gel due to the use of the 10% gel rather than the earlier 15% gels. Still no other protein spots can be seen. This lead to the conclusion that a more sensitive stain than Coomassie Blue was needed.



9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5

pH range

Figure 4.4 Typical scan of a 10% gel loaded with right eye tears of Val and creatine phosphokinase carbamylate. Once again the only proteins seen are the standard carbamylates. These are still not satisfactorily separated.

The gels shown in figures 4.3 and 4.4 indicate the effect of using 10% gels under otherwise identical conditions to those outlined above. Lines representing the same carbamylate standard were significantly closer to the bottom of the gel. This shows that these gels retard protein movement to a lesser degree than the 15% gels (as expected due to their lower amount of cross-linkage).

No spots other than the carbamylate standard can be clearly distinguished, an indication that a more sensitive stain may be required. In a further refinement due to the results obtained with the two gel recipes investigated it was decided to use only the 10% gels for further work.

4.4 Analysis of Standard Protein Solutions

As mentioned above, it appeared that the Coomassie Blue staining system was not sensitive enough to detect tear proteins in these 2-D analyses. It was thus decided to utilise the silver staining method of Heukeshoven and Dernick outlined in section 2.7. This stain is reported to be up to one hundred times more sensitive than the Coomassie Blue method represented in the previous section[135]. At this point it was decided that the sensitivity of the silver stain system should be checked to establish if it would be sensitive enough for tear analysis.

The method used to test sensitivity involved treated samples of standard solutions of the main tear proteins. Solutions were made that represented both physiological and ten times physiological concentrations. Each protein should resolve either as a single spot, an extended smear or a series of spots of equal molecular weight depending on the existence of isoforms possessing different pIs. In addition to checking sensitivity, it was hoped that running standard proteins would allow the spots seen in subsequent gels loaded with tear samples to be conclusively identified.

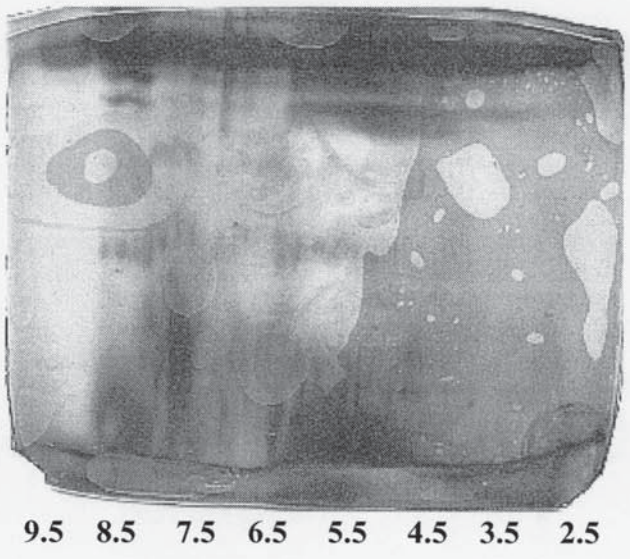


Figure 4.5 Gel loaded with a solution containing ten times physiological concentration of lactoferrin (20 mg/ml). The large amount of background noise seen on this gel made it difficult to associate any spots/features seen with the protein loaded with any degree of confidence. For this reason no analyses were made on this gel.

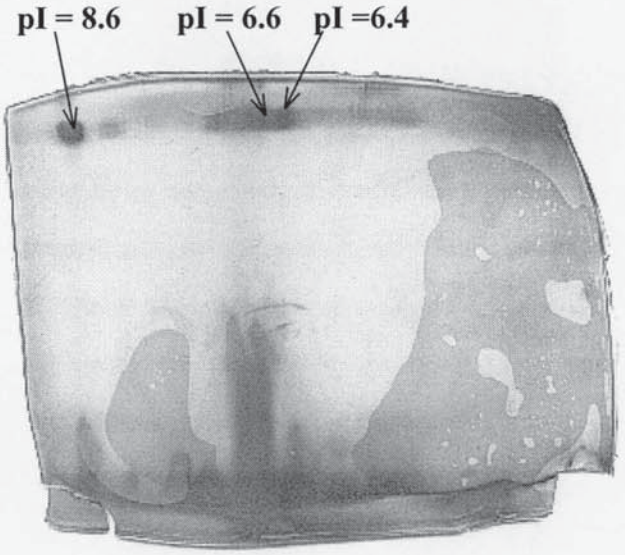
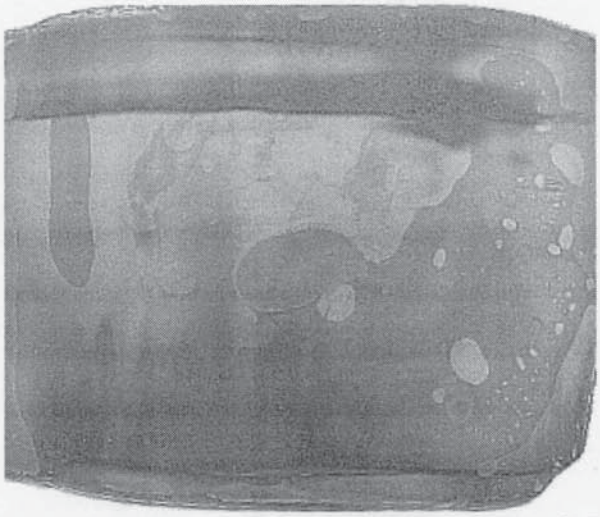


Figure 4.6 Gel loaded with physiological concentration of lactoferrin (2.1mg/ml). The pIs of the clearest spots are indicated on the figure. The shrunken appearance of these gels is a side effect of the staining system which makes the gels very difficult to store and scan. Three clear spots were seen in near identical positions on duplicate runs of these samples. The position of these spots would seem to correspond with lines of dark staining on the ten times physiological gels, but the large amount of background impeded any true correlation.

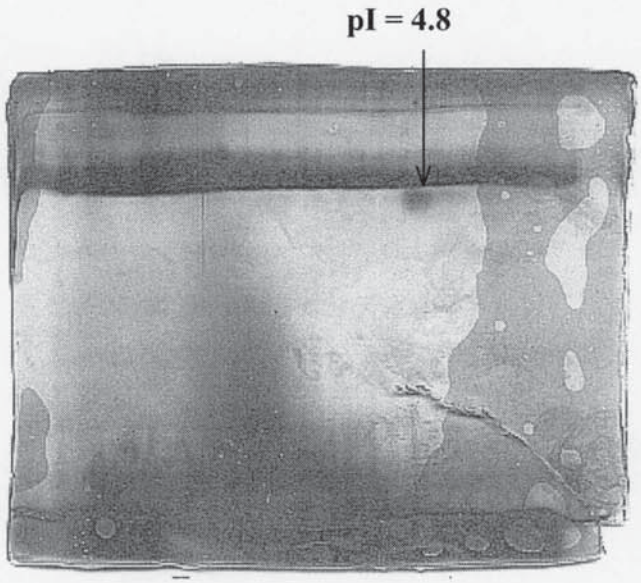
These gels display two distinct features. The first is a line of large spots at the top of the gel. In the more concentrated example, the amount of protein involved has led to the spots merging to form a line of darkly stained protein. The physiological concentration exhibits three clear spots in the same area. The second shared feature is a number of 'peaks' close to the bottom of the gel where it appears that protein spots have been dragged and merged with the background stain. It is unclear whether this second feature is an anomaly or if it truly represents lactoferrin in some way.



9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5

pH range

Figure 4.7 Gel loaded with ten times physiological concentration of albumin (13mg/ml). As with the other ten times physiological samples there is a large amount of background that masks the protein spots. The one spot that might be associated with albumin is stained so darkly that it has partially merged with the dark line. It is not possible to accurately determine a value for isoelectric point as a result.



9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5

pH range

Figure 4.8 Gel loaded with a solution containing physiological concentration of albumin (1.3mg/ml). The isolectric point (pI) of the distinct, fully resolved spot is clearly marked. The spot indicated was seen on duplicate runs and could be partially seen on ten times physiological run.

As with the lactoferrin examples, there are several interesting features in these gels. The repeated appearance of a peak or peaks at the bottom of the gels would seem to indicate that this is in fact an anomaly resulting from the staining technique or some other source and not indicative of any protein. In addition, the apparatus seems to be overwhelmed by high protein concentration, with both solutions of ten times physiological concentration demonstrating intense bands of staining at their upper extremes. In the case of albumin this intense staining conceals a clear, well-defined spot that may represent albumin which can be easily visualised in Figure 4.8.

The nature of the 'peaks' observed at the bottom of these gels is further clarified by its existence on those that were run with lysozyme standards (Figures 4.9 and 4.10, below). The presence of this feature on all the gels which were loaded with solutions of pure protein means that they can not represent a particular protein and so must be attributable to the stain.

The lysozyme gels also highlight the problem of overloading and seem to prove that this technique should be perfectly capable of demonstrating the human tear proteins at unconcentrated/physiological levels. Lysozyme seems to give a clear spot at physiological concentration, with the overloading associated with greater concentration leading to several spots of similar pI but apparently varying molecular weight. These spots can be considered to be 'ghosts' of the actual protein (i.e. protein which has not fully resolved and then stained to give a false positive).

Overall the results obtained from these 2-D runs of standard protein solutions are helpful in two ways. The main benefit is in establishing that the staining system should be more than sensitive enough to measure tear proteins at unconcentrated levels and that certain problems might be resolved by actually diluting the tear samples loaded. In addition, the rough position of these proteins can be visualised and this should allow the nature of spots seen in the running of tears to be broadly identified. The main source of worry is the distance moved by lysozyme on these gels. With a molecular weight of just 14.6kDa compared to albumin at 68kDa and lactoferrin at 82kDa, lysozyme should be seen very close to the bottom of the gel. The intense background could mask this presence. It is uncertain what the spots represent.

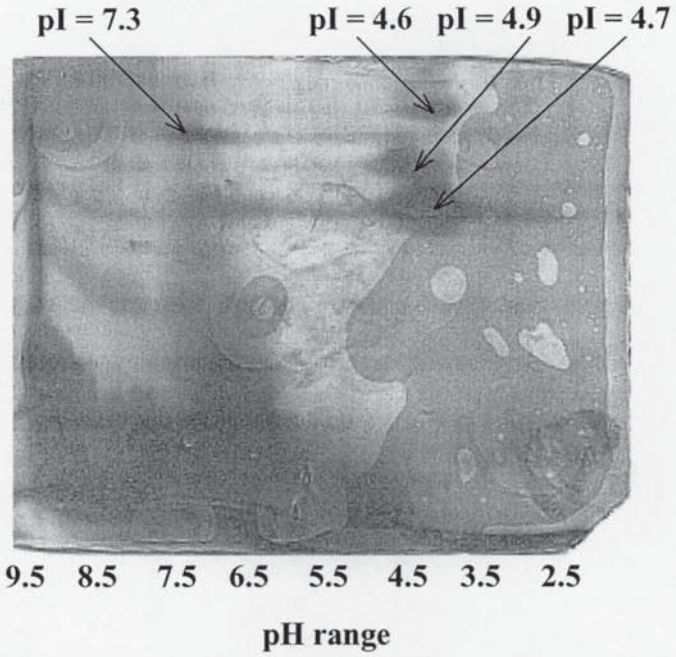


Figure 4.9 Gel loaded with ten times physiological concentration of lysozyme (18.5mg/ml). pI values for the various spots are indicated. Of all the ten times physiological samples, only lysozyme gave levels of background staining low enough to determine individual spots. The actual identity of these spots is unclear.

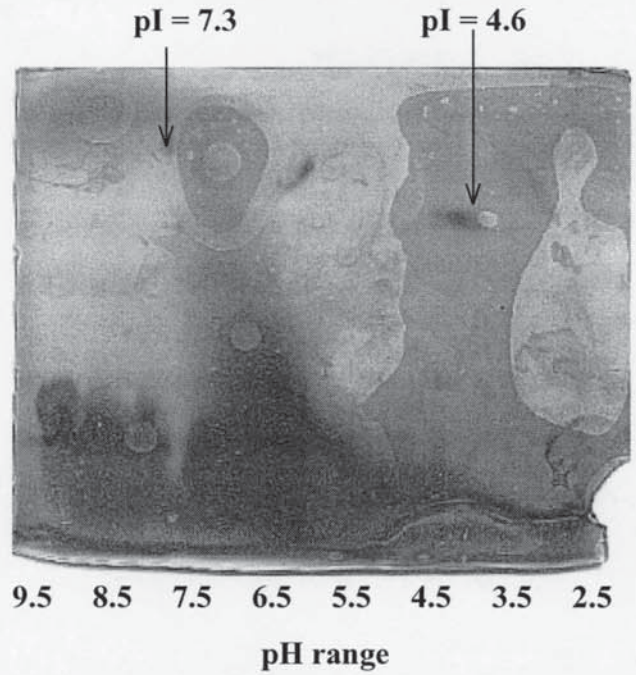
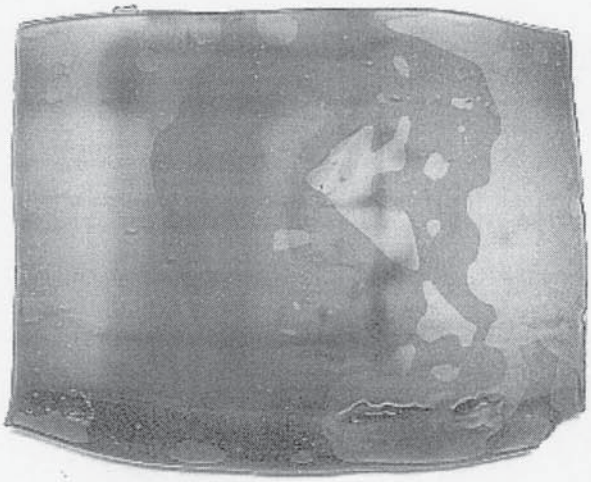


Figure 4.10 Gel loaded with a solution containing physiological concentration of lysozyme (1.85mg/ml). pI values are shown. The nature of the spots is clearer in the lower concentration protein standard. The three spots seen with pIs around 4.8 have resolved to just one of 4.6. This suggests that the three spots seen on the ten times physiological gels were 'echoes' of the same protein spot.

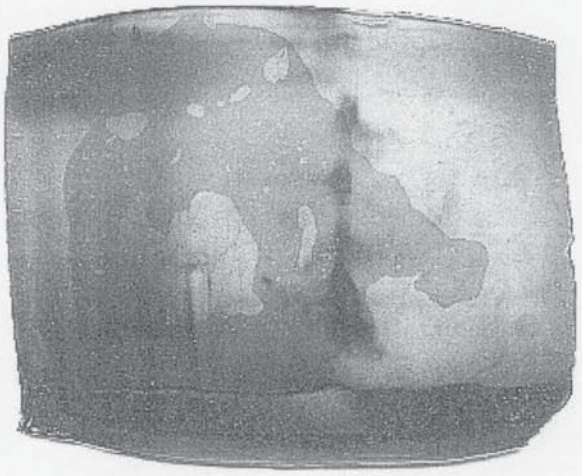
4.5 Silver Staining of 2-D Gels Loaded with Pooled Tears



9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5

pH range

Figure 4.11 Silver stained gel loaded with pooled left eye and right eye tears from one individual. A large number of spots can be seen, but they all appear to have a similar pI range of 5.0 to 5.8. The number of spots sharing the same pI value makes it difficult to attach values to the individual spots on the gel.



9.5 8.5 7.5 6.5 5.5 4.5 3.5 2.5

pH range

Figure 4.12 Silver stained gel loaded with pooled left eye and right eye tears from one individual. This gel shares a common set of first and second dimension conditions to figure 4.11. The reproducibility of the result is clearly seen with the protein spots all being present around the same pI of 5.0.

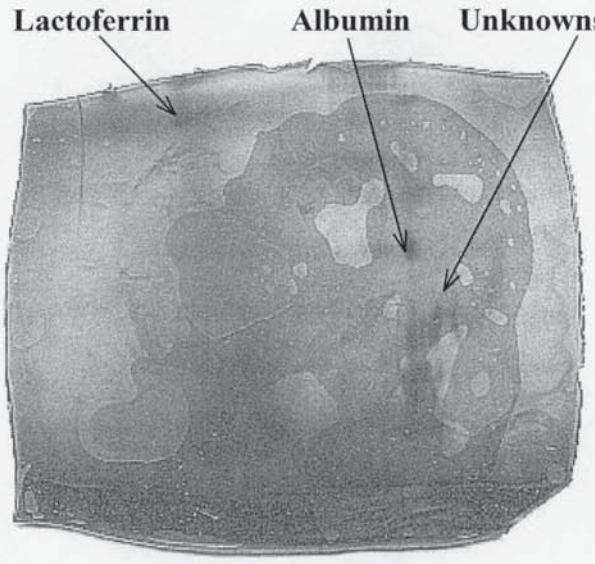


Figure 4.13 Silver stained gel loaded with pooled tears as above. The possible nature of some of the spots is as indicated. This identification is based on the results seen earlier with standard protein solutions. The other spots remain unidentified due to a lack of knowledge available in the current literature.

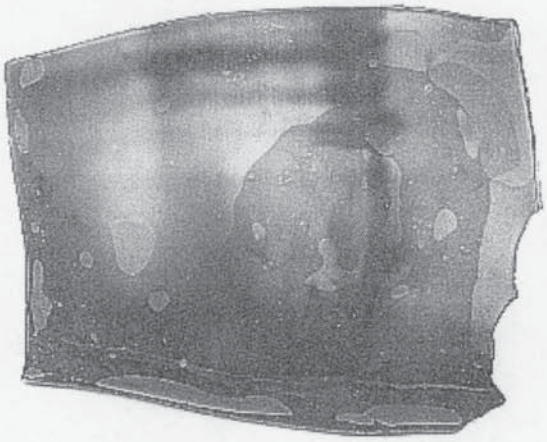


Figure 4.14 A combination of tears and creatine phosphokinase carbamylate. The carbamylates were loaded to assess their impact on the results obtained. No pI values are shown due to the inability to distinguish individual spots. The loading of the carbamylates has lead to large amounts of background that have obscured the tear protein spots. At this point it was decided that the benefits provided by the carbamylate standards were vastly outweighed by the loss of data.

The four gels depicted above (Figures 4.11-4.14) represent first dimension tube gels that were run at the same time and were thus loaded with aliquots of the same pooled tear samples that had been treated under identical sample preparation conditions. Figures 4.11 and 4.12 are a matched pair of tube gels that were subsequently run in the second dimension at the same time, and thus under as close to identical conditions as possible in the two dimensional separation. As a result of this they show a very similar pattern of staining. Both gels are reproduced here to indicate the level of reproducibility that can be achieved.

The gel in figure 4.14 was additionally loaded with a standard carbamylate to see if this interfered with the tear proteins in any way. The results indicate that the carbamylate standard lead to overloading effects similar to those seen with ten times physiological protein standards. At this point it was decided to stop loading these carbamylate standards as the potential benefits supplied by these standards was far outweighed by the problems associated with overloading and masking of the proteins of interest.

In comparison to the Coomassie Blue stained gels, a number of spots can be seen on these gels (e.g. Figures 4.6, 4.8 and 4.9) which can be assumed to represent the tear proteins. The fact that spots were seen in similar positions on each of these gels indicates a degree of reproducibility of this technique. As already mentioned, addition of a carbamylate standard appears to mask some of the spots seen and some are also hidden by the dark staining of the bottom of the gels. The cause of this darkly stained background could not be discerned, but is similar to that seen in the running of protein standards and can be assumed to be a feature of the staining system employed.

Whilst conclusive identification of the tear proteins cannot be made at this stage, comparison with the aforementioned runs of standard proteins does allow a degree of elucidation. Where considered applicable, the likely nature of a particular spot as a result of homology to a standard protein pattern is indicated on the scans. In spite of this a number of protein spots remain 'unknowns' and categorical analysis of the proteins making up the normal protein pattern cannot be achieved without further steps such as immunostaining or sequencing.

4.6 Narrow-band Isoelectric Focusing

In all the results shown so far the pH gradient used in the first dimension separation was derived from a broad-band ampholyte solution which gives a pH range from 2.5 to 9.5. As can be seen, this results in the majority of protein spots observed being very close together and displaced towards the right of the gel in one small area with the rest of the gel being mostly free and clear. This is the acidic end of the range.

Ampholytes can also be produced that form narrower pH ranges thus giving greater separation over the same distance. It was therefore decided to run first dimension tube gels which covered shorter pH ranges. Due to the fact that the main spots seen to this point were at the right-hand (acidic) side of the gel an ampholyte solution covering the range of 2.5-5.5 was chosen. Again, all the gels represented here are displayed with their acidic ends on the right, all gels were 10% cross-linked and all samples were treated as outlined in Chapter 2. Pooled tears from one individual (i.e. left eye tears + right eye tears) were used in each case.

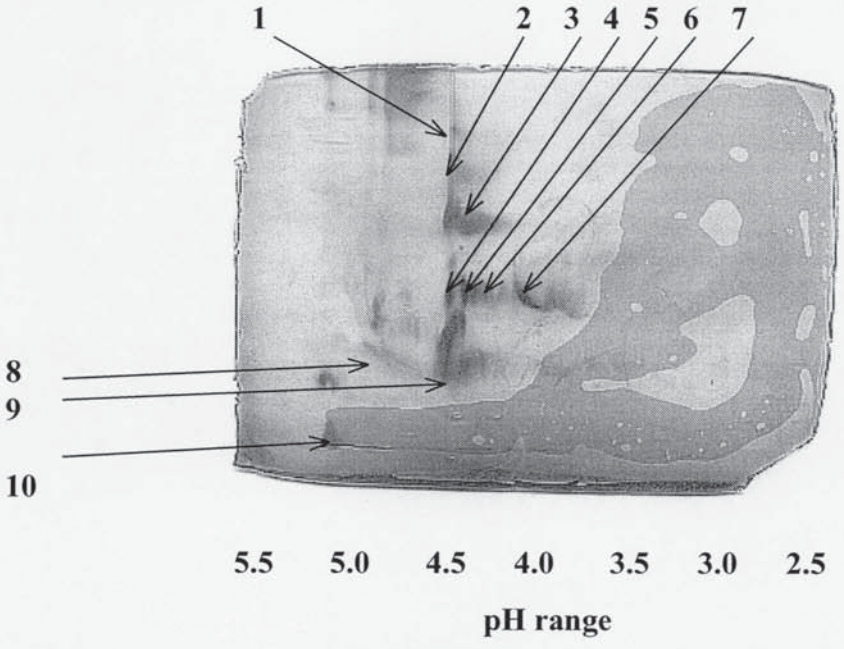


Figure 4.15 Typical gel representing a narrow-band first dimension (pH 2.5-5.5) loaded with pooled tears. The use of a smaller range of pHs over the same distance has resulted in a much better separation of the spots seen earlier in figures 4.11 and 4.12. This means that pI values can now be easily attributed to individual spots. These pI values can be found in Table 4.1 where they are linked to the identity of the proteins.

In the gel depicted in Figure 4.15, the benefits of the narrow-band pH range can be clearly seen. Spots are nicely separated, and the pIs (isoelectric points) of the individual spots are easy to measure. Calculating the pI is achieved by measuring the position of the protein relative to the pH gradient. Ampholytes deliver a linear pH gradient which in this case is from 5.5 at the left-hand side of the gel to 2.5 on the right. The measured isoelectric points are indicated on the figure, and the possible identity of the proteins is reported in Table 4.1 below. The identification of these proteins is based on comparison of the pIs and position of the spots with those reported in the work of Molloy[127] and Mii[126]. These groups have identified proteins seen in 2-D maps via protein sequencing.

Spot Number (as Fig. 4.15)	Possible Identity of Protein Spot[126, 127]	Isoelectric Point (pI)
1	IgA	4.5
2	Haptoglobin	4.5
3	Albumin	4.5
4	Tear lipocalin	4.6
5	Tear lipocalin	4.5
6	Tear lipocalin	4.4
7	Tear lipocalin	4.2
8	Processed lipocalin	4.9
9	Processed lipocalin	5.0
10	Unknown	5.1

Table 4.1 pI of proteins separated with narrow-band isoelectric focusing.

4.7 Discussion

Having assessed the utility of the technique of 2-D electrophoresis in tear analysis, a number of areas arose. Foremost amongst these were problems relating to direct comparison of the protein patterns of individual patients. Although a large degree of reproducibility was established after much practice with the equipment, the system could not allow for a number of samples to be run side by side. Each sample loaded on to a particular set of tube gels could safely be considered to have been subjected to the same sample treatment, loading and running conditions in the first dimension. Subsequent second dimension separation could only be carried out on two tube gels per day, leading to differences in storage conditions (from 24 hours to three days in the freezer), running time in the second dimension, staining of the gels and finally time stored before the gels were scanned into a computer. This meant that direct comparison could only be made between tube gels that were run on the same day. Any other collation has to consider all of the many possible variables that could exist between days.

Amongst other problems was the degree of fragility of the tube gels used in the first dimension separation. Upon expression from their glass tubes these gels had a tendency to fold up and stick to themselves. Straightening them often lead to breaks in the gel causing further problems when they subsequently had to be laid onto slab gels for molecular weight separation. In addition, the force needed to express the gels from these tubes often lead to the gels breaking apart into several pieces. Such problems often meant that a truly linear pH gradient was not produced with pieces being placed incorrectly (either back to front or in the wrong order) or indeed such small fragments being produced that they could not be used in the subsequent steps of the process. Misplacement could only be seen once the entire experiment had been run and the final gel was stained and this lead to a lot of wasted time.

Additionally, the equipment was new both to the research group and myself. A great deal of trouble was encountered in getting to know where sources of error could creep in and how to address problems such as gels not setting, samples not loading properly, tube gels fragmenting, etc. Optimising running times and conditions took much repetition as did selecting the best time to leave tube gels in equilibration buffer and time allowed for gels to polymerise.

Even after these variables had all been selected, the staining system used had to be explored. Coomassie Blue proved to be ineffective in visualising tear proteins and the more sensitive silver stain comprised of many time dependent steps all of which could introduce variability between gels. Silver stain was particularly sensitive to overloading and subject to intense non-specific background stain which obscured proteins of low molecular weight. Silver staining was ultimately chosen over Coomassie Blue as it actually meant that some proteins could be seen. The lack of familiarity with the method also meant that it was difficult to assess what was a good result.

Another area, which caused problems, was the inability to state the nature of the spots seen. Isoelectric point is easily measured on 2-D gels due to the linear nature of the pH gradient. Molecular weight determination on the other hand needs the introduction of a ladder of markers. The length of the tube gels in relation to the second dimension slab gels was such that it proved difficult to introduce a well containing molecular weight markers and the loading of the carbamylate standards caused a large number of the proteins spots to be obscured. Exploration of another carbamylate (carbonic anhydrase carbamylate – data not shown) lead to a similar problem.

At best estimation of the identity of the spots could be achieved by reference to work done by other researchers, but even this was inadequate due to the large number of proteins that were left as 'unknowns' in these reports[126, 127]. Positive identification would only be truly possible if a further, time-consuming and complicated step such as protein sequencing was carried out. This was outside the scope of this research.

In conclusion, this method of 2-D electrophoresis is one which addresses a number of the problems experienced when attempting to analyse tear proteins. Amongst these are the need for only relatively small sample volume, separation of proteins of similar molecular weight, the ability to detect very small concentrations of protein and the production of a relatively reproducible pattern (or 'map') of an individuals tear proteins. However there are also many areas where the technique does not fulfill the necessary criteria for the work proposed in this thesis.

The main area where the technique of two-dimensional electrophoresis falls short is in the lack of a method to run tear samples of a number of individuals side by side to ensure exact matches in running conditions. As a result of all the difficulties involved outlined above in combination with the lack of direct comparison it was decided to move on from 2-D electrophoresis and to determine whether 1-D electrophoresis could answer this concern and thus was a more viable option. The evaluation of 1-D electrophoresis is fully covered in Chapter 5.

Chapter 5

Evaluation of the Effect of Several Variables on the Protein Pattern Observed with One Dimensional Polyacrylamide Gel Electrophoresis

5.1 Introduction

Following on from the results of the 2-D PAGE work outlined in the preceding Chapter, it was decided to explore the technique of 1-D PAGE. Although a great deal of work has been done using 1-D PAGE for tear analysis, the evolution of the technique in recent years has meant that its' potential still remains to be fully explored. The majority of previous work done by other researchers was limited by the equipment available at the time, with a requirement for large sample volumes that could only be obtained by pooling of samples or stimulation of lacrimation being chief among the problems encountered[55, 138]. The production of mini systems, such as the one produced by Hoefer and employed in this study, have addressed this problem allowing smaller volumes and thus individual non-stimulated samples to be studied. In addition, the establishment of methods involving gradient gels, new staining systems and advances in sampling techniques mean that 1-D PAGE is a technique that deserves fresh consideration when choosing methods for analysing tear proteins.

The selection of the particular apparatus used was made as a result of it matching a number of criteria. These included the ability to analyse small sample volumes, the option of assessing up to ten samples on one gel (thereby allowing direct comparison of individuals and rapid sample processing), the chance to develop the gels in a number of ways such as direct staining or Western blotting and the flexibility of the system allowing both tear samples and lens extracts to be analysed. In addition, the large number of variables that could be tweaked to give a variety of end products was appealing.

As mentioned earlier, a number of studies exist evaluating 1-D PAGE, but this has been in comparison to other techniques[128, 132, 139] or focusing on parameters such as sample treatment[131] and storage time[130]. The goal of this study was to explore a number of variables within the technique in order to obtain the best possible representation of the tear protein pattern that was both easy to interpret and easily reproducible. These variables included the use of stacking gels, the staining method, the influence of running voltage and the impact of gradient gels.

5.2 Method

(see Sections 2.3, 2.4, 2.7, 2.8 and 2.9)

Each gel scan presented in this chapter is loaded with a combination of molecular weight standards, tear samples, standard protein solutions and lens extracts. Lens extracts were produced following the method outlined in Section 2.4 and all tears were collected as described in Section 2.3. Standard protein solutions were made by dissolving protein in distilled water and whirlimixing to achieve uniform concentration.

All samples were treated with an equal amount of 2X treatment buffer and boiled for 5-10 minutes before loading. Unloaded samples were frozen at -20°C until needed. These storage conditions were followed based on earlier work carried out in our lab that showed that tear samples could be stored at this temperature for extended periods with no major alteration to the tear protein pattern observed[62]. Wherever these conditions were not followed it is indicated in the title of the scan. The natures of the samples loaded on a particular gel are also indicated in the title, as is the nature of the gel recipe and the staining system employed.

5.3 Evaluation of Stacking Gels

The use of stacking gels in 1-D PAGE is common place and acts to tighten the bands before they pass onto the resolving area of the gel. The following gels are 10% to 20% gradient gels that had an additional stacking gel of 4% (see Table 2.3). These particular formulations were chosen according to the guidelines provided for new users in the protocols supplied with the electrophoresis equipment. The stacking gel was removed prior to scanning as no bands were seen in this area and also because of the fragility of the stacking gel.

The name of each extraction sample indicates the duration of lens wear. For example, 1 day is a lens that has been worn for 1 day, removed and then boiled in extraction buffer for three hours (as described in Section 2.4). The lens type involved is indicated where applicable. In all cases the name is shortened to just the time period.

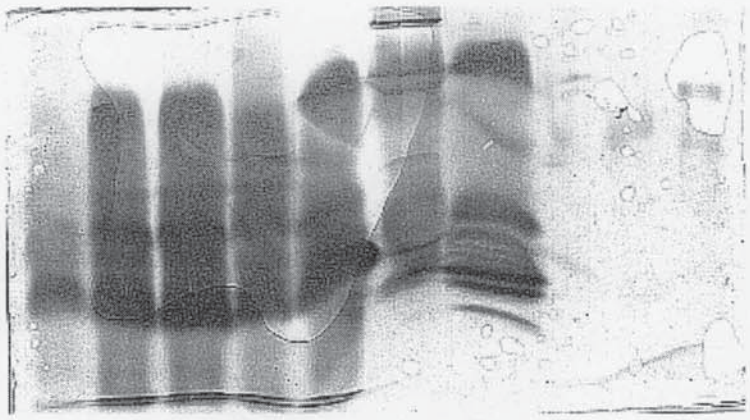


Figure 5.1 Typical scan of a 10-20% gradient gel that has had its' stacking gel removed. Lane 1 = 1 day, Lane 2 = 1 week, Lane 3 = 2 weeks, Lane 4 = 3 weeks, Lane 5 = 4 weeks, Lane 6 = Broad-band molecular weight markers, Lane 7 = Tears, Lane 8 = Kininogen (1.0mg/ml), Lane 9 = Albumin (1.5mg/ml), Lane 10 = Lactoferrin (2.0mg/ml). The gel was run at 40 mA and stained with Coomassie Blue. All lenses that were extracted were Acuvue Monthly Disposable lenses.

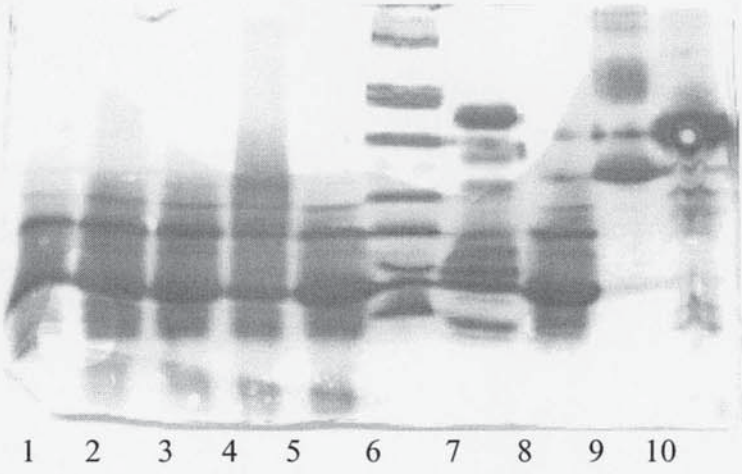


Figure 5.2 Typical scan of gel that was run using a 4% stacking gel and a 10-20% gradient resolving gel. The stacking gel has been removed. Lane 1 = 1 day, Lane 2 = 1 week, Lane 3 = 2 weeks, Lane 4 = 3 weeks, Lane 5 = 4 weeks, Lane 6 = Broad-band molecular weight markers, Lane 7 = Tears, Lane 8 = Extended wear extraction, Lane 9 = Albumin (1.5mg/ml), Lane 10 = Lactoferrin (2.0mg/ml). The gel was run at 40mA and stained with Coomassie blue. The extended wear extract was from a FOCUS monthly disposable lens. All other extracts were produced from Acuvue Monthly Disposable lenses.

After several runs utilising stacking gels it was decided to see what effect dispensing with them would have. The next two gel were stained in the same way as those depicted in Figures 5.1 and 5.2 and were loaded with similar samples. These gels were gradient gels consisting of a gradient from 7.5% to 20%. The gel in Figure 5.3 was also loaded with a sample of the extraction buffer used on worn contact lenses to ensure that this did not contribute to the protein patterns seen with lens extracts.

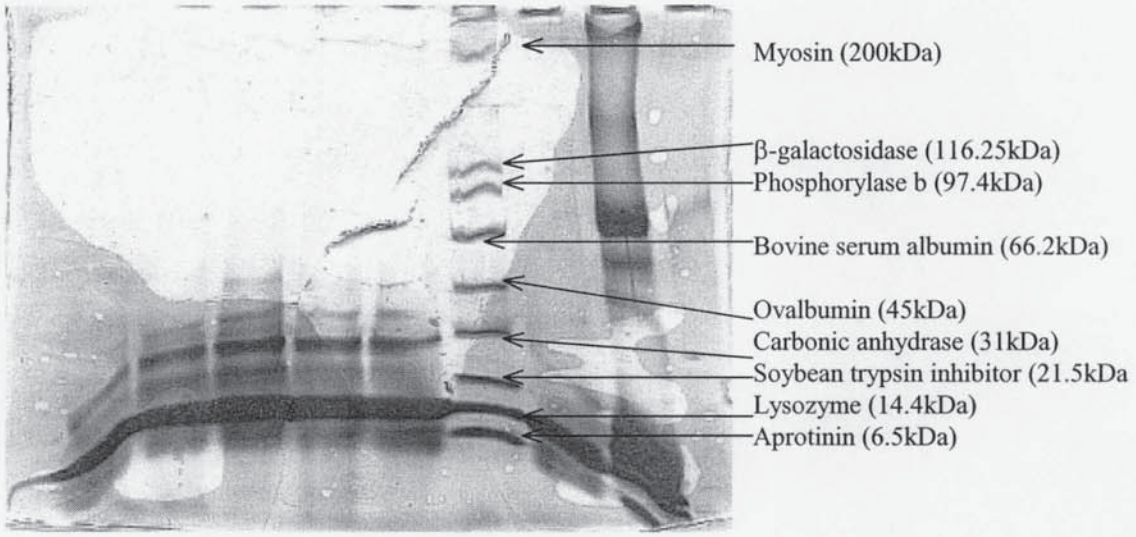


Figure 5.3 7.5-20% gradient gel run at 40mA and Coomassie Blue stained with extreme case of 'smiling'. Lane 1 = Extraction Solution, Lane 2 = 1 day, Lane 3 = 1 week, Lane 4 = 2 weeks, Lane 5 = 3 weeks, Lane 6 = 4 weeks, Lane 7 = Broad-band molecular weight markers, Lane 8 = 1 day disposable extraction, Lane 9 = Tears, Lane 10 = Albumin (1.5mg/ml). Lanes 2-6 contained extracts from Acuvue Monthly Disposable lenses that are worn during the day, removed, cleaned overnight then worn again the next day. After 28-30 days of such wear they are thrown away. The extract in lane 8 termed 1 day disposable extraction was produced from a Ciba Dailies lens. These are normally worn for one day before disposal.

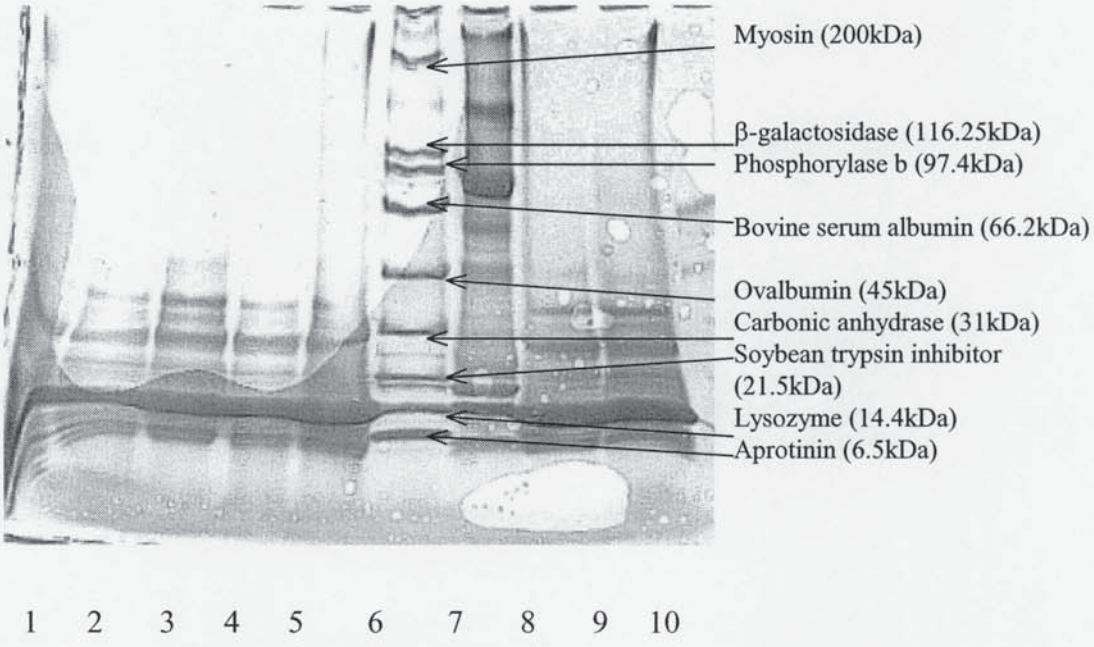


Figure 5.4 Typical example of a 7.5-20% gradient gel run at 40mA and Coomassie Blue stained. Whilst the smile effect is not as bad as that seen in Figure 5.3, it is still clear. Lane 1 = 1 day, Lane 2 = 1 week, Lane 3 = 2 weeks, Lane 4 = 3 weeks, Lane 5 = 4 weeks, Lane 6 = Broad-band molecular weight markers, Lane 7 = Tears, Lane 8 = 1 day disposable lens extraction, Lane 9 = Extended wear extraction, Lane 10 = Albumin (1.5mg/ml). The lens extract in Lane 8 was produced from a Ciba Dailies lens, the extract in Lane 9 was made from a FOCUS monthly disposable lens.

Having observed the results achieved with and without stacking gels, it was decided that from now on no stacking gels would be used. The potential benefits of tightening bands through the stacking gels were far outweighed by the loss of resolving power resulting from losing the top 2cm of a gel whose total possible length available to separate proteins is around 7cm.

5.4 Reducing the 'Smile effect'- An Optimisation of Running Current

Having evaluated stacking gels, the next area to be addressed at this stage was the problem clearly indicated on these gels of a 'smile' pattern being seen in the dye front and the protein bands. This is where the samples resolve in a semi circular pattern, with proteins at the edges running further down the gel than those in the central lanes. An extreme example of this phenomenon can be seen in Figure 5.3. From reference to the troubleshooting guide provided with the equipment it was determined that this well-known problem was a result of overheating of the system. In response to this the current at which the gels were run was reduced from 40mA to 30mA. The direct effect of reducing the current on the gel pattern can be observed in Figures 5.5 and 5.6 (below). Where previously the bands had been resolving in a semi circle, now they were in well ordered straight lines across the gel.

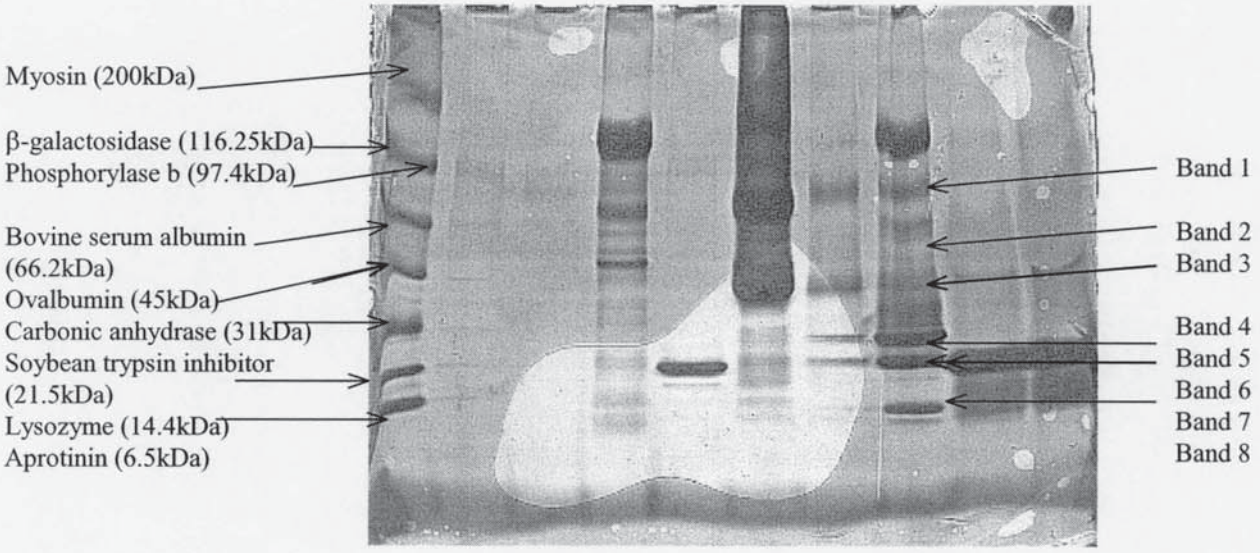


Figure 5.5 7.5-15% gradient gel run at 30 mA to reduce 'smile'. Lane 1 = Broad-band molecular weight markers, Lane 2 = Albumin (1.5mg/ml), Lane 3 = Kininogen (1.0mg/ml), Lane 4 = Lactoferrin (2.0mg/ml), Lane 5 = Lysozyme (1.0mg/ml). Lane 6 = IgG (1.0mg/ml), Lane 7 = IgA (1.0mg/ml), Lane 8 = Tears, Lane 9 = 1 day, Lane 10 = 3 weeks. Gel was stained with Coomassie Blue. The extracts were obtained from Acuvue monthly disposable lenses.

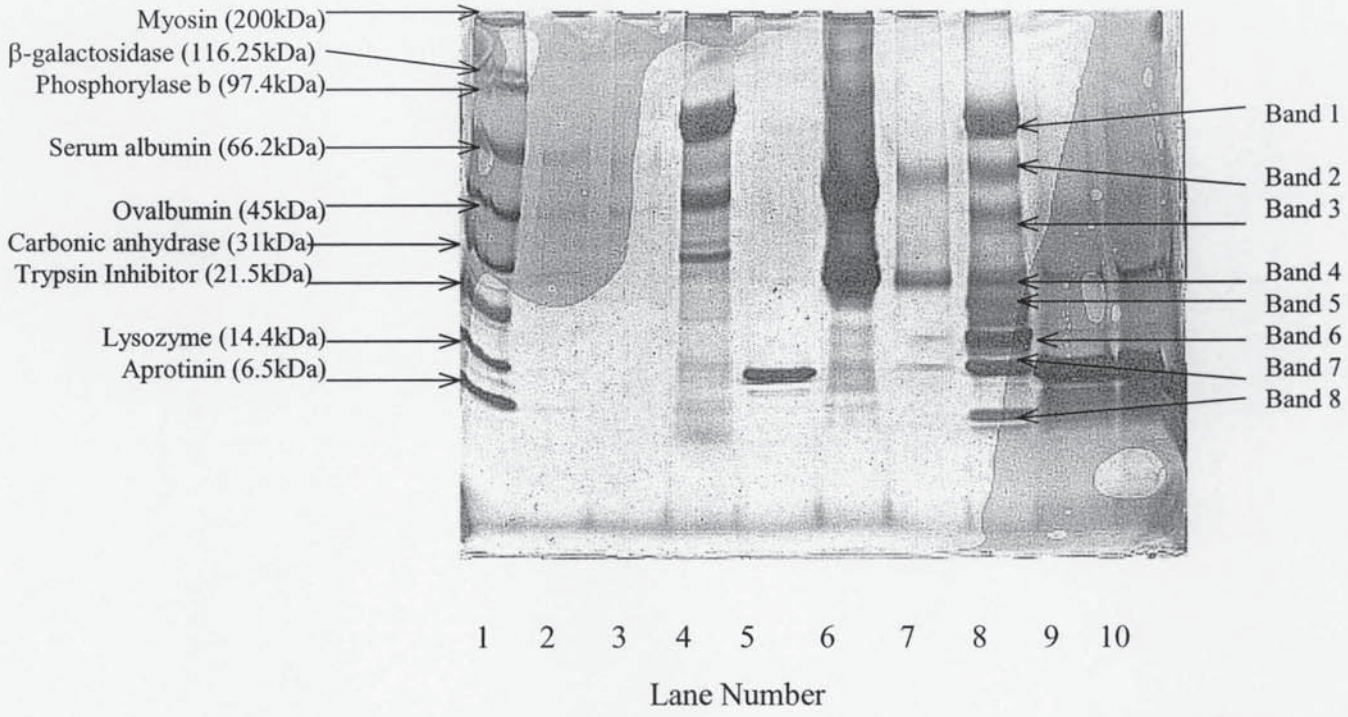


Figure 5.6 7.5-15 % gradient Gel run at 30 mA. This gel was loaded, run and stained under identical conditions to the gel in Figure 5.5. The molecular weight markers are the same as those in Figure 5.5.

Although reducing the current greatly reduced the amount of 'smile' seen when running the gels, some effect was still occurring in the outside lanes (1 and 10). As a result of this these lanes were abandoned for sample loading from this point forward.

5.5 Calculation of the Molecular Weights of the Protein Bands Seen in Figures 5.5 and 5.6

The molecular weight of the unknown bands seen in the tear sample and the extractions depicted in Figures 5.5 and 5.6 were calculated by plotting a graph of log MW (molecular weight) against Rf (relative mobility) for the molecular weight markers. Rf is calculated by dividing the distance moved by the protein by that distance migrated by the dye front. These values give a straight line from which molecular weight can be derived from distance moved by a protein band in the gel.

The calibration curve for calculating molecular weights can be seen in Figure 5.7 and the molecular weights of the bands in Table 5.1. The number of the bands corresponds to their position in the gel i.e. band one is that closest to the top, band two next closest, etc. Where it is unclear as to whether a band is one distinct entity or a blur of several bands it is taken to be one band and the distance traveled measured to the centre of the band.

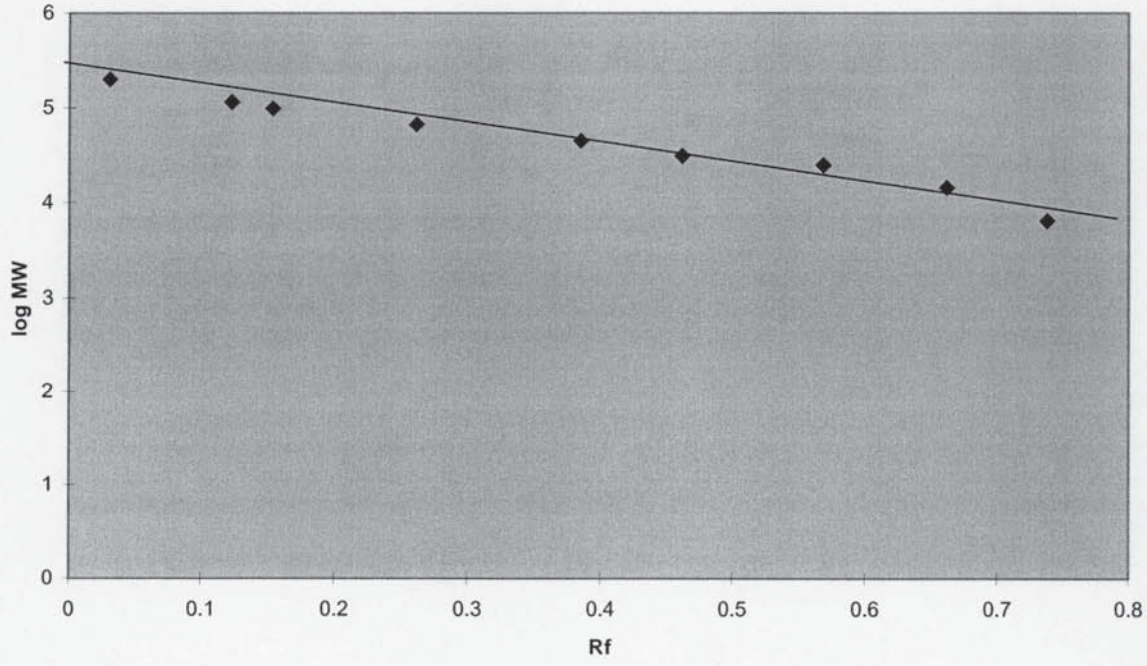


Figure 5.7 Calibration curve showing log MW and Rf for broad-range molecular weight standards

Protein/Band Name	Rf	Log MW	MW (Da)
Myosin	0.031	5.30	200,000
β-galactosidase	0.123	5.06	116,250
Phosphorylase b	0.154	4.99	97,400
Bovine serum albumin	0.262	4.82	66,200
Ovalbumin	0.385	4.65	45,000
Carbonic anhydrase	0.462	4.49	31,000
Soybean trypsin inhibitor	0.569	4.40	21,500
Lysozyme	0.662	4.16	14,400
Aprotinin	0.738	3.81	6,500
Tears. Band 1	0.215	5.00	100,000
Tears. Band 2	0.308	4.80	63,095
Tears. Band 3	0.400	4.60	39,810
Tears. Band 4	0.507	4.40	25,120
Tears. Band 5	0.462	4.25	17,780
Tears. Band 6	0.631	4.15	14,125
Tears. Band 7	0.692	4.05	11,220
Tears. Band 8	0.785	3.90	7,940
1 day extraction. Band 1	0.507	4.40	25,120
1 day extraction. Band 2	0.692	4.05	11,220
1 day extraction. Band 3	0.769	3.90	7,940
3 week extraction. Band 1	0.507	4.40	25,120
3 week extraction. Band 2	0.692	4.05	11,220
3 week extraction. Band 3	0.769	3.90	7,940

Table 5.1 Molecular weight of unknown bands as derived from the calibration curve shown in Figure 5.7. Also indicated are the values used to plot the graph which were obtained from molecular weight markers.

The molecular weights of the marker proteins reported in Table 5.1 are as given in the details supplied by Bio-Rad with their Broad Range Molecular Weight Standards. These values may vary from those found in proteins derived from human tears.

5.6 Influence of Gel Recipe: Simple 7.5% and 12.5% Gels versus Gradient Gels

In the previous sections we established that a running current of 30mA was preferable to 40mA and that the purported benefits derived from the use of a stacking gel were largely outweighed by its detrimental effects. The next variable that was taken into consideration was the difference that could be achieved by running wide-range gradient gels as opposed to simple narrower focusing gels. In order to do this, duplicate runs of samples were undertaken on 7.5-15% gradient gels loaded with broad range markers and on gels whose recipe was reported by the suppliers (Bio-Rad) to be ideal for a particular narrower range marker mixture. These mixtures were 7.5% for high range markers and 12.5% for low range markers (see Table 2.3).

At the same time as assessing the different recipes, the benefits of using the silver staining method of Heukeshoven and Dernick[135] described in section 2.7 was evaluated, with the narrower focus gels being silver stained whilst the broad-range gradient gels were Coomassie Blue stained. Again the molecular weight markers used were designed for the particular stain used.

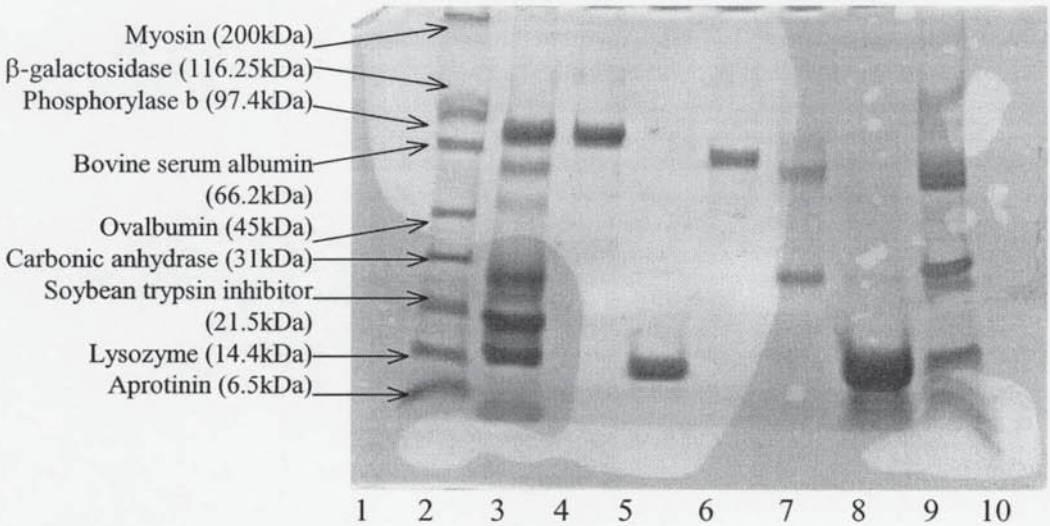


Figure 5.8 Broad range gel consisting of a 7.5-15% gradient. Lane 1 = Empty, Lane 2 = Broad range markers, Lane 3 = Tears, Lane 4 = Lactoferrin (2.0mg/ml), Lane 5 = Lysozyme (1.0mg/ml), Lane 6 = Albumin (1.5mg/ml), Lane 7 = IgA (1.0mg/ml), Lane 8 = Extended wear extract, Lane 9 = Saliva, Lane 10 = Empty. The gel was Coomassie Blue stained. The extract was from a monthly disposable FOCUS lens.

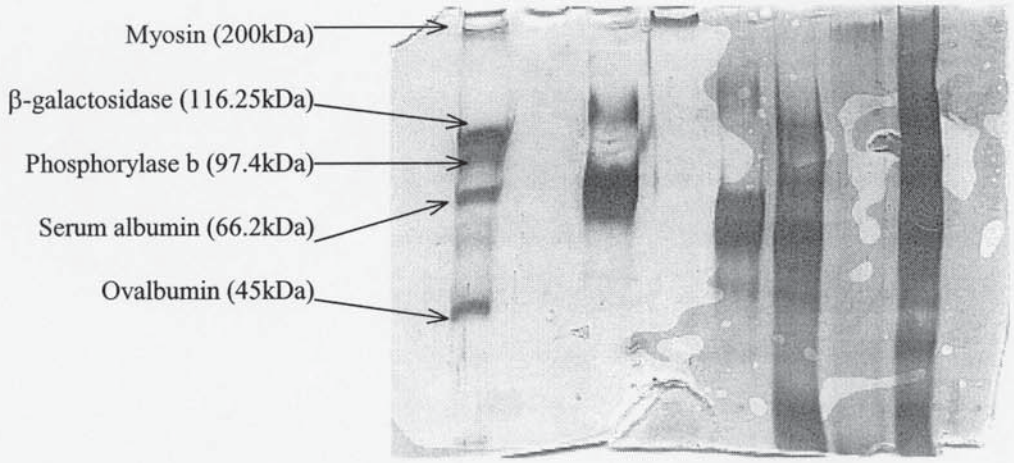


Figure 5.9 High molecular weight analysis using a 7.5% gel. This gel was loaded as that in Figure 5.8 except that Lane 2 is loaded with high molecular weight standards. The gel was silver stained following the method of Heukeshoven and Dernick[135].

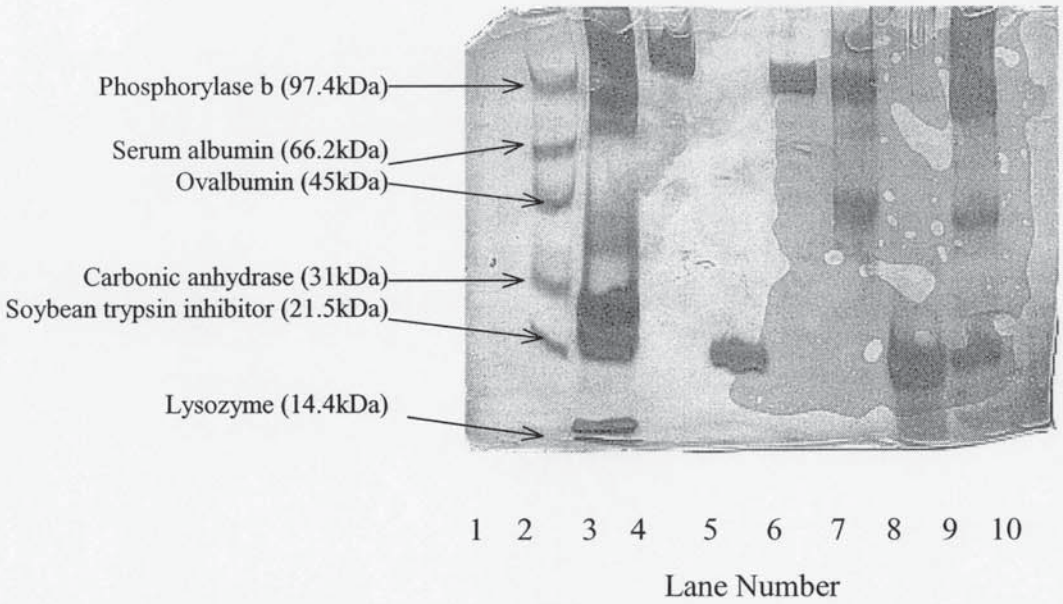


Figure 5.10 Low molecular weight (12.5%) gel. This gel was loaded as that in Figure 5.8 except that Lane 2 is loaded with low molecular weight standards. The gel was silver stained following the method of Heukeshoven and Dernick[135].

The contents of the three mixtures of standards described above and the molecular weight of these proteins is shown in Table 5.2.

Name	Molecular Weight (Daltons)	Found in Broad mixture	Found in Low mixture	Found in High mixture
Myosin	200,000	Yes	No	Yes
β-galactosidase	116,250	Yes	No	Yes
Phosphorylase b	97,400	Yes	Yes	Yes
Serum albumin	66,200	Yes	Yes	Yes
Ovalbumin	45,000	Yes	Yes	Yes
Carbonic anhydrase	31,000	Yes	Yes	No
Trypsin inhibitor	21,500	Yes	Yes	No
Lysozyme	14,400	Yes	Yes	No
Aprotinin	6,500	Yes	No	No

Table 5.2 Content of molecular weight standard mixtures used. The terms 'Broad', 'Low' and 'High' used in this table are the names given to these molecular weight markers by the company that they were purchased from (Bio-Rad, UK)

To fully assess the impact of narrow focus compared to broad the procedure of running duplicate samples was performed several times. A second set of these runs with slightly different samples is indicated in Figures 5.11-5.13 below to indicate that the results described above were the norm and not a one off.

By direct comparison of the silver stained gels seen in Figures 5.9 and 5.10 to the identically loaded broad-range and Coomassie blue stained scan seen in Figure 5.8, it can be clearly seen that the silver stain method of Heukeshoven and Dernick is more sensitive than Coomassie Blue staining. In each case the silver stain gives much darker bands for the standards and samples. The clearest example of the increased sensitivity of the silver staining method is seen in the IgA samples in Figures 5.11, 5.12 and 5.13 (Lane 7 in each gel). In the Coomassie Blue stained gel this sample is only very lightly stained whilst in the two silver stained examples the protein bands are much darker.

However, although this staining method is more sensitive it also has several negative features. Amongst these are the fact that the silver based method involves a much larger number of steps (fifteen compared to just three with Coomassie Blue), it includes several points where a single miss-timing can completely ruin the results, is comparatively time consuming and also needs certain chemicals which are highly toxic. In addition the process is light sensitive, causes the gels to shrink severely and makes gels a lot harder to store (they dry out faster than usual).

The shrinking of the gels is a serious problem as it makes bands difficult to determine. Other problems with this system that make the gels difficult to analyse include the degree of background staining that occurs and the intensity of staining at even low concentrations of protein.

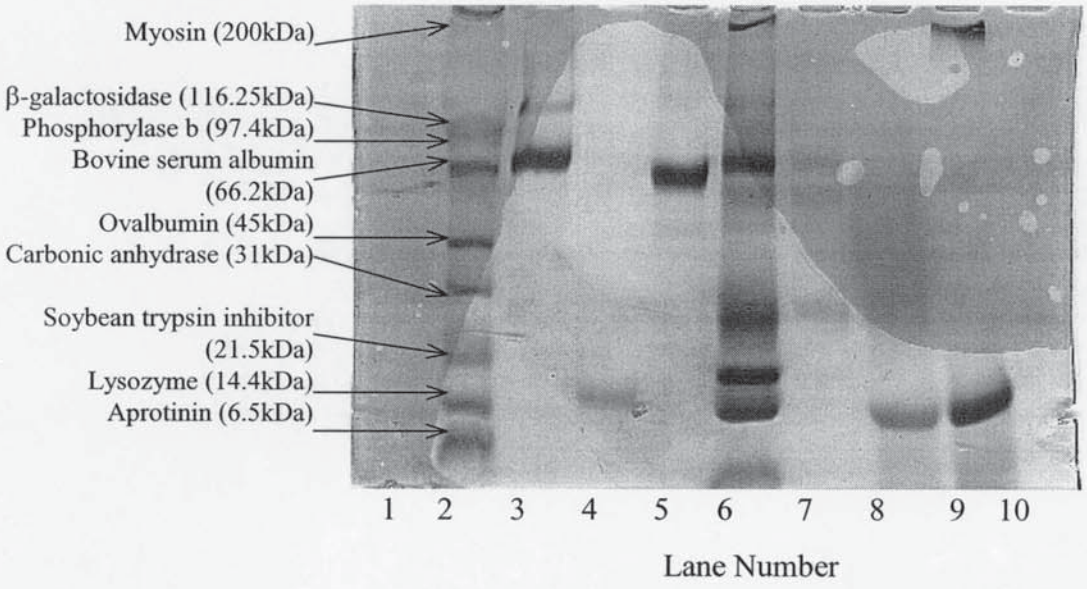


Figure 5.11 A broad range, gradient gel (7.5-15%) that has been Coomassie Blue stained. Lane 1 = Empty, Lane 2 = Broad range markers, Lane 3 = Lactoferrin (2mg/ml), Lane 4 = Lysozyme (1.0mg/ml), Lane 5 = Albumin (1.5mg/ml), Lane 6 = Tears, Lane 7 = IgA (1.0mg/ml), Lane 8 = 28 day FOCUS monthly disposable extraction, Lane 9 = 1 day CIBA daily disposable extraction, Lane 10 = Empty.
***N.B** Lane 7 (1mg/ml IgA) is only faintly stained compared to Figures 5.12 and 5.13*

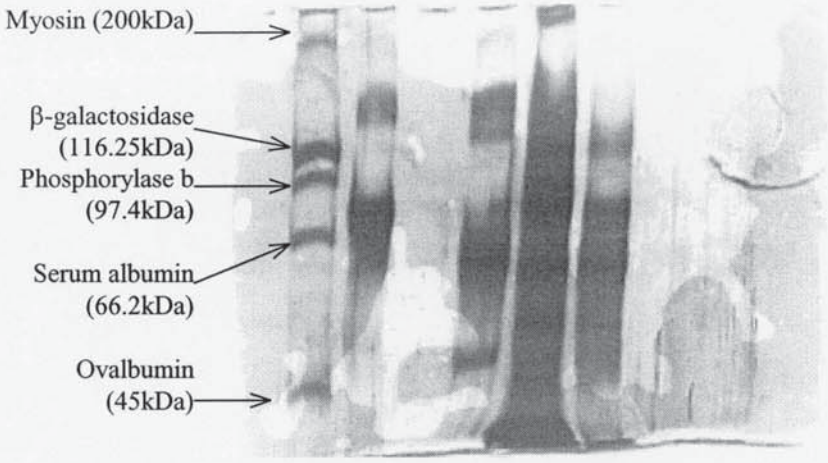


Figure 5.12 A high range gel (7.5%) that has been silver stained. This gel was loaded in an identical manner to that shown in Figure 5.11 except that lane 2 was loaded with high range molecular weight markers. It was silver stained following the method of Heukeshoven and Dernick[135]. Lane 7 (IgA) is very darkly stained.

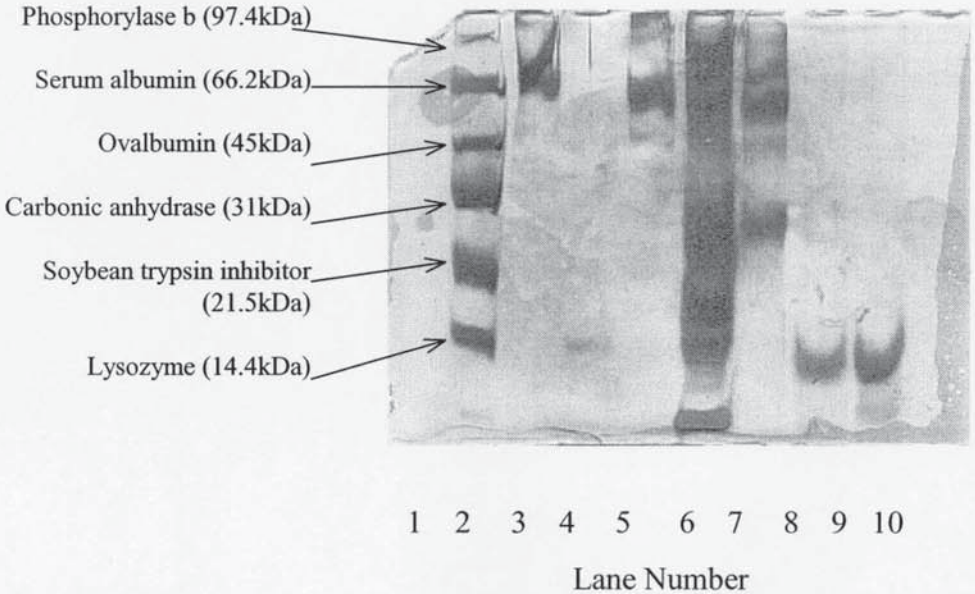


Figure 5.13 A low range gel (12.5%) that has been silver stained. This gel was loaded in an identical manner to that shown in Figure 5.11 except that lane 2 was loaded with low range molecular weight markers. This gel was silver stained following the method of Heukeshoven and Dernick[135]. Lane 7 (IgA) is very darkly stained indicating the sensitivity of this staining method compared to Coomassie Blue (see the corresponding Lane in Figure 5.11).

As a result of all the problematic factors associated with this particular staining system (discussed earlier in this Section) it became apparent that an alternative was needed that combined the enhanced sensitivity of Heukeshoven and Dernicks' silver staining method with the relative simplicity and clarity provided by Coomassie Blue. A description of the appraisal of such a system can be seen in section 5.6.

The problems encountered with the staining system are not the only visible/obvious drawback associated with using narrow range gels as opposed to gradients. The advantage supplied by running narrow band gels should be greater separation over the same distance, allowing bands that are too close together to distinguish on the broad range gels to be seen as individual species. What was actually seen was that virtually none of the proteins of interest from tears and contact lens extracts were seen in high molecular weight gels. This was due to the fact that they would have run off the bottom of these gels. Where proteins were seen they were so darkly stained that separate bands could not be distinguished.

In low molecular weight gels, more of the proteins of interest such as lysozyme, albumin, lactoferrin and the immunoglobulins were present but again there was no real measurable enhancement of separation. Also, the higher molecular weight proteins were isolated at the extreme top of the gel. Taking into account the disappointing effect of using narrow range gels and the original concept of viewing the complete tear pattern at once, it was decided that the best possible option in terms of what we wanted to achieve was to continue by using the gradient gels and to abandon the use of the narrow range recipes.

5.7 Alternative Silver Staining Method

Following on from the work done using both Coomassie Blue staining and the silver staining method of Heukeshoven and Dernick[135] described in Section 5.6, it was decided that a new method epitomising the best features of both systems was needed. After researching the available staining systems, it was decided to try the method devised by Gottlieb and Chavko[136] that is outlined in Section 2.9. This method involves a relatively small number of individual steps (five compared to the fifteen used in the Heukeshoven and Dernick silver based system), is reported by the suppliers to be fifty times more sensitive than Coomassie Blue and is supposedly less sensitive to timing errors, background staining and shrinkage. Thus, it can be seen that this staining method comprises the desired features of the two previous stains.

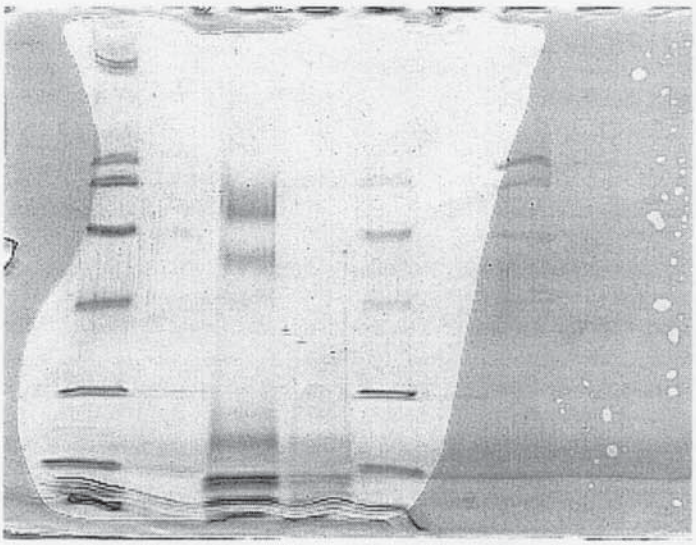
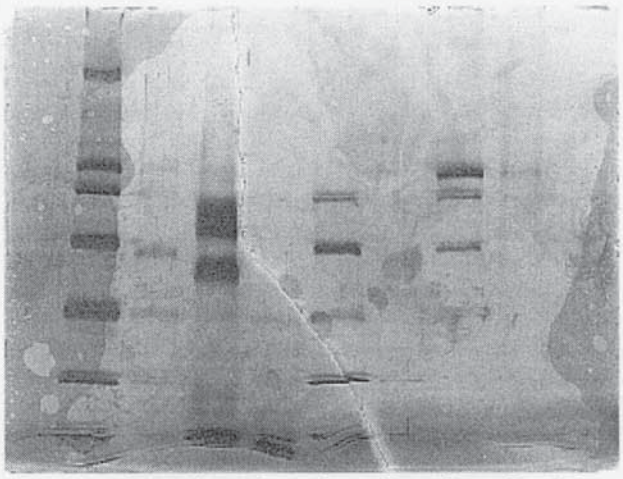


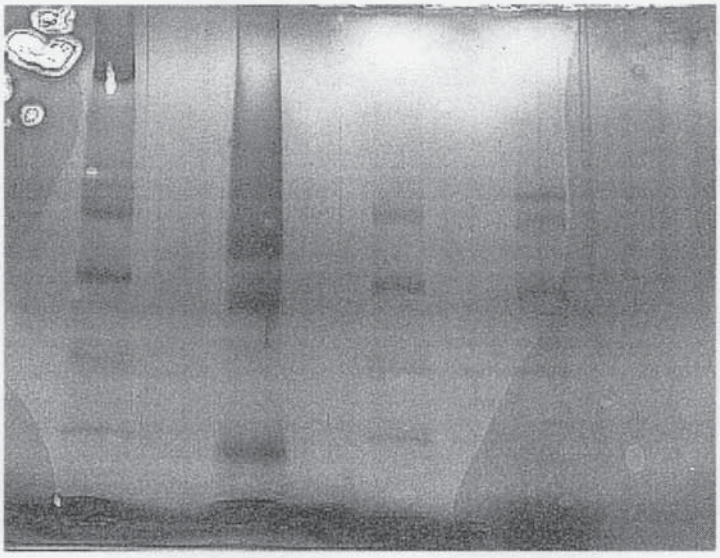
Figure 5.14 Typical Coomassie Blue stained gel. This gel was loaded with: Lane1 = Empty, Lane 2 = Broad range markers, Lane3 = Markers diluted 1/10, Lane 4 = Tears, Lane 5 = Tears 1/10, Lane 6 = Low range markers, Lane 7 = Low range markers 1/10, Lane 8 = High range markers, Lane 9 = High range markers 1/10, Lane 10 = Empty.



1 2 3 4 5 6 7 8 9 10

Lane Number

Figure 5.15 Example of a gel stained using the silver staining method of Heukeshoven and Dernick[135]. This gel was loaded identically to those in Figures 5.14 and 5.16.



1 2 3 4 5 6 7 8 9 10

Lane Number

Figure 5.16 Example of a 7.5-15% gel stained using the silver staining method of Gottleib and Chavko[136]. This gel was loaded identically to those in Figures 5.14 and 5.15. A colour scan is shown here because the presence of some bands is lost on grey scans of these gels.

The gels depicted above represent duplicate aliquots of samples run under identical conditions on the same gel recipe (i.e. 7.5-15% gradient gels). The only difference between the gels is whether they were stained using Coomassie Blue, the older silver staining method (Heukeshoven and Dernick) or the newer method (Gottleib and Chavko). Dilutions were made using distilled water and the density of the samples was kept high enough to allow loading by addition of urea. The dilutions were used in an attempt to gauge sensitivity of the separate staining methods.

Although they were loaded with aliquots of the same samples, the gels depicted above were run on different days. This lack of parallel running conditions could mean that the absence of differences in the intensity of staining observed might be due to slightly different running times, to differences in how long the samples had been stored for, etc. rather than being indicative of the stain used. Any such differences would be minor, however, and it is quite clear that the new silver stain method of Gottleib and Chavko gives no increase in sensitivity when compared to Coomassie Blue let alone the silver staining method of Heukeshoven and Dernick. In other words, no additional protein bands were seen when this method was used.

In order to rule out any possibility that the lack of sensitivity was due to the gels being run on different days, two gels of identical recipe were run at the same time ensuring identical running time, running current and properties of the samples loaded. After the run, one gel was stained with Coomassie Blue whilst the other was silver stained using the alternative method of Gottleib and Chavko. This was done to see whether this silver staining method could provide any improvement in sensitivity compared to the Coomassie stain. The results of this can be seen in Figures 5.17 and 5.18.

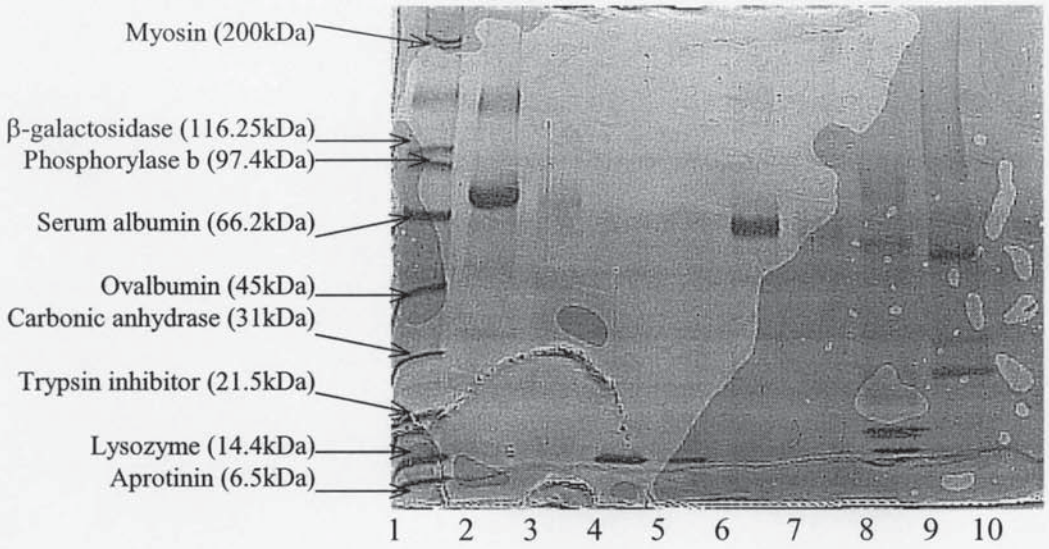


Figure 5.17 7.5-15% gel stained with Coomassie Blue. Lane 1 = Broad range markers, Lane 2 = Lactoferrin (2.0mg/ml), Lane3= Lactoferrin (0.2mg/ml), Lane 4 = Lysozyme (1.0mg/ml), Lane 5 = Lysozyme (0.1mg/ml), Lane 6 = Albumin (1.5mg/ml), Lane 7 = Albumin (0.15mg/ml), Lane 8 = Tears diluted 1/10, Lane 9 = IgG (1.0mg/ml), Lane 10 = IgG (0.1mg/ml)

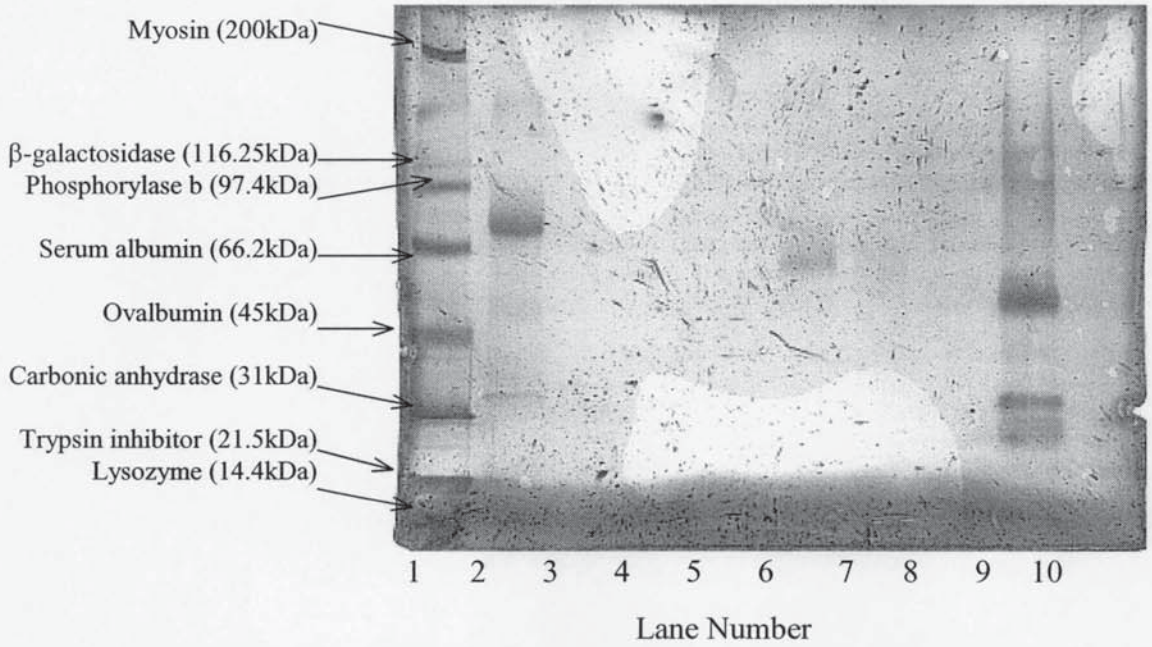


Figure 5.18 Gel subjected to silver staining. The silver staining system used was that of Gottleib and Chavko[136]. This gel was identical to that in Figure 5.17 in terms of the samples loaded, the gel recipe and the running conditions. The Aprotinin band is lost in the dark staining area at the bottom of the gel.

After comparing the results achieved using the various stains, the advantages derived from silver staining are hard to determine. In some cases the staining system of Gottlieb and Chavko did appear to stain certain bands darker than Coomassie Blue, making them easier to see. However on the same gels other bands were lighter than those seen with the Coomassie stain indicating that the two systems have different affinities for different proteins. What causes this affinity and what the identities of the proteins involved are remains unclear. Such factors as pI, folding pattern and the nature of the protein structure (i.e. the balance of positive and negatively charged amino acids) could all influence the degree to which a protein takes up a particular dye and hence the intensity of staining.

Overall, the alternative silver stain system of Gottleib and Chavko did not appear to be as sensitive as reported and in fact as can be seen by a comparison of Figures 5.17 and 5.18 the Coomassie Blue stain actually manages to show more distinct bands and much less background. In addition, although this alternative silver stain is simpler than the previous silver system, it is still more complicated than Coomassie Blue and is also prone to problems with overdeveloping and background staining.

The main problem of the Gottleib and Chavko system is the need for glassware to be specially cleaned because the stain reacts with residues left behind by lab detergents. Successful cleaning is both time consuming and difficult to judge until the gel is developing, at which point a mistake is brought home by large areas of non-specific staining and nothing can be done to remedy the problem. In conclusion it appears that Coomassie Blue should remain the stain of choice due to its simplicity, safety and indeed the relative sensitivity compared to the other systems.

5.8 Summary of Optimal Conditions

Having fully explored all the variables described in this chapter a set of conditions were selected which would give the best possible resolution of the pattern of tear proteins we could achieve. These were :

- **No stacking gels**
- **Run at 30 mA**
- **Stain with Coomassie Blue**
- **Use gradient gels (either 7.5-15% or 7.5-20%)**
- **Treat samples with an equal volume of 2X treatment buffer**
- **Scan gels as soon as possible after run**
- **Depth of running buffer in lower chamber kept as low as possible**

Other factors were also optimised through practice of using the apparatus such as gel pouring, sample loading, gradient formation and gel storage method. Having established this 'best method', a number of gels were run following these conditions. An example of the best pattern obtained is shown in Figure 5.19. Full analysis of this gel was carried out using a calibration curve (Figure 5.20). The values used to plot this curve along with the molecular weights of the individual unknown protein bands seen in tears and in the contact lens extracts run on this gel are found in Table 5.3.

Possible identities of these proteins based on the molecular weights of the major tear proteins as reported in the literature are assigned where possible. Although these indicate the possible/probable identity of the protein bands, conclusive identification would only be possible with a further analytical step such as Western blotting coupled with immunostaining or protein sequencing. In other words we can say what the protein is likely to be based on its' molecular weight compared to what we would expect to find in a tear sample, but we cannot be certain without further analyses.

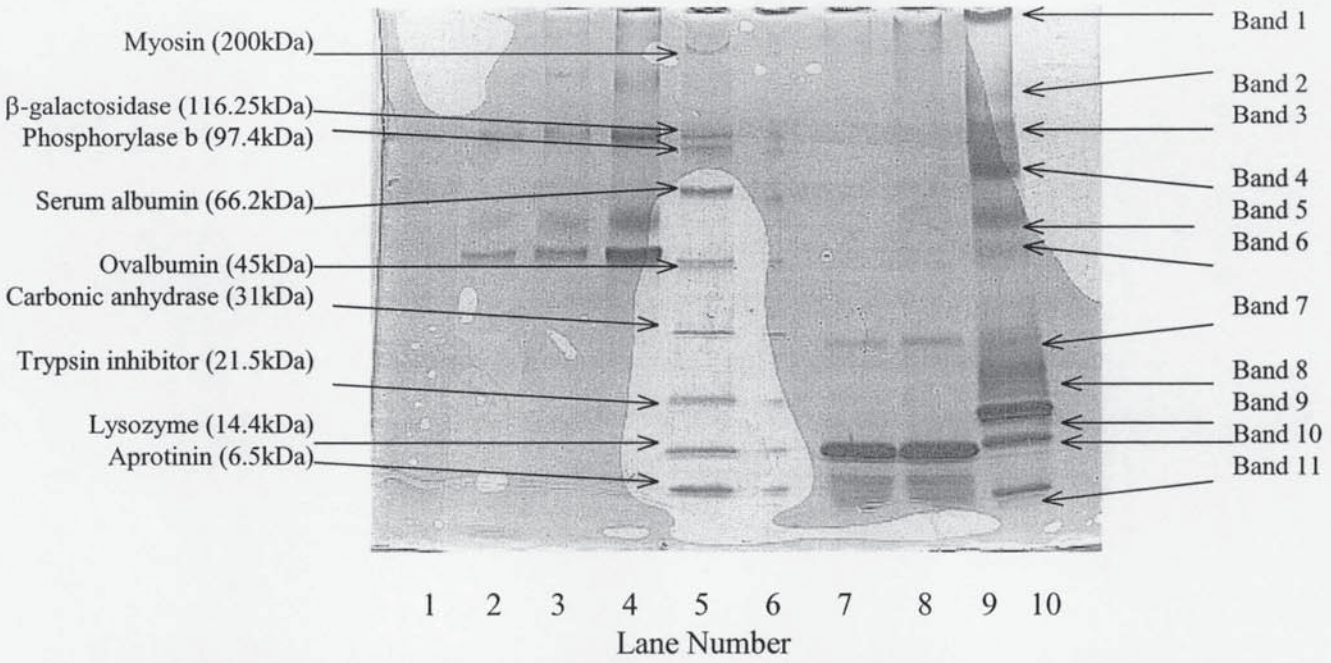


Figure 5.19 Optimised gel showing the best possible example of the tear protein pattern. Lanes 1 & 10 = Empty, Lane 2 = Kininogen (0.5mg/ml), Lane 3 = Kininogen (1.0mg/ml), Lane 4 = Kininogen (2.0mg/ml), Lane 5 = Broad range markers, Lane 6 = 1 month old 1-day Acuvue extraction, Lane 7 = Fresh-1 day Acuvue extraction from left eye lens, Lane 8 = 1-day Acuvue extract (left and right lens extracts pooled), Lane 9 = Tears

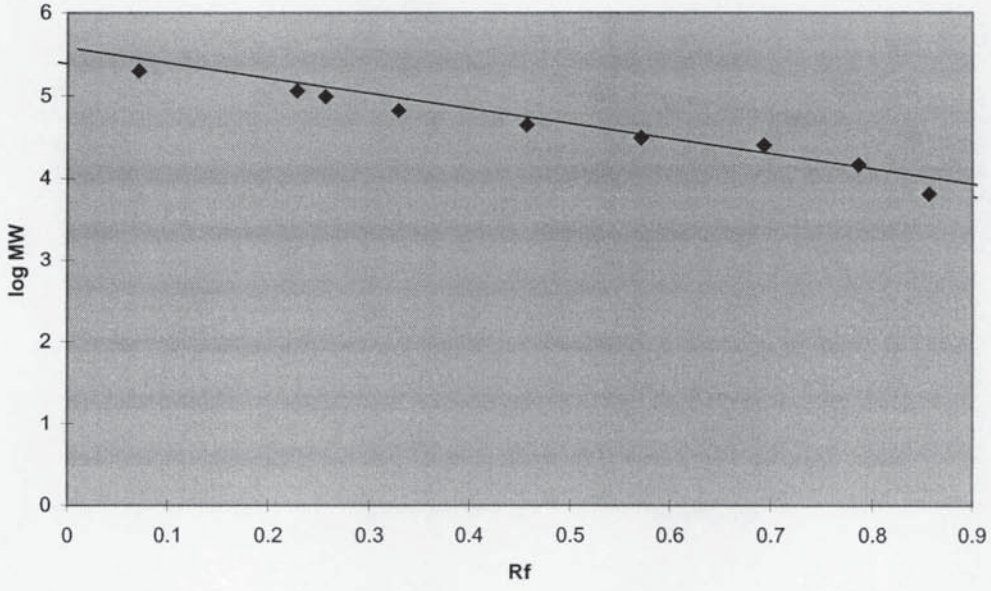


Figure 5.20 Graph showing a plot of Rf against log MW for a set of broad range markers. This can be used to determine the molecular weights of unknown bands

Name of protein/band	Rf	log MW	Molecular Weight /Daltons	Possible Identity
Tears 1	0.029	5.500	316,230	IgA dimer
Tears 2	0.171	5.225	167,880	IgA/IgG
Tears 3	0.229	5.100	125,893	Kininogen
Tears 4	0.300	4.950	89,125	Lactoferrin
Tears 5	0.386	4.800	63,096	Albumin
Tears 6	0.457	4.650	44,668	Kininogen subunit
Tears 7	0.600	4.350	22,387	Lipocalin
Tears 8	0.671	4.275	18,836	?
Tears 9	0.729	4.175	14,962	Lysozyme
Tears 10	0.786	4.025	10,593	?
Tears 11	0.871	3.900	7,943	β-lysin
Kininogen 1	0.229	5.100	125,893 (120,000)	N/A
Kininogen 2	0.314	4.910	81,283 (120,000)	N/A
Kininogen 3	0.443	4.650	44,668 (120,000)	N/A
Extraction 1	0.600	4.350	22,387	Lipocalin
Extraction 2	0.800	4.050	11,220	Lysozyme
Extraction 3	0.857	3.950	8,913	β-lysin

Table 5.3 Rfs, log MWs and molecular weights of the bands seen in Figure 5.19. For the standard protein solution (kininogen) the reported molecular weight[74] is shown in brackets under the calculated molecular weight determined using Figure 5.20. The values determined for the molecular weight markers used to plot Figure 5.20 are not shown in the table. It is possible that the three bands seen with kininogen represent its two forms (see Section 1.4.3.4) or individual subunits of the protein. The two forms are High Molecular Weight Kininogen (120kDa) and Low Molecular Weight Kininogen (68kDa). The most likely explanation is that High Molecular Weight Kininogen has been cleaved by the sample treatment process into constituent subunits.

In addition to calculating values for a gel showing the best tear protein pattern achieved it was decided to carry out similar processing for a gel loaded with some proteins whose presence in tears has been well established (namely lactoferrin, lysozyme, albumin and IgG). Figure 5.21 represents such a gel and is a duplicate of Figure 5.17. The reasoning behind this data analysis was to compare the variation between known, reported molecular weights for these proteins and the values determined using our technique. Table 5.4 shows the comparison for the physiological concentration samples. Values for the tear protein bands are also included although this run is not particularly good due to the 1/10 dilution of the tear sample that was carried out.

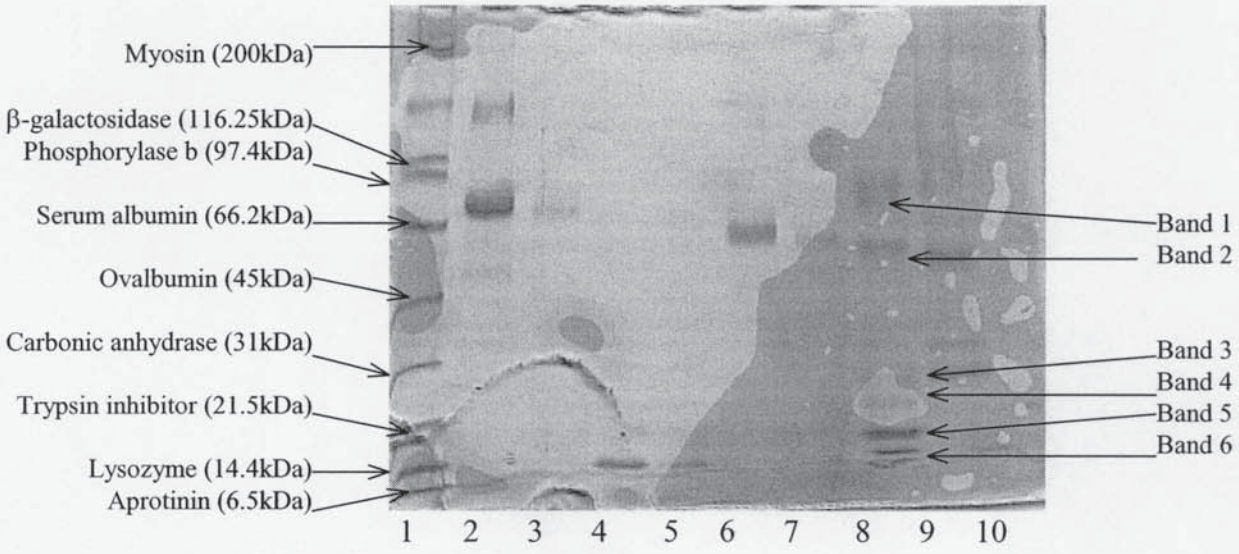


Figure 5.21 Example of a gel used to measure the difference between reported values for some common tear proteins to those obtained using our optimised technique Lane 1 = Broad range markers, Lane 2 = Lactoferrin (2.0mg/ml), Lane 3 = Lactoferrin (0.2mg/ml), Lane 4 = Lysozyme (1.0mg/ml), Lane 5 = Lysozyme (0.1mg/ml), Lane 6 = Albumin (1.5mg/ml), Lane 7 = Albumin (0.15mg/ml), Lane 8 = Tears/10, Lane 9 = IgG (1.0mg/ml)and Lane 10 = IgG (0.1mg/ml)

Name of protein/band	Rf	log MW	MW/Daltons (calculated)	MW/Daltons (reported)	Difference in values
Lactoferrin band 1	0.207	5.200	158,489	164,000 (dimer?)	3.4 %
Lactoferrin band 2	0.410	4.900	79,432	82,000[35]	3.1%
Lysozyme	0.910	4.100	12,589	14,600[29]	13.8%
Albumin	0.459	4.825	66,834	68,000[46]	1.7%
IgG band 1	0.525	4.700	50,118	150,000[48]	
IgG band 2	0.705	4.375	23,713	150,000	4.8%
IgG band 3	0.770	4.275	18,836	150,000	
Tears band 1	0.377	4.950	89,125	N/A	
Tears band 2	0.492	4.825	66,834	N/A	
Tears band 3	0.770	4.290	19,498	N/A	
Tears band 4	0.820	4.200	15,848	N/A	
Tears band 5	0.902	4.050	11,220	N/A	
Tears band 6	0.974	3.925	8,414	N/A	

Table 5.4 Comparison of reported molecular weights to measured values.

The bands seen for IgG are likely to represent the separate chains of this immunoglobulin. A normal immunoglobulin molecule consists of two identical heavy chains (as represented by the single, thick 50,000 Dalton band) plus two different light chains (as indicated by the two bands of around 20,000 Daltons). Adding these values together gives a total of 142,785 Daltons which is very close to the reported value of 150,000. The percentage accuracy in Table 5.4 for IgG is based on the combined molecular weight of the subunits.

5.9 Conclusions

The establishment of an optimal set of parameters for running tear samples using 1-D polyacrylamide gels for protein analyses was achieved following the exploration of a wide number of variables. Influences such as running current, gel recipe, running time and staining system were all assessed and conditions selected that would deliver the clearest reproducible pattern that could be visualised and stored using the scanners available. The necessity to have a pattern that could be 'seen' by the scanner meant that it was not always the gels that looked clearest to the naked eye that were selected. Gels were ultimately chosen according to such criteria as tight and defined bands, minimal smiling effects, reduced background staining, eases of reproducibility lack of shrinking/tearing and clearest attainable separation of proteins.

Having determined these optimised conditions (an outline of which can be seen earlier in this Chapter) they were applied to the analysis of both tear proteins and contact lens extracts. By using the technique along with marker proteins of known molecular weight and previously published molecular weight values of some common tear proteins determined using a variety of methods, the possible nature of some of the protein bands observed in the tear patterns and lens extracts can be suggested, although conclusive identification would require a further step. It appears that the identification/separation of a number of important tear proteins is enabled by the use of the parameters determined through the course of this evaluation process.

In addition to the demonstration that the technique is viable for separating the complex mixture of tear proteins, the relative accuracy of using this technique to determine the molecular weight of a particular band has been investigated. Figure 5.21 and Table 5.4 show that when stock solutions of some of the proteins whose presence in tears is well established were run along with some molecular weight markers, and the usual calculations and data analyses were performed, the calculated molecular weight values were very similar to the reported values.

This information lends further weight to the suitability of 1-D PAGE for our investigation into the tear proteins in normals and lens wearers. Such accuracy would be invaluable in tentatively classifying any unknown bands: at the very least the molecular weight of each and every band can be reported as shown in Table 5.3.

The only dilemma that remains in the use of 1-D PAGE is the lack of conclusivity when it comes to identifying a protein. It is much more valuable to be able to say with conviction what a particular band is or whether a particular protein is present. It is for this reason that it was decided to develop the established, optimised electrophoresis technique into the area of Western Blotting and Immunostaining and Chapter 6 sets out the experimental work involved.

Chapter 6

Western Blotting and Immunostaining of One Dimensional Gels: Conclusive Identification of Protein Species

6.1 Introduction

Following on from the work done on establishing a method of 1-D PAGE which gave optimal results with regards to a pattern of tear proteins, it was decided that it was important to have a method whereby the identity of the proteins seen could be stated in such a way as to not be open to argument. Whilst the identity of a particular protein band can be estimated in 1-D PAGE by comparing its' molecular weight (as determined from a graph of mobility and molecular weight of marker proteins) to the known molecular weights of the proteins usually found in tears, this only suggests what the protein is likely to be. In order to determine exactly what the bands represented, it was decided to Western blot the gels and then use antibodies to identify the protein species. The use of antibodies in detecting proteins on Western Blots is also called immunostaining.

Compared to 1-D PAGE, immunostaining gives irrefutable proof as to the identity of a particular protein as well as establishing the presence, or otherwise, of a particular species. In 1-D PAGE where only Coomassie Blue or silver staining is used, the absence or presence of a protein may be masked by the existence of another protein or proteins of similar molecular weight which would occupy the same position in a gel.

2-D PAGE may overcome this particular problem because two parameters/dimensions are used to separate the proteins, molecular weight AND isoelectric point (pI). This means that proteins having the same molecular weight but different pIs will be separated and can be seen as distinct entities. The difficulties encountered with this technique have been documented earlier (see Chapter 4) and if both the molecular weight and pI are similar for two or more proteins the same problem as 1-D PAGE will be seen.

The main benefit of Western blotting with immunostaining is that the fairly simple technique of 1-D PAGE, which allows the complete pattern of the tear proteins to be recorded, can be adapted to establish the precise nature of any alteration in this pattern. This is invaluable when trying to determine the influence of proteins on (or indeed the impact upon proteins of) something such as lens wear or disease.

Western blotting involves the transfer of the separated protein spots from the gels they are run in to a membrane under the influence of an electric current. This is done to remove the proteins from the confines of the gel where they are relatively inaccessible to a number of species, such as antibodies and other large molecules. Once on the membrane surface, the proteins are free to react and the highly specific antibody/antigen reaction can be exploited to assess the presence of a particular protein. Such a reaction can be highly sensitive with detection down to nanogram levels compared to Coommasie Blue which has a lower limit of around 0.01mg in our experience. As a result of this sensitivity, the ability to explore the importance of a huge range of individual proteins in the tear film using this technique is only confined by the availability of antibodies raised to them.

Once the technique of immunostaining had been applied to the established tear proteins and its achievable level of sensitivity had been ascertained the hope was to utilise the system in the identification of some of the unknown protein bands seen and in reinforcing the identification of certain proteins previously reported in our group using other techniques[62]. An outline of the work carried out to confirm the presence of one particular protein, kininogen, can be found in Chapter 7 whilst the results presented here in this chapter demonstrate the initial appraisal of the system. Other applications of the method would include its' utility in revealing the identity of any protein anomalies seen in lens wear or eye disease that were displayed by 1-D PAGE but could not be determined by other methods.

6.2 Method

(see Section 2.10 and 2.11)

6.3 Quality of Protein Transfer

Before proceeding with the immunostaining of blots prepared from gels run under optimum conditions, it was decided to investigate the amount of protein that was actually transferred using the Kem-En-Tec horizontal Semi Dry Blotter 2. This method is called 'semi dry' because it uses filter papers soaked in the various anode and cathode buffers instead of liquid filled buffer chambers. Using filter papers in this way greatly reduces the amount of buffer needed compared to 'wet' blotting systems. The reason for investigating the amount of protein transferred was to minimise expense incurred and to establish if this technique was worthwhile in terms of time consumption compared to observable results. Antibodies are expensive due to their method of production and time is always invaluable so there would be no point carrying out further blotting and immunostaining if the majority of proteins were not seen to transfer from the gels.

The method used to evaluate the relative efficiency of blotting involved running two gels simultaneously which had been loaded with identical volumes of the same eight samples. After electrophoresis, one gel was placed in Coomassie Blue stain as usual whilst the other was subjected to the blotting method outlined in Section 2.10. Following the blot the gel was stained and destained under matching conditions to its partner and the pattern of bands was compared.

In some early experiments an additional step was performed wherein the PVDF membrane was stained with Ponceau red immediately after the blot. PVDF was chosen over the more traditional nitrocellulose membrane because of the reported fragility of nitrocellulose and increased performance associated with PVDF systems. Ponceau stain highlights bands on the membrane surface but can be subsequently removed by washing in distilled water so that it does not interfere with subsequent immunodetection. In this case a number of bands corresponding to those that were 'missing' from the blotted gel were seen upon treatment with the Ponceau reagent.

This data is not presented due to the transient nature of the stain and the need to wash the membrane as soon as possible after staining to minimise any residual pinky red colour. After a number of blots had been carried out successfully the Ponceau staining step was ceased as the unavoidable pink background obscured some of the weaker positive results obtained.

An example of the 'Before and After' picture obtained from a blotted gel compared to an unblotted one can be seen in Figures 6.1 and 6.2. The contrast in the staining pattern between these two gels is a clear indication of the high efficacy of the process of blotting proteins from gels to the PVDF membrane.

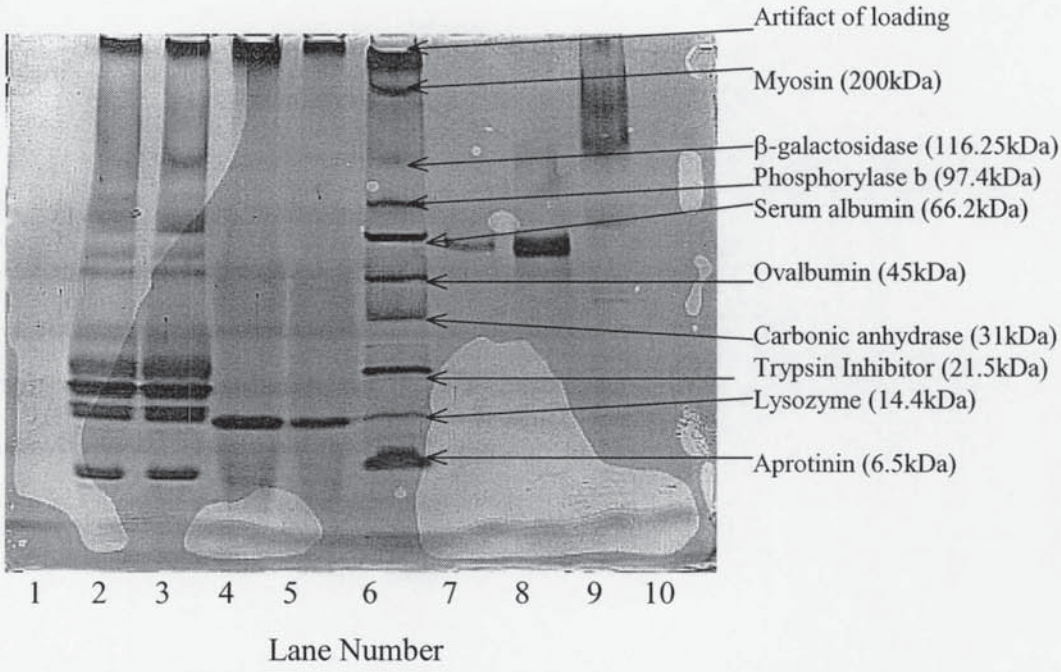


Figure 6.1 Unblotted gel stained using Coomassie Blue. This gel was a 7.5-20% gradient gel ran at 30mA. Lane 1 = Empty, Lane 2 =Ais tears collected via microcapillary, Lane 3 = Helena tears collected via microcapillary, Lane 4 = 7 day Acuvue extraction, Lane 5 = 28 day Acuvue extraction, Lane 6 = Broad band MW markers, Lane 7 Albumin (0.15 mg/ml), Lane 8 = Albumin (1.5 mg/ml), Lane 9 = Lactoferrin (0.5 mg/ml) + Lysozyme (1.0mg/ml), Lane 10 = Empty.

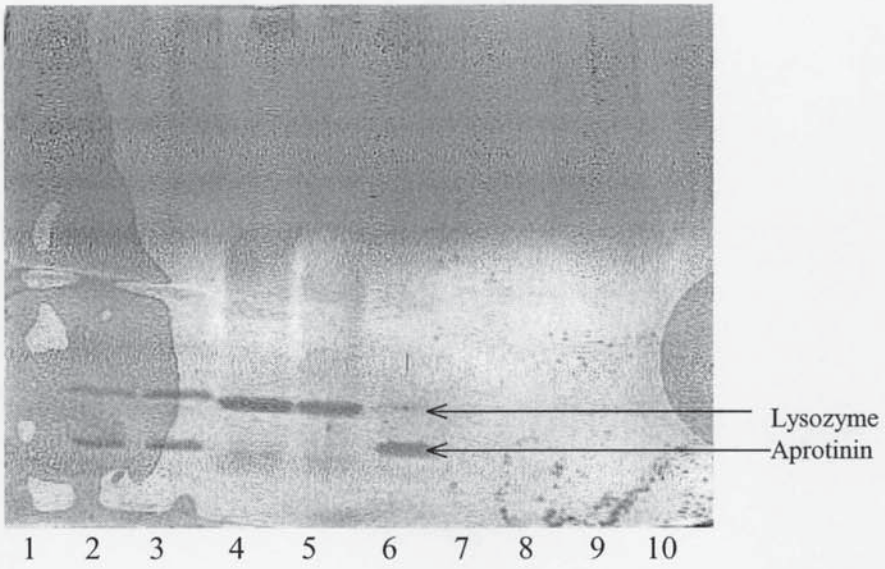


Figure 6.2 Gel loaded and run under identical conditions as that shown in Figure 6.1, but subjected to Western Blotting. This image shows the proteins left behind after the gel has been blotted for 2.5 hours. After blotting the gel has been subjected to Coomassie Blue staining for the same duration as that in Figure 6.2. The identity of the remaining proteins is unknown, but they appear to be of low molecular weight and probably have a net charge which has prevented them from being transferred by the current to the PVDF.

As can be seen from a comparison of Figure 6.1 to Figure 6.2 the vast majority of protein has been transferred from the gel. In a couple of instances, particularly darkly stained bands from the first gel have not completely transferred. This is not too much of a problem because generally the darker the staining, the more protein present and by contrast to the bands seen in the gel shown in Figure 6.1 relatively little proteins is left behind. There are a number of reasons why certain protein bands may not transfer as well as others. For example, particularly large proteins are difficult to blot and so will remain to some extent in the gel. Also, the native charge of the proteins becomes a factor once the protein leaves the influence of the SDS found in the gel and in some cases this can also impede transfer.

Overall, following a number of such experiments to evaluate the efficiency of Western Blotting, it was decided to go ahead with immunostaining for a number of the commonly found and reported tear proteins. In each case, the samples were run in duplicate with one gel being Coomassie Stained in order to obtain a pattern of tear and

lens extract proteins whilst the other was blotted and then stained. This acted as a method for assessing the continuing viability of the blots as well as giving a wider picture of the protein pattern that could be related to the conclusive identification of the proteins.

6.4 Immunostaining for Albumin

The first common tear protein whose presence we chose to demonstrate was albumin. Using the blot generated from the gel depicted in Figure 6.2, immunostaining was started by adding an antibody raised against human albumin in goats (i.e. Goat anti-human albumin antibody). This step was followed by addition of an antibody raised against general goat antigens in mice (i.e. mouse anti-goat antibody) to which an alkaline phosphatase (AP) sidechain is attached. The AP subsequently reacted with BCIP/NBT to give an insoluble blue colour wherever albumin was present. The pattern of distribution of albumin in the tears of the volunteers depicted in Figure 6.1 and the relative sensitivity of this technique as indicated by the strength of staining of albumin solutions of two different concentrations is shown in Figure 6.3.

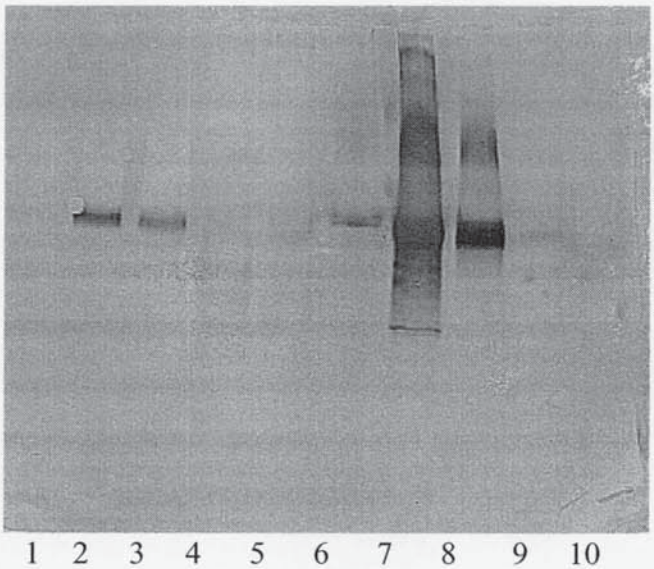


Figure 6.3 Western blot immunostained for albumin. This is the blot taken from the gels seen in Figures 6.1 and 6.2. Strong positive bands can be seen for the tear samples and standard solutions. A weak band can be seen in each of the contact lens extracts. This proves the presence of albumin in tears and extracts.

The immunostained membrane clearly shows the presence of albumin in tears as expected. By comparing the intensity of the positive bands in tears to those of the standard solutions, the concentration in tears can be estimated. The estimated concentration would be 0.075mg/ml. In addition there is some faint staining of the lens extracts proving that there is at least a low level of albumin deposited onto contact lenses during wear.

The staining of the two albumin samples indicates that the immunostaining system is conclusively more than sensitive enough to be able to detect protein concentrations as low as 0.15mg/ml, and the staining is dark enough to suggest that it may be sensitive enough to detect even lower levels.

In fact, the 1.5mg/ml sample of albumin appears to have been too concentrated with severe smearing and interference of the desired distinct bands. This suggests that there is an upper limit to immunostaining above which no viable information will be generated. The staining of a band in the molecular weight marker lane comes as no surprise as one of the components of this marker solution is albumin.

In addition to the data about sensitivity, the specificity of the immunostaining process with regards to albumin in particular using this antibody is also demonstrated by this experiment. Despite the presence of lactoferrin and lysozyme standards at concentrations similar to those that gave deep staining for albumin, there is no significant staining in the lane containing these samples. The results confirm not only that the method will only detect albumin but also show that the positive results seen in the other lanes were due to albumin and not any other protein.

6.5 Immunostaining of Lactoferrin

Another of the most widely reported and well-established proteins found in tears is lactoferrin. This protein has been shown to be present in tears at concentrations of around 1.5mg/ml[34] and is known to be particularly important in the antibacterial defense of the eye. A more complete description of the nature and role of lactoferrin can be found in Section 1.4.1.2.

In this experiment a number of tear samples collected using microcapillaries were loaded onto a normal 7.5-20% gradient gel. In addition, standard lactoferrin samples of both physiological concentration (i.e. 1.5mg/ml) and 1/10 physiological concentration (0.15mg/ml) were loaded as were some standard solutions of some other common tear proteins including albumin (1.5mg/ml) and IgA (1.5mg/ml). The reason for loading these other standard proteins was for them to act as controls and demonstrate that the staining was an indication of lactoferrin and nothing else. In other words, they were included to prove that the immunostain was specific for lactoferrin and that no non-specific or background staining occurred.

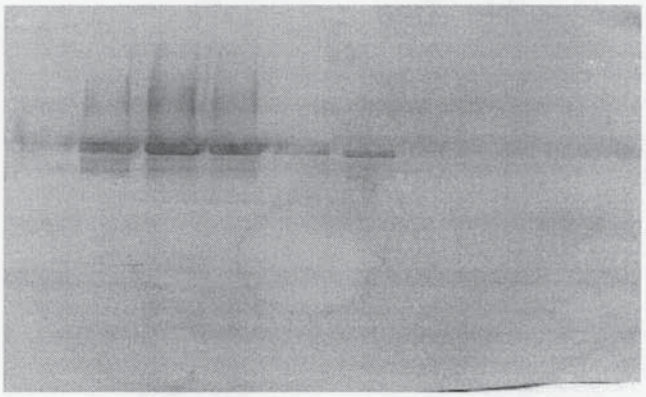


Figure 6.4 PVDF membrane immunostained for lactoferrin[140]. The samples seen here are: Lanes 1 and 10 = Empty, Lane 2 = Ais tears, Lane 3 = Helena tears, Lane 4 = Matthew tears, Lane 5 = lactoferrin (0.15mg/ml), Lane 6 = lactoferrin (1.5mg/ml), Lane 7= albumin (1.5mg/ml), Lane 8= IgA (1.5mg/ml), Lane 9 = Broad molecular weight markers. Tear samples were collected with microcapillaries. By comparing the intensity of the lactoferrin bands to the bands seen for the tear samples, the concentration of tears can be estimated as being in the region of 3.0mg/ml.

The results shown above in Figure 6.4 again demonstrate the specificity of the method. Taken in isolation the immunostain of albumin samples could only be seen as an indication of the specificity of this particular version of the method. In combination with the result seen for lactoferrin it would be fairly safe to say that immunostaining was sufficiently specific with regards to whatever protein was being explored, although this should be clearly demonstrated before any conclusions are drawn for a particular immunostaining result.

In terms of sensitivity, this result also offers support to the value indicated with albumin. The lactoferrin sample of 1.5mg/ml concentration is very distinctly and darkly stained whilst the 0.15mg/ml sample gives a clear positive that seems to indicate a lower limit of detection. In other words, although this is clearly a positive result the band is relatively lightly stained.

The level of sensitivity appears to differ between albumin and lactoferrin as the intensity of staining of the 0.15mg/ml samples is lighter for lactoferrin than for albumin. This could be a reflection of the antibodies involved or due to minor variations in the periods of incubation and staining of the membranes or due to some other factor we have not considered. Further work would be needed to explore this point of interest and it has only been raised here as a fact that cannot be explained at this time. Of greatest importance is the fact that these samples gave a visible positive result with the samples and thus displayed that the technique is capable of detecting proteins at these concentrations and indeed possibly lower concentrations.

6.6 Problems Associated with Immunostaining Immunoglobulins

When it comes to the application of blotting and immunostaining of the immunoglobulins, there are some particular circumstances that have to be taken into consideration. The roles and importance of the immunoglobulins in tears were outlined in Chapter 1, and it is clear that their study would be of particular interest to us in our investigation of the reaction of the eye and tear film to lens wear. The reason for this interest centres around the fact that these proteins can act as indicators of immune responses and indeed inflammation (for example the presence in the tears of immunoglobulins normally found predominantly in the plasma such as IgG can be used as evidence of elevated permeability of the blood-tear barrier which increases with inflammatory episodes)[66].

It was also thought that because of the demonstrated sensitivity and specificity of immunostaining it might be useful in indicating the presence of some of the immunoglobulins whose contribution to the tear film is still cause for discussion. In particular, the immunoglobulins IgE whose concentration in tears is particularly low (around 400ng/ml) and IgD are poorly reported when studies of tear proteins are made.

The issues with regards to immunostaining of immunoglobulins stem from the need for specific binding of the antibody to the protein of interest for the technique to be successful. Specificity is derived from the interaction of the antibody with unique features of the target protein, which act as binding/recognition sites (i.e. antigenic epitopes). The problem with antibodies is that they are constructed in a modular fashion and consist of four domains. Two of these domains are common to every type of immunoglobulin (i.e. IgA, IgG, IgM, IgE and IgD) and this causes cross reactivity between antibodies that are supposed to be raised against one type in particular and so immunostaining results using these antibodies cannot be seen to be conclusive.

An example of a membrane which has been taken from a gel loaded with standard solutions of both IgA and IgG and then immunostained for IgA can be seen in Figure 6.5 which clearly demonstrates that the specificity of the technique has been compromised. Both IgA and IgG have been stained with equal intensity and so another answer to our need to analyse the immunoglobulin content of tears had to be determined.

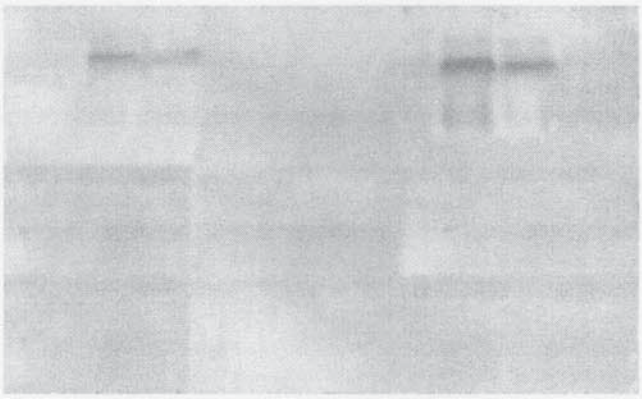


Figure 6.5 PVDF membrane immunostained for IgA. The samples that were initially loaded were Lane1 = Empty, Lane 2 = Ais Tears collected by microcapillaries, Lane 3 = Acuvue Janet 1 day wear extract, Lane 4 = Purevision Julie 1 month wear extract, Lane 5 = Broad range markers, Lane 6 = Albumin (1.5mg/ml), Lane 7 = Lactoferrin (1.5mg/ml), Lane 8 = IgA (1.5mg/ml), Lane 9 = IgG (1.5mg/ml), Lane 10 = Empty. It is clear that both immunoglobulin standards have been stained equally which is not desirable. The identity of the immunoglobulins that have stained positively in the tears cannot be stated conclusively due to the cross reactivity. The concentration of immunoglobulin in tears appears to be approximately 0.05mg/ml.

The answer to this dilemma may come from the fact that each immunoglobulin has a unique heavy chain from which it determines its' name and so antibodies raised against these sub units should show minimal or indeed no cross reactivity and so no false positive results. These subunits are termed α, γ, μ, ε and δ and at this time not all of them have corresponding antibodies that are commercially available. The full investigation as to the success of using antibodies targeted against these immunoglobulin specific sub units is ongoing within the laboratory at this time.

6.7 Discussion

There were two main aims to the work outlined in this chapter:

1. To determine the broad parameters of the technique in terms of its general specificity and sensitivity
2. To finally demonstrate the true nature of some of the protein bands which had previously been seen when samples were Coomassie Blue stained in polyacryalmide gels.

The need for determining sensitivity was particularly important due to the obvious requirement for an enhanced level of detection when the technique of immunostaining was compared to Coomassie Blue staining. In addition, the more sensitive the stain the greater the level of confidence that the results obtained are a true picture of events and that proteins only present at low level concentrations have all been highlighted. The less sensitive the stain, the higher the possibility of missing proteins which are present only at low concentrations and the more likely it is that an important aspect of the protein profile is not seen. The technique of immunostaining was seen to be up to 100 times more sensitive than the results obtained with Coomassie Blue.

The level of detection we have seen using this method is 0.05mg/ml and it would seem that it could prove to be more sensitive than this. The depth of staining/intensity of signal provided by protein solutions of this concentration as a result of immunostaining was such that a further tenfold dilution would likely be detected. The ultimate lower limit of detection was not explored beyond this point as it was decided that such sensitivity was sufficient for our further work and for us to be confident of our results.

Without an evaluation of the specificity of the antibody reaction upon which immunostaining is based, the results generated cannot be regarded as scientifically sound. The possibility of bands being produced because of cross-staining or non-specific staining of proteins other than the protein of interest has to be removed. One way to do this is to compare the staining of the target protein with that seen with a standard solution of a different protein whose presence in tears has been widely reported.

In each of the previous sections of this chapter at least one negative control was included to demonstrate that only the target protein was being stained and thus that no non-specific staining occurred. The observed lack of staining of the control proteins demonstrated that specificity of the antibodies was high in each case and that the bands observed were true representations of the proteins we were trying to stain.

Overall, in combination the observed high level of sensitivity and degree of specificity of immunostaining provides extreme confidence that we are seeing as close to the true nature of the samples we are studying as possible. We can also be confident that the results generated are due to the proteins we are trying to show and nothing else. Both of these values were seen as encouraging with regards to our application of the technique to the subsequent study of kininogen that is discussed in the next chapter.

The problems associated with using an antibody-based method for staining of antibodies are also highlighted here. Common subunits between the types of immunoglobulin lead to a high level of cross specificity wherein an antibody targeted to one immunoglobulin will generate a visible signal for all the other immunoglobulins in the sample to some degree. Work investigating the utility of trying to immunostain samples with antibodies raised against subunits of the immunoglobulins, which are specific to each type, are ongoing in our research group. In other words, antibodies raised against the μ chain of IgM, the ε chain of IgE, the δ chain of IgD, the γ chain of IgG and the α chain of IgA will be examined and these should only react with the corresponding immunoglobulin. It is hoped that this will provide an answer to the encountered problem of cross reactivity and allow a detailed study of the immunoglobulins found in the tears to be made.

In summary, it would seem that due to its sensitivity and specificity this technique is ideal for our study of a tear protein that has not been widely investigated in the current literature. This protein is kininogen and the experiments within which its existence in tears and lens extracts was explored are shown in the following chapter.

Chapter 7

Identification of Kininogen in Tears and Contact Lens Extracts

7.1 Introduction

In the previous chapter the ability of Western Blotting coupled with immunostaining was demonstrated as a useful method to conclusively identify some of the protein bands seen when tear samples and lens extracts are run on SDS polyacrylamide gels. In order to evaluate the technique it was used to identify a number of proteins whose existence in tears has been widely and conclusively reported on many occasions in the past such as albumin and lactoferrin. The sensitivity was also assessed on a basic level by the staining of standard protein solutions whose concentration varied by a factor of ten. The results of this analysis showed that concentrations of around 0.1 mg/ml could be clearly identified and indeed that concentrations of 1-2 mg/ml appeared to be too concentrated for this method of analysis leading to a large amount of non-specific background staining.

As a result of the data obtained regarding the sensitivity, specificity and clarity of this method, it was decided to use it to confirm a previous observation within the group as to the presence of an unusual protein in tears and contact lens extracts whose influence could be dramatic but whose presence is poorly reported. This unusual protein is called kininogen and the reasons that its presence in tears has not been widely reported include its' relatively low concentration in tears and the difficulty in determining the nature of one particular unknown protein band amongst the sixty or so that have been observed[25].

Kininogen is of particular interest to us because it is a member of the kinin family and has been shown to play important roles in inflammatory responses and blood coagulation events when studied in plasma and saliva. Its presence in tears could indicate a number of things including localised production in response to insult of the ocular surface (for example by the introduction of a contact lens) and/or an increase in permeability of the blood-tear barrier allowing kininogen from the plasma to leak into the tears as a result of some kind of inflammatory response. The role of kinins in allergic responses is also important when considered alongside our interest in the response of the eye and tears to the contact lens.

The levels of kininogen produced over the course of a normal day of lens wear and indeed following overnight wear wherein the eye is considered to be in a state of sub-clinical inflammation[23] would also be of great interest, although difficulties in collecting a true 'nocturnal tear' are well reported and were explained in Section 1.5.3. Development of novel techniques that could be especially useful for harvesting the tears immediately upon waking are explored in the following chapter.

The aim of this chapter was to apply all of the techniques that have been optimised and evaluated thus far to an investigation of the presence of kininogen in tears collected via microcapillaries and in extracts produced from lenses of varying wear modalities and type. In addition it was hoped to confirm previous work that had shown the presence of kininogen using the technique of counter immunoelectrophoresis gel assays[62]. By confirming and analysing the relative presence of kininogen in these various samples it was hoped that a possible role of kininogen in the predisposition of lens wearers to various ocular diseases might be postulated.

7.2 Methods

The methods used for this study, namely 1-D polyacrylamide gel electrophoresis and Western Blotting/immunostaining, were fully described in Sections 2.8.1, 2.10 and 2.11 respectively. Evaluations of these techniques were discussed in Chapters 5 and 6 and the optimal conditions for electrophoresis are highlighted in Section 5.6.

7.3 Confirmation of Results Produced using Counter Immunoelectrophoresis

As mentioned previously, it was hoped that these methods would be capable of confirming the results obtained using another method within the lab, namely that of counter immunoelectrophoresis (CIE). In order to explore this, samples that had been shown to contain kininogen by CIE (unpublished data) were subsequently run via electrophoresis before being blotted and immunostained.

Figure 7.1 shows the result of running a number of samples that had given a positive result with immundiffusion along with some standard protein solutions. These standards included one of kininogen of very low concentration that had also given a measurable signal with counter immunoelectrophoresis. It is very clear from looking at this membrane that no immunostaining has occurred.

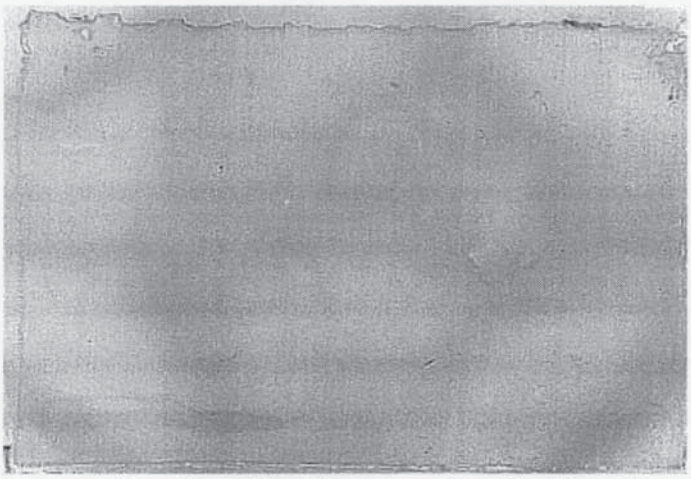


Figure 7.1 Initial immunoblot of kininogen and confirmation of counter immunoelectrophoresis. This PVDF membrane was immunostained for kininogen. Lane1 = Empty, Lane 2 = Ais tears collected via microcapillaries, Lane 3 = Ciba vision daily disposable extract concentrated twice, Lane 4 = Untreated Ciba vision daily disposable extract*, Lane 5 = Ciba vision 1 day Janet extract, Lane 6 = Broad MW markers, Lane 7 = 0.025 mg/ml Kininogen, Lane 8 = 2mg/ml Albumin, Lane 9 = 2.5mg/ml Lactoferrin, Lane 10 = Empty. The samples marked with a * were previously shown to contain kininogen by counter immunoelectrophoresis.*

One possible reason for this lack of positive signals could be the age of the extract samples. When samples were first analysed using counter immunoelectrophoresis methods, they were freshly made. However it was several weeks before they were studied using the methods described here. In addition it was reported that these samples gave relatively weak signals when run on counter immunoelectrophoresis (CIE) gels and it is reasonable that the lower limit of detection of CIE technique may exceed the lower limit of the immunostaining method.

One final possible source of error/reason that no results were obtained is that this represents the first time the kininogen immunostain was attempted and so some user error is possible. It is known that the lack of a positive result was not caused by a failure in transfer of proteins across to the membrane because the PVDF was treated with Ponceau red stain following the blotting step and several bands were clearly seen on the membrane. In addition, a comparison between the blotted and unblotted gels indicated that the majority of the bands had been transferred from the gel.

Initially it had been hoped that immunostaining would be able to indicate samples of concentrations in the order of 0.025mg/ml and thus have comparable sensitivity to counter immunoelectrophoresis. However, the lack of staining of the sample shown in the above figure seemed to indicate that maybe sensitivity was not this high. It was also the aim of this experiment to indicate the specificity of the immunostain with an ideal outcome being that the very low concentration kininogen was stained whilst the higher concentration albumin and lactoferrin sample lanes remained clear.

Unfortunately, because absolutely nothing stained it meant that the specificity needed to be confirmed by a separate study. Positive results from such studies would also remove the worry that maybe the antibody was degraded or non-functional due to some error in shipping or storage which could be another source of error. As a result of needing to assess specificity, sensitivity and viability of the antibody and therefore the technique before applying it to any further precious samples, it was deemed essential to carry out a series of experiments to determine whether the immunostain was specific for kininogen and to obtain a guide to its lower limits of detection. These experiments are described in Section 7.4.

7.4 Analysis of Sensitivity and Specificity of Available Antibodies to Kininogen

When using antibodies for visualisation of proteins on Western Blots, the sensitivity and specificity of the antibody is all important. Commercially available antibodies will vary in these two parameters, although most will have been optimised for both factors to ensure customer satisfaction. Although we had previously determined overall sensitivity of immunostaining, using antibodies raised against albumin and lactoferrin, it was felt that it was important to complete a similar trial on the antibodies that were to be used in kininogen immunoanalyses. This seemed particularly important after the disappointment encountered when trying to confirm results obtained via immunodifussion studies described earlier. In addition, the success of transfer of kininogen from gel to blot was also measured by observing gels that had been blotted and then Coomassie stained with those that had not been subjected to blotting. The success of transfer can be observed by a direct comparison of Figures 7.2 and 7.3 below.

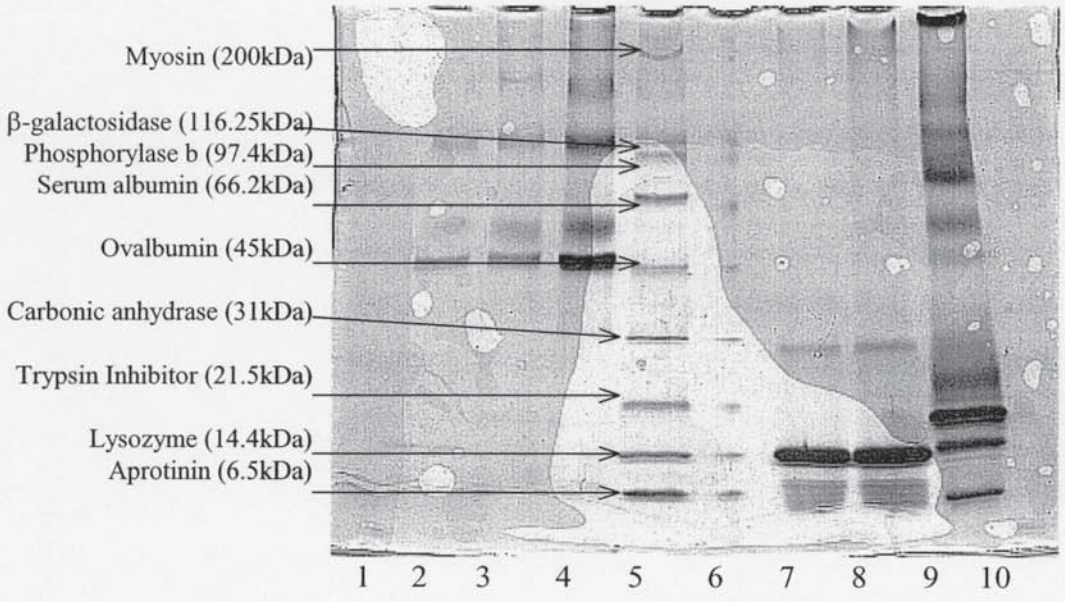


Figure 7.2 Gel run under optimal conditions showing the position of kininogen. The gel was loaded with: Lane 1 = Empty, Lane 2 = 0.5 mg/ml Kininogen, Lane 3 = 1 mg/ml Kininogen, Lane 4 = 2 mg/ml Kininogen, Lane 5 = Broad band MW markers, Lane 6 = Acuvue Janet 1 day No. 1 (1 month old extract), Lane 7 = Fresh extract of Acuvue Janet 1 day (Left lens), Lane 8 = Pooled Acuvue fresh extracts, Lane 9 = Ais tears collected via microcapillary, Lane 10 Empty

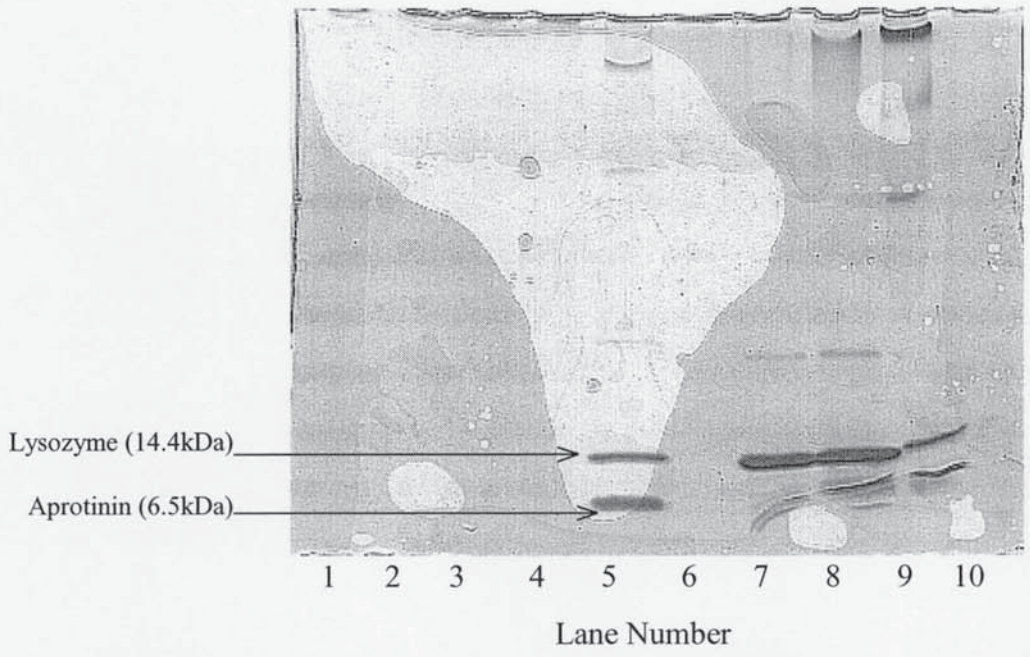
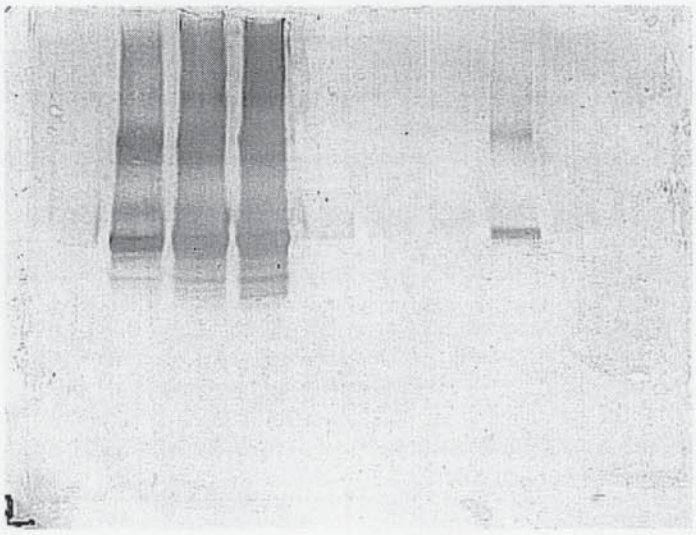


Figure 7.3 Blotted gel loaded and run identically to that seen in Figure 7.2

Transfer of the kininogen bands can be seen to have been highly efficient with complete disappearance of the bands that correspond to kininogen from the blotted example. In addition to highlighting the success of transfer, it can be seen that the above gels were loaded with serial dilutions of kininogen (0.5mg/ml, 1.0mg/ml and 2.0mg/ml). Following the transfer step this allowed the sensitivity of the immunostaining to be determined. The inclusion of a number of lens extracts and tear samples collected via micro capillaries meant that along with evaluation of the method an initial study of the presence of kininogen in tears/extracts could be made.

The results of this experiment are shown in Figure 7.4 and indicate that kininogen concentrations as low as 0.5mg/ml can be easily detected. Indeed the intensity of the staining of the 0.5mg/ml sample makes it reasonable that lower concentrations are also likely to be detectable. In addition to the clear staining of the standard protein samples, a small but significant amount of kininogen was also seen to be present in the pooled extract sample run in lane 8. Very faint bands in lanes 5, 6 and 7 also seem to show the existence of kininogen in these samples, but staining is not intense enough to be truly confident about this conclusion. The possibility is that this is background staining or non specific and so these results would need to be checked by further runs of these samples.



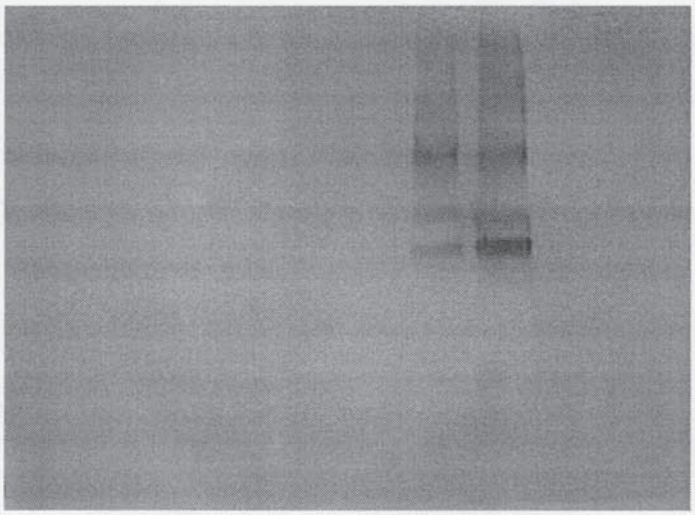
1 2 3 4 5 6 7 8 9 10

Lane Number

Figure 7.4 Western Blot of the gel depicted in Figure 7.3 which has been immunostained for kininogen. Note the three clearly stained bands in Lanes 2,3 and 4 that correspond to the kininogen standard solutions. In addition, the sample in lane 8 which is a pooled extract also stains positively and some very faint staining can be observed in lanes 5, 6 and 7.

Having shown that the immunostain was sufficiently sensitive for our purposes with a lower limit under 0.5mg/ml, it was also thought to be appropriate to test the specificity of the antibodies. The method used to determine this was to load a gel with a number of extracts and tear samples along with standard solutions of kininogen of 0.25mg/ml and 0.5mg/ml concentration and an albumin standard solution of 2mg/ml. The aim was to run and blot these samples and complete the immunostaining step using the kininogen antibody. If the albumin sample was also seen to stain following incubation with the antibody, no matter how faintly this was, then we would know that staining was not kininogen specific. If, however, the albumin sample did not stain we could progress with our studies confident that the samples we were seeing positive kininogen results for did indeed contain kininogen itself.

This assessment of specificity also enabled us to further explore the sensitivity of the immunostain. Two kininogen samples were run on this gel, one of 0.5mg/ml the other of 0.25mg/ml. Again, both of these dilutions can be clearly seen enhancing our previous knowledge as to the theoretical lower limit of detection provided to us. Such a sensitive proven lower limit of detection increases the confidence when determining the presence or otherwise of kininogen in our samples.



1 2 3 4 5 6 7 8 9 10

Lane Number

Figure 7.5 Evaluation of kininogen antibody specificity. This Western Blot has been immunostained for kininogen. The gel this blot was taken from was run under the usual optimised conditions and loaded with: Lane 1 = Empty, Lane 2 = Ais tears collected with microcapillaries, Lane 3 = Acuvue Janet 1 day extract, Lane 4 = Pooled Acuvue Janet 1 day extracts, Lane 5 = Hydron Sharen extract 3 years continued wear, Lane 6 = Broad MW markers, Lane 7 = Kininogen (0.25 mg/ml), Lane 8 = Kininogen (0.5 mg/ml), Lane 9 = Albumin (2mg/ml), Lane 10 = Empty. The staining pattern seen shows that there is no cross reactivity between kininogen and albumin. This is clearly indicated by the fact that the kininogen samples are both darkly stained whilst an albumin sample of much greater concentration is not stained at all.

As can be seen from the membrane shown in Figure 7.5, the albumin did not stain at all despite its high concentration and so it appears that the antibody is specific enough for our experiments in addition to being sensitive enough.

The next step was to apply this method to the study of a number of samples. The results seen for the extracts on this example were disappointing, but were not the same batch as those used in the gels from Figures 7.2-7.4 which explains the variation. We would have hoped to see the presence of kininogen in the 'Pooled Extract' sample in particular following its intense staining in the previous example.

Overall, the results clearly show that the antibody is highly specific when determining between albumin and kininogen and that it will be possible to detect kininogen in samples containing concentrations of the protein as low as 0.25mg/ml.

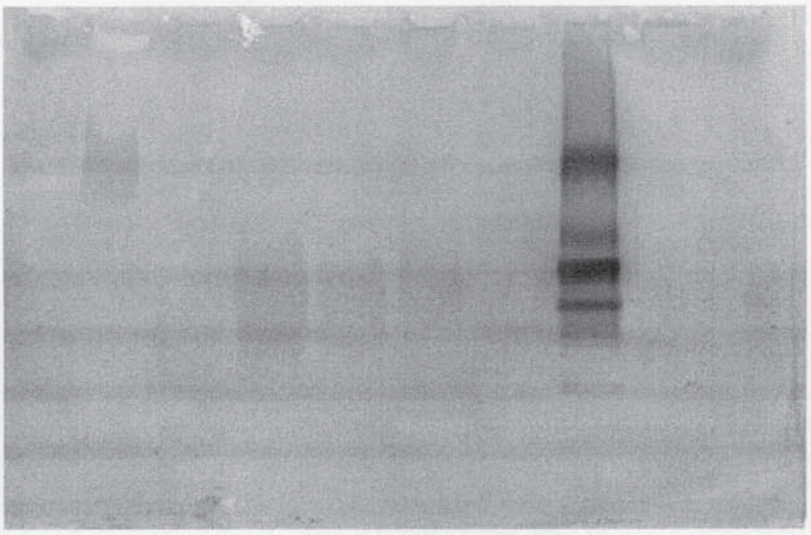
7.5 Analysis of Lens Extracts and Tear Samples for the Presence of Kininogen

The Western Blotting and immunostaining method involving this antibody raised against kininogen had now been shown to be suitable for analysing samples which contained relatively small concentrations of kininogen. In addition, confidence could be placed that positive results were due solely to kininogen and not to other components of the tear or extract.

The next step was to analyse some tear samples and extracts to determine the distribution of kininogen (i.e. which types of lenses produced extracts which tended to contain kininogen) and to attempt to establish the existence of kininogen in the basal tears of a non contact lens wearer. The following figures outline the results of this study.

From the results shown it is clear that the positive result seen for kininogen in tears is different in some way to that seen for the extracts and standard solution which was included as a positive control. The band is stained a different colour as clearly demonstrated in Figure 7.7 and the reason for this is thought to be a result of the very low concentration represented. The actual colour of the BCIP/NBT staining reaction used in our immunostaining technique should be blue (as described in Chapter 2.11).

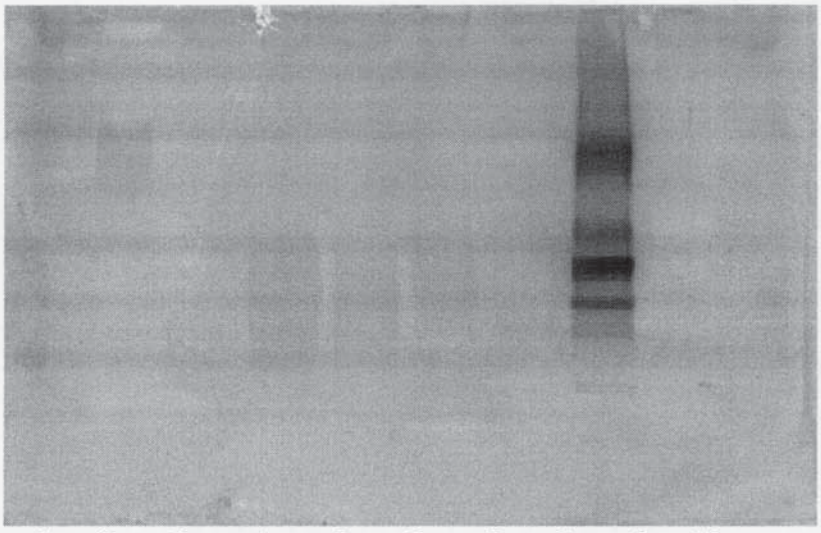
The reason that other bands appear almost black in colour is because they represent relatively high concentrations. The positive result for kininogen in tears being a light blue is indicative of this being a much lower concentration of kininogen and suggests a reason why the presence of this protein in tears is not widely reported.



1 2 3 4 5 6 7 8 9 10

Lane Number

Figure 7.6 Positive demonstration of the presence of kininogen in a tear sample. Lanes 1 and 10 = Empty, Lane 2 = Ais tears collected via microcapillaries, Lane 3 = Ciba 'Dailies' Janet 1 day wear, Lane 4 = Purevision Julie 1 month continuous wear, Lane 5 = Hydron Sharen 3 year continued wear, Lane 6 = Acuvue Janet 1 day wear, Lane 7 = MW markers, Lane 8 = Kininogen (0.2mg/ml), Lane 9 = Albumin (2mg/ml). From these bands it is clear that kininogen is present in tears and in the lens extracts. It would appear from the difference in intensity of the staining that kininogen is more concentrated in the tear sample.



1 2 3 4 5 6 7 8 9 10

Lane Number

Figure 7.7 Colour version of Figure 7.6. This colour scan has been included to show the difference in colouration between the kininogen band seen in the tear sample and those seen in the extracts. The molecular weights of the various bands were subsequently calculated and this data can be found in Section 7.6.

The other obvious difference is in the position of the positive band. In all the other positive results seen the stained species is about halfway down the blot. With the tear sample on the other hand the band is much closer to the top. With regards to the technique of SDS polyacryalmide gel electrophoresis, the further a protein migrates in a gel the smaller it is with larger species being retained closer to the top. In this case, the tear sample result would appear to represent either an intact form of kininogen compared to a degraded or denatured form produced in the lens extracts or possibly a dimerised form. Both of these would cause the kininogen species in the tear sample to have a higher molecular weight and so would explain the difference in the distribution of the positive results.

Another possibility for the difference is that the binding of kininogen to the lens may bring it into close proximity to some other protein or proteins that could trigger cleavage of kininogen into its' active form. Free circulating kininogen is less likely to encounter such a protein and so remains intact. In other words, the contact lens surface may act as a binding and activating site for kininogen.

In a further observation, by comparing the band seen with the tear sample to the four bands generated by the standard kininogen solution there is a corresponding band in the pure standard solution so it is unlikely that the result for the tear sample represents anything other than kininogen. In other words it is not an erroneous result. The bands seen for the extracts also correspond to a band seen with the standard solution, so we can be confident that all our results are true positives.

In order to assess what has generated the difference in the bands between the tear sample and the extracts it was decided to calculate the molecular weights of all the positive bands.

7.6 Measurement of the Molecular Weight of the Positively Stained Bands

As mentioned in the previous section, it was decided to determine the cause of the difference seen between the tear samples and the lens extracts by calculating the molecular weights of the observed bands. In order to do this a graph has to be generated using the values of log molecular weight and the relative mobility of the protein (also known as the Rf value) for the molecular weight standards that are run on every gel. Once this plot has been generated, the molecular weights of any unknown bands can be calculated by measuring their Rf value and cross-referencing this to a log Molecular Weight value and so to an actual Molecular Weight.

In this example, the molecular weight markers used to generate the graph were taken from the gel that was run in parallel with that which was blotted and subsequently immunostained to give the results seen above. The molecular weight markers from the blotted gel were not available as a direct result of the blotting. These values can be used with extremely high confidence because the two gels were loaded with identical samples, run under identical conditions at the same time and so the pattern generated for the unblotted gel would be nearly identical to that which would have been seen with the blotted gel. The gel used to generate the graph is shown in Figure 7.8, the graph itself is depicted in Figure 7.9 and the Rf values for the standard markers and the bands seen in the immunoblot along with the known and calculated Molecular Weights can be found in Table 7.1.

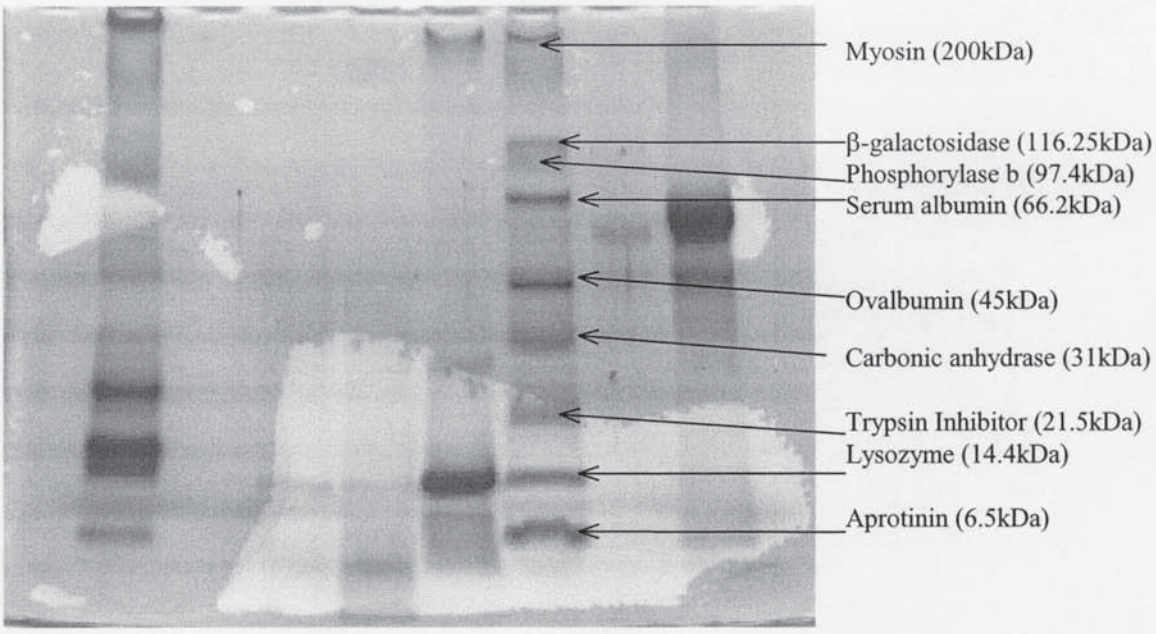


Figure 7.8 Gel used to generate values for the calculation of the Molecular Weights of the bands seen on the positive kininogen immunoblot. This gel was loaded and run identically to that used to generate the immunoblot seen in Figure 7.6. The Molecular Weight markers used to generate the calibration curve are in Lane 7.

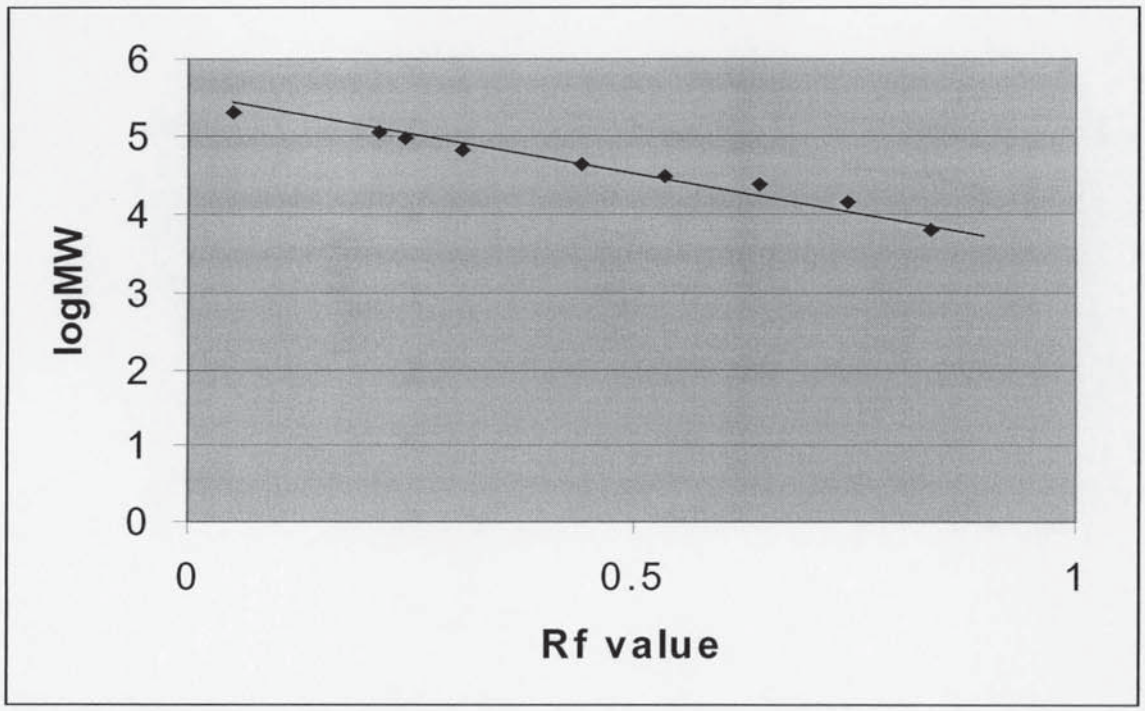


Figure 7.9 Graph showing logMW versus Rf for the Molecular Weight markers in Figure 7.8. This graph was used to calculate molecular weights of the positively stained bands seen on the kininogen immunoblot by measurement of their Rf values.

Name of protein/band	Rf value	LogMW	Actual MW (kDa)	Calculated MW (kDa)
Myosin	0.050	5.30	200.00	
β-galactosidase	0.214	5.06	116.25	
Phosphorylase b	0.243	4.99	97.40	
Serum albumin	0.307	4.82	66.20	
Ovalbumin	0.443	4.65	45.00	
Carbonic anhydrase	0.536	4.49	31.00	
Trypsin inhibitor	0.643	4.40	21.50	
Lysozyme	0.743	4.16	14.40	
Aprotinin	0.836	3.82	6.50	
Positive band in Tears	0.245	5.08		118.85
Positive band in Purevision extract	0.493	4.55		35.48
Positive band in Hydron extract	0.493	4.55		35.48
Positive band in Acuvue extract	0.493	4.55		35.48
Kininogen Standard Band 1	0.243	5.08		118.85
Kininogen Standard Band 2	0.300	4.93		84.14
Kininogen Standard Band 3	0.436	4.70		50.12
Kininogen Standard Band 4	0.493	4.55		35.48

Table 7.1 Rf values and calculated Molecular Weights for the bands seen in the immunoblot shown in Figure 7.6.

The previously reported values of the molecular weight of kininogen as described in Chapter 1 say that there are two forms, a low molecular weight form of 68kDa and a high molecular weight version of 120 kDa. The calculated value for the molecular weight of the band that stained positively in the tear sample was 118.85 kDa which allowing for slight margins of error in measurements is incredibly close to the value of the high molecular weight form of kininogen. This is further clear evidence to support the fact that kininogen is present in tears in an intact form.

With regards to the bands seen with the contact lens extracts, the calculated molecular weight was 35.48 kDa which would appear to correspond to neither of the established forms of kininogen. So what do these bands represent?

The most obvious answer would be that these bands represent a breakdown product or subunit of the intact kininogen produced as a direct result of the harsh extraction process. By looking at the bands produced by the 0.2mg/ml kininogen standard solution it can be seen that the 35.48 kDa band is also present in the standard protein. Further manipulation of the values obtained from calculating the molecular weights of the four bands seen with the kininogen standard provide us with one explanation of what these bands represent. Band 1 would appear to represent the intact high molecular weight form of kininogen, with bands 3 and 4 representing the subunits of this protein. The molecular weight calculated for Band 2 is very close to the molecular weight of one Band 3 added to one Band 4, and the molecular weight of Band 1 is equivalent to either one Band 3 added to two of Band 4 or Band 2 plus Band 4.

One posibility is that the structure of kininogen consists of three subunits, one of 50kDa and two identical subunits of 35kDa each and that the bands seen with lens extracts are the 35kDa subunits. The lack of the 50kDa subunit in the extracts could mean that this is too strongly attached or too deeply imbedded into the contact lens for removal whilst the 35kDa subunits were only attached to the lens because they were linked to the 50kDA subunit. Upon extraction therefore the link between the 35kDA subunits were broken freeing them up into solution were they could be analysed and give a positive signal while the 50kDa subunit was left behind.

One other possibility is that only the active, smaller breakdown product (35kDa subunit) is capable of sticking/binding to a lens. Cleavage of kininogen into its' active form may expose some site on the 35kDa chain that has an affinity for the lens. In the intact form this site might be covered by other parts of the protein or be hidden by some structural feature that is lost when the protein is cleaved. In this case the 50kDa subunit would never have been associated with the lens if it doesn't possess the same functional group and this would provide another reason why this subunit is not seen in the extract.

In summary, our results show that the kininogen seen in tear samples is the intact, fully functional high molecular weight species and so conclusively proves that kininogen does exist in the basal tears of non contact lens wearers. In addition, it appears that the structure of kininogen consists of three subunits (one of 50kDa plus two of 35kDA which may or may not be identical) which combine together to give the 120kDa high molecular weight form.

Finally it would appear that there are a couple of potential explanations as to why we only see the 35kDA subunits in the lens extracts. One possibility is that the 50kDa domain is what actually binds to the contact lens so when extractions are made this subunit remains tightly bound and is left behind. The 35kDa species are only bound to the lens via bonds with the 50kDa subunit and when extraction occurs these bonds are broken, freeing the 35kDa subunits. Alternatively, there may be a different explanation which is also based on affinity of the subunits for the lens. Upon cleavage caused by lens wear the 35kDA subunit may become attracted to the lens due to exposure of some reactive site. The 50kDa subunit is not attracted because it does not have this particular feature. Both these hypothetical models would explain the absence of the 50kDa subunit from the extract samples but are based on very different ideas of which subunit actually binds to the lens.

The actual cause of the differences seen between the lens extracts and the tear samples would need a great deal of further investigation that is beyond the scope of this research project.

7.7 Discussion

The results achieved by the application of Western blotting and the antibody based staining of kininogen to both tear samples and contact lens extracts were remarkable for a number of reasons. For example, the ability to demonstrate the presence of kininogen in lens extracts using an additional technique to the previously successful counter immunoelectrophoresis studies[62] enhances the validity and importance of the results derived using both of these methods. To be able to obtain the same data using a number of techniques is clearly encouraging when considering the legitimacy of the result.

Overall, it would appear that the counter immunoelectrophoresis method is significantly more sensitive than immunostaining with sensitivity around 0.01mg/ml compared to that of immunostaining which is somewhere under 0.2mg/ml, although the ultimate lower limit of detection was never determined conclusively. This raises the question: Why use immunostaining if it is not as sensitive as an existing technique? The answer to this lies in the fact that immunostaining of Western blots has some very tangible benefits of its own that counter immunoelectrophoresis simply cannot provide.

One major benefit is in the ability to run two gels which have been loaded with aliquots of the same sample in parallel and then to use one for blotting and immunostaining whilst the other is stained using a method such as that involving Coomassie Blue. This means that whether a particular protein is present in a sample or not can be studied in comparison to the overall pattern of proteins in that sample and so broad relationships can be postulated. In addition, the technique of counter immunoelectrophoresis is unable to report the presence of a number of subunits of a particular protein on one gel or whether distinct, different species were generating a positive result. It also cannot be used to calculate the molecular weight of the positively staining entity unless it is assumed that the sample giving the positive result is the intact protein. The danger of making such an assumption is clearly shown by the difference between kininogen positives from tears and lens extracts reported in this Chapter.

In a very clear demonstration of this point, results like those seen in Figure 7.6 where distinct bands/species all give a positive result but of varying molecular weights would not be possible with counter immunoelectrophoresis. All that would have been measurable would be that kininogen was present in each of the samples. None of the different bands would have been shown, the variation between the tear sample and the lens extracts would not have been highlighted and the existence of four bands in the standard solution would also have been missed. The ability to measure the molecular weight(s) of what has given a positive signal is very important in terms of achieving a complete picture of what is happening in the ocular environment and so the value of a method which can provide this data is obvious. The potential application of this technique to further studies where positive results obtained using counter electrophoresis could be derived from more than one protein or subunit of a protein is very exciting.

Another reason that the results described in this chapter are so exciting is in the positive result obtained with tear samples collected using microcapillaries. To our knowledge this is the first time that such a result has been achieved which clearly demonstrates the presence of the intact form of high molecular weight kininogen in a tear sample using this particular combination of techniques. The value of such a result comes from the knowledge of the part that kininogen plays in inflammation and allergy (see Chapter 1). It is possible that studies of a patients tears using these methods may provide a diagnostic tool for determining the existence of an allergy without the need for painful or invasive tests such as drawing a blood sample.

In addition to the contribution of this method to our knowledge of kininogen in the tears there could be some value in this technique in terms of testing patients for protein species that could lead to contact lens intolerance. Prior to dispensing lenses an optician could take a tear sample and analyse it for any proteins that were indicators of such a problem. The decision whether to recommend lenses could then be taken based on the results obtained. Existing lens users presenting with various diseases could also be tested to check for any change from normal levels of proteins

such as kininogen and this could be used to devise strategies for treating contact lens associated diseases and disorders.
For any of these possibilities to become reality a great deal of further work would be needed and a large number of clinical samples would need to be analysed. Ultimately, however, the techniques are in place to enable such a study.

One final area where the results generated by immunostaining of these samples for kininogen is of particular merit is in the difference observed between the positive band seen for tears and that seen in the lens extracts. On one hand this variation could be interpreted as an explanation of the method by which kininogen is taken up by the contact lens. The bands seen in the extracts could represent breakdown products of kininogen caused by the extraction method while other parts of the protein remained either tightly bound to the surface or too deeply embedded in the body of the lens to be removed.

On the other hand, the bands seen for kininogen with the extract samples show a higher concentration of the protein than that seen in the tears of a non contact lens wearer. This result demonstrates a possible pathway by which lens wear might cause increased inflammation and by which some lens wearers might become intolerant of their lenses. The higher level of kininogen could be due to the contact lens removing kininogen from the tears and concentrating it in the lens. In the tears of non lens wearers, the kininogen is free to circulate and the concentration will remain relatively constant as the tears are regenerated and passive transport occurs across the blood tear barrier. In lens users the release of kininogen will remain at normal levels but instead of being removed during regular tear turnover events the kininogen will be trapped in the tear film by the lens.

It can be seen from this theoretical course of events that the longer the lens is in place, the higher the concentration of kininogen will become and the increased probability of an inflammatory or allergic episode. This could obviously result in the symptoms described for various contact lens associated problems discussed in Chapter 1. Understanding this potential cause of lens wear problems could enable opticians to suggest methods which would alleviate the problems such as shortened wear periods,

more thorough cleaning of lenses, use of anti allergy medication, etc. although again much further study is needed.

In conclusion, the results we have obtained indicate that this technique provides an invaluable extra dimension to antibody based analysis methods in that it allows the nature of the protein that has generated the positive result to be more comprehensively explored. In addition we have shown the presence of kininogen in the tears of non lens wearers conclusively for the first time using this particular combination of methods and tentatively explored the relationship between the kininogen species seen in tear samples to that found in lens extracts. Further, we have applied this observed difference to explanations of the nature of kininogen's binding/take up by contact lenses and the putative role of kininogen in lens wear intolerance.

These results open up a number of areas of research which have great potential in the study of the impact of the contact lens on the tear film and ocular environment and the tools needed to carry out these studies have all been optimised and are in place.

Chapter 8

A Novel Method for the Removal of the 'Tear Envelope' From Worn Contact Lenses

8.1 Aim

The aim of this Chapter was to devise an alternative method or methods that would enable us to remove the 'tear envelope' for subsequent analysis via the electrophoresis methods described previously. By tear envelope we mean the portion of the tear film that is left attached to the contact lens upon removal from the eye (see Figure 8.1).

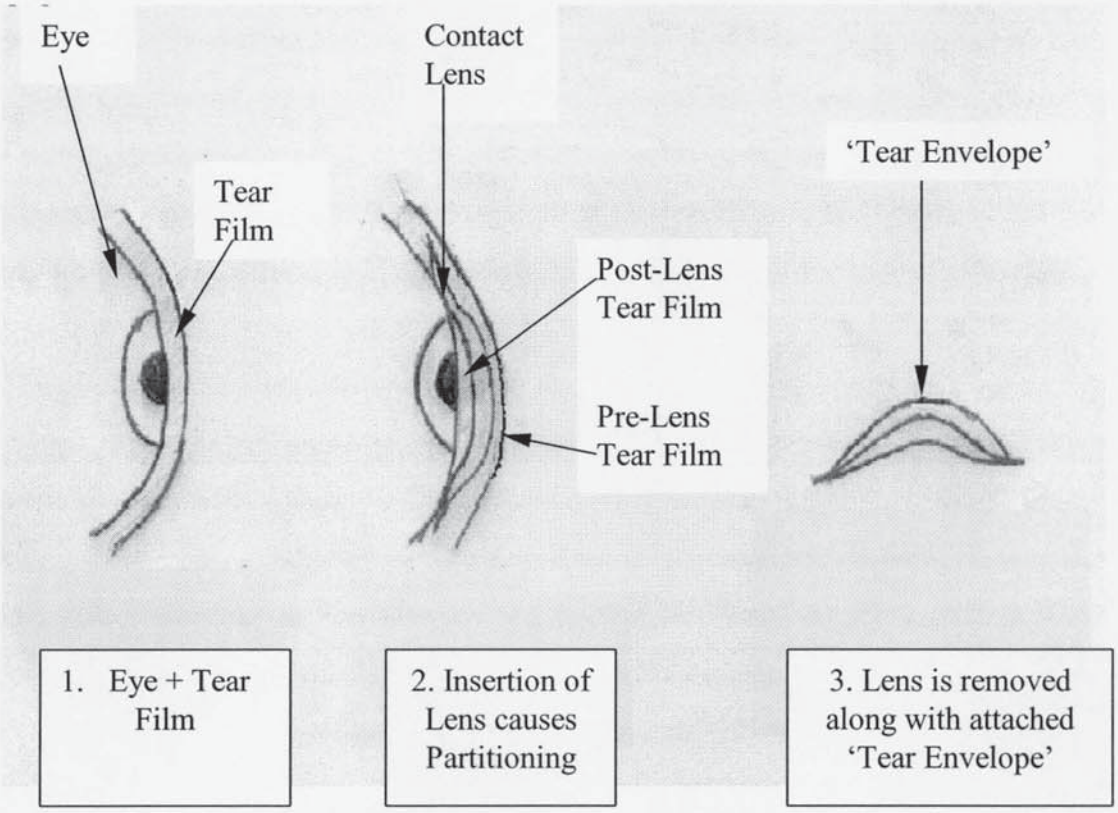


Figure 8.1 The 'Tear Envelope'. This figure is meant to be illustrative and is not to scale. In real life the lens would be comparatively much thicker than indicated.

The reasons for exploring this alternative to the extraction method outlined in Section 2.4 include the relative harshness of the extraction method, which is bound to have an influence on the protein pattern observed causing native proteins to be broken down into separate chains/subunits. Another reason for exploring an alternative to extraction is the fact that the extractions include proteins that have been absorbed into the lens matrix. Such proteins would not normally be present in samples taken of the tear film or tear envelope of a lens wearer and so their presence in lens extracts gives a misleading picture of the actual nature of the relationship between the lens and tears.

Extracting proteins from a lens involves an incredibly stringent method featuring extended periods of boiling and destructive chemical treatments. For previous work on the influence of the lens on the tears and the nature and quantity of proteins removed into the lens matrix by different types of lens materials this method was ideal[62]. However it did not suit our needs due to the influence on protein pattern and the fact that the proteins taken up by the lens are what is ultimately studied rather than the tears.

Our need was for a kinder method which would allow removal of the 'tear envelope' with the minimal amount of interference to the pattern of proteins seen and left those proteins taken up by the lens inside the lens matrix. The idea was that such a method would show a more accurate picture of the pattern of proteins in the tears of contact lens wearers and would allow this pattern to be more easily studied in terms of lens users greater tendency to suffer from a variety of ocular diseases. A comparison of the characteristics of each sampling method can be found in Table 8.1.

In summary the aim was to allow the analysis of the tears associated with the surface of a contact lens rather than the analysis of the proteins that had been removed from the tears and taken up into the lens. A number of methods were investigated for the removal of the tear envelope of which the two most successful are described in this chapter.

8.2 Removal Method A

The first partially successful method for envelope extraction was carried out using immersion of the lenses into 2X buffer immediately upon removal from the eye, followed by maximisation of the contact between the lens surface and the buffer and subsequent centrifugation to dislodge any buffer that clung to the lens. The problems that had to be overcome mostly centered around the incredibly small volume of tears to be dealt with and the fact that the tear film on a lens taken from the eye would quickly rupture and/or evaporate making obtaining a viable and worthwhile sample difficult. This meant that the lens had to be rapidly placed into buffer directly following removal. The other big dilemma involved choosing the optimal volume of buffer to use. Too much 2X buffer would lead to poorly visible protein bands upon Coomassie Blue staining whilst too little buffer would end up either drying out in the tube or just being absorbed by the lens.

In practice a series of volumes of 2X buffer was added to a number of 2ml eppendorf tubes. These volumes were 50µl, 100µl, 150µl and 200µl. When the lens was removed from the eye it was pushed into the buffer in the bottom of the tube so that as much of the lens was covered as possible. The lenses were then left in the buffer until samples were to be run. At this time the lenses were again pushed into the buffer before being whirlimixed for ten seconds to try and get as much of the lens in contact with buffer as possible. The tubes were then left to stand so that the majority of the liquid collected in the bottom of the tube. This pattern of whirlimixing and standing was repeated up to five times at which point the liquid was drawn off using a 100µl pipette and put into a fresh tube.

It was seen that some liquid remained attached to the lenses, an observation made easy by the blue dye component of the 2X buffer and so it was decided to try and remove this residual sample material by use of a centrifuge. The lenses were placed on top of a porous support in a 5ml sample tube and spun at 2000g for ten minutes (see Figure 8.2). The liquid displaced by this treatment was then added back to the corresponding tube containing the liquid drawn off previously.

Samples were then whirlimixed to ensure the two liquids were well mixed together before each sample was treated as usual for loading (i.e. boiling at 95°C for five minutes) and run on 1-D electrophoresis gels. An example of the pattern obtained using this method is shown in Figure 8.3.

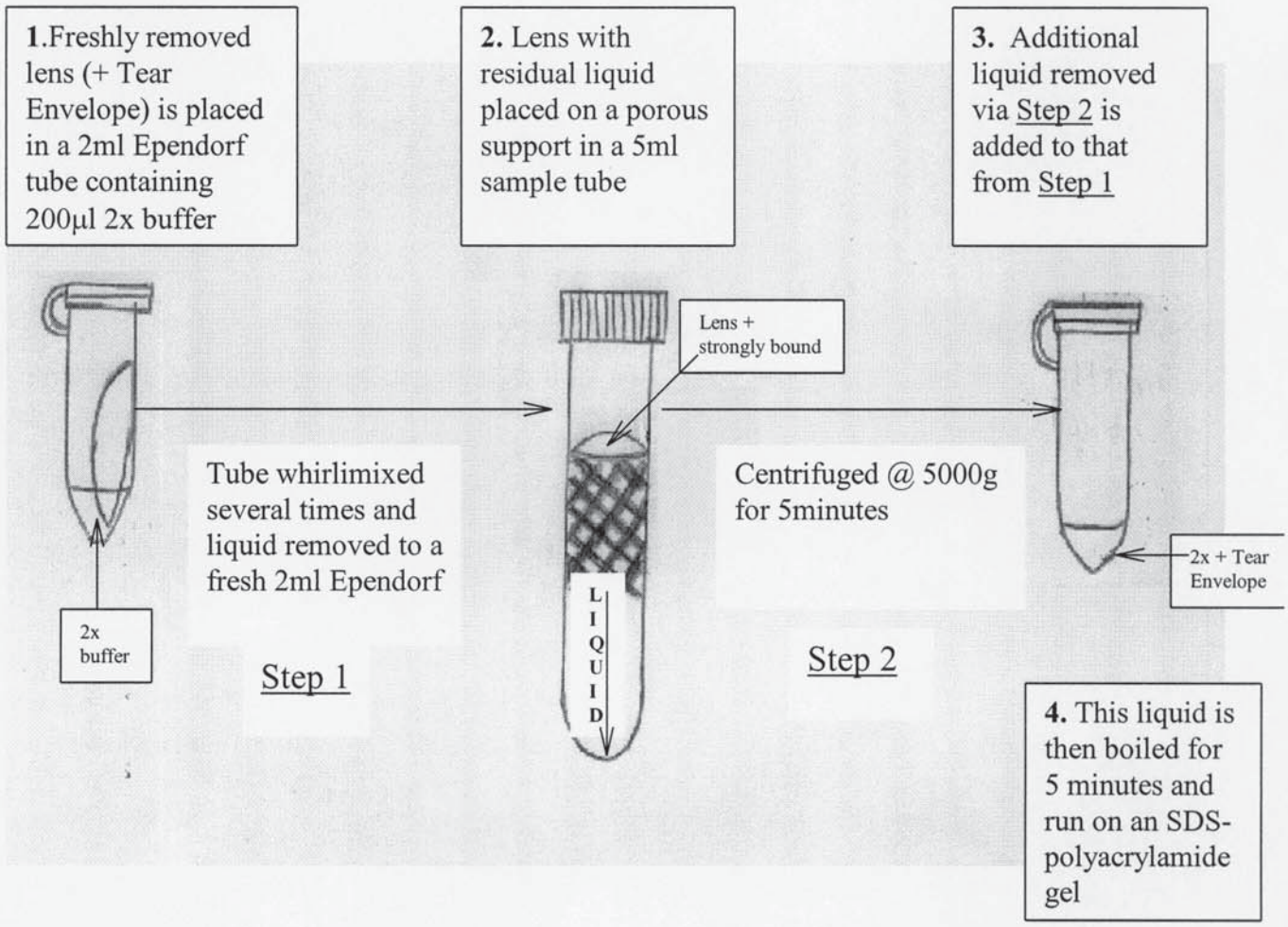


Figure 8.2 Representation of Method A for removal of the tear envelope

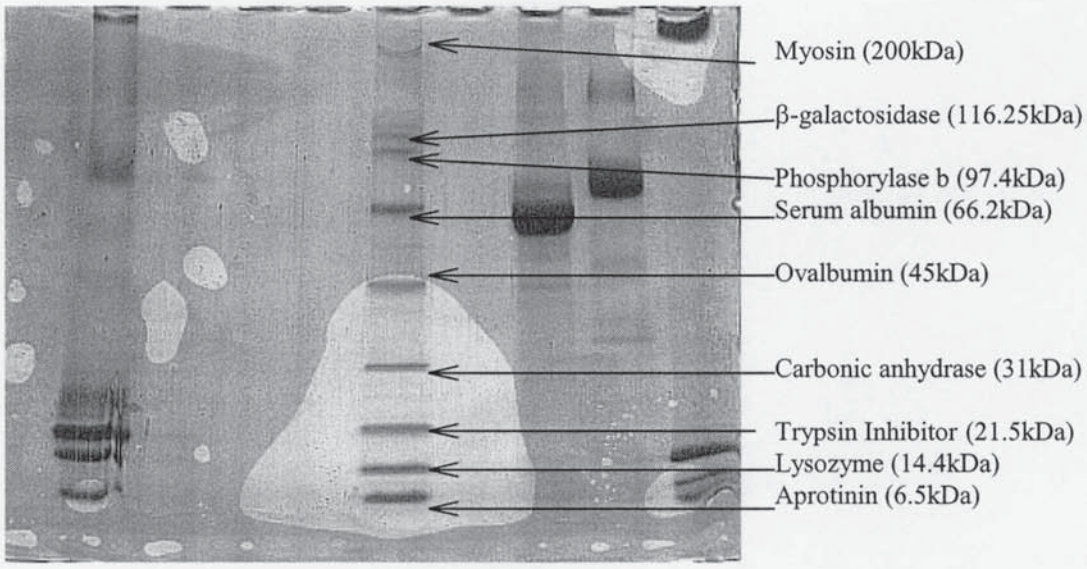


Figure 8.3 Typical example of sample collected via Removal Method A. The samples represented here are: Lane 1 = EMPTY, Lane 2 = Ais tears collected via microcapillaries, Lane 3 = Ciba vision daily disposable extract concentrated twice, Lane 4 = Untreated Ciba vision daily disposable extract, Lane 5 = Ciba vision 1 day Janet extract, Lane 6 = Broad MW markers, Lane 7 = 0.025 mg/ml Kininogen, Lane 8 = 2mg/ml Albumin, Lane 9 = 2.5 mg/ml Lactoferrin, Lane 10 = Sample collected by treating a freshly worn Acuvue 1 day disposable contact lens of a different type with Removal Method A. All samples were treated with an equal volume of 2X buffer and boiled for 5 minutes. They were then run at 30mA and subsequently stained in Coomassie Blue for 2 hours and destained overnight.

8.3 Assessment of Method A

As can be seen in Figure 8.3 this method for envelope removal was viable to some degree. By comparison to the protein pattern seen for fresh tears collected using microcapilaries, it is clear that a lot fewer bands are seen (Lane 2). However there are a few distinct bands which indicates that some degree of tear protein removal has occurred from the lens. By contrasting this pattern to those generated by the extraction method on a lens of the same material and make worn for a comparable period, it is clear there is a similar number and distribution of bands. Whilst this data is not shown here, reference to the Figures depicting extraction samples of Acuvue lenses shown in Chapter 6 clearly highlight this fact.

In conclusion it would appear that this method merits further investigation as to its utility in monitoring the effect of lenses on tear protein patterns. It allows a method of removal that is much less destructive to the proteins themselves and should only allow removal of proteins from the lens surface not embedded in the lens. However there were one or two problems when applying this method. The biggest problem was that the forces exerted on the lens coupled with the large holes in the porous support tended to lead to the lens being shredded. This could lead to small amounts of lens material ending up in the sample liquid that might give erroneous results and/or affect the proteins in the sample. In addition the size of the eppendorf was large compared to the volumes of liquid involved leading to some of the sample drying onto the tube surface. The only way to remedy this would be to redissolve the lost sample by addition of more water and hence result in sample dilution. Overall this method was promising but required further refinement before it could be considered truly useful.

8.4 Removal Method B

Following on from the results we achieved using the method described above it was decided that certain aspects should be adjusted in order to maximise the potential of what was a promising technique. Firstly, the size of the tube to which the 2X buffer and the lens would be added was changed. Instead of the 2ml eppendorf used previously, a 0.5ml tube was chosen. Although this caused some handling difficulties for the lens wearers in terms of placing the lens in the tube, the advantages of minimising evaporation of the sample and increasing the proportion of the lens immersed in buffer were produced. The cause of more lens being immersed was that an equal volume of buffer in a smaller tube produces a greater depth in which to dip the lens. In a couple of samples the volume was varied to assess the ideal volume to use.

Following immersion a similar pattern of whirlimixing and standing of the tube to that explored in Method A was used to ensure that the lens surface was well rinsed with buffer. At this stage the standing liquid was again drawn off with a pipette and moved to a fresh 0.5ml tube.

The main difference in this method aside from the choice of sample tube size was in the method used to remove the stubborn portion of liquid that remained associated with the lens even after standing. Now rather than remove the lens and put it in a 5ml tube on a support, a hole was made with a pin in the 0.5ml eppendorf and this was stood in a 2ml tube. A schematic of the whole process is shown in Figure 8.4. The two tubes were then spun at 5000g for five minutes and displaced liquid added back to the corresponding sample of liquid isolated earlier. These were briefly whirlimixed to ensure complete integration of the samples.

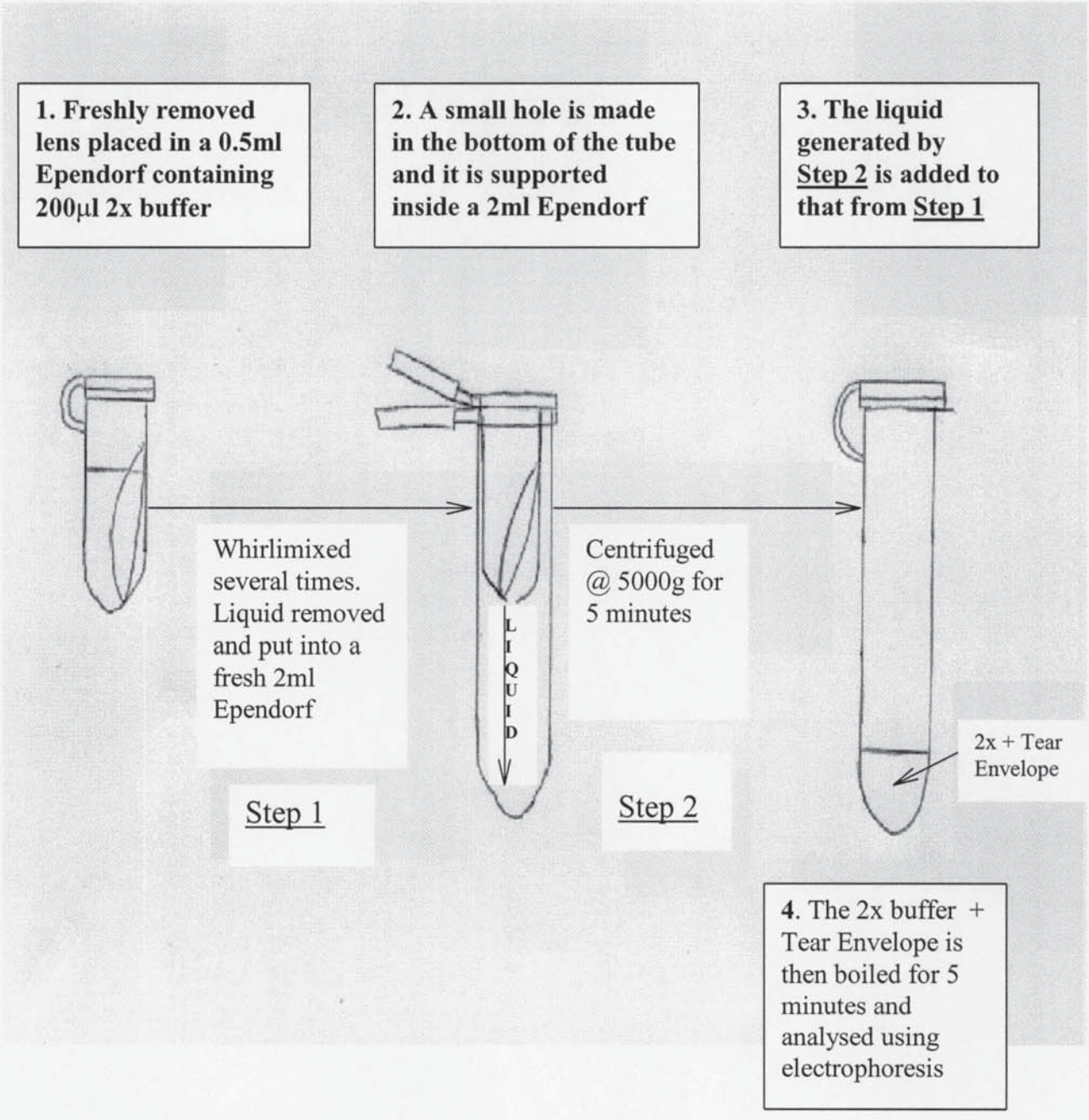


Figure 8.4 Removal Method B

Following collection and treatment the liquid was run as normal on SDS polyacrylamide gels. Figure 8.5 shows a direct comparison between a sample produced by the extraction method and this method of 'removal'.

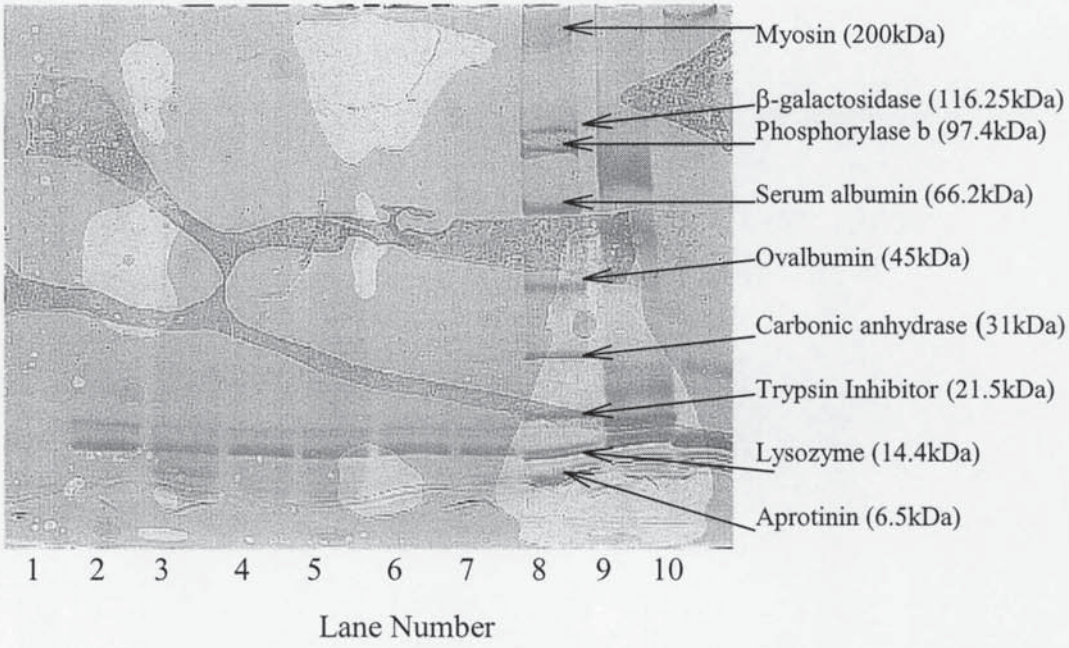


Figure 8.5 Comparison of a 'traditional' extract to samples produced using removal method B. The lanes on this gel are loaded with: Lane 1 = Empty, Lanes 2-7 = Alternative extractions of Janet Lenses, Lane 8 = Broad MW markers, Lane 9 = Ais Tear Sample collected using microcapillaries, Lane 10 = Janet Acuvue extraction. Insufficiently cleaned staining trays caused background staining. There are clear bands in the tear sample, the lens extract and the samples produced by 'tear removal' using method B.

In addition to the assessment of this method shown in Figure 8.5, this method was repeated and samples were run on a gel with a number of extracts from a variety of lenses. The results of such a run are depicted in Figure 8.6.

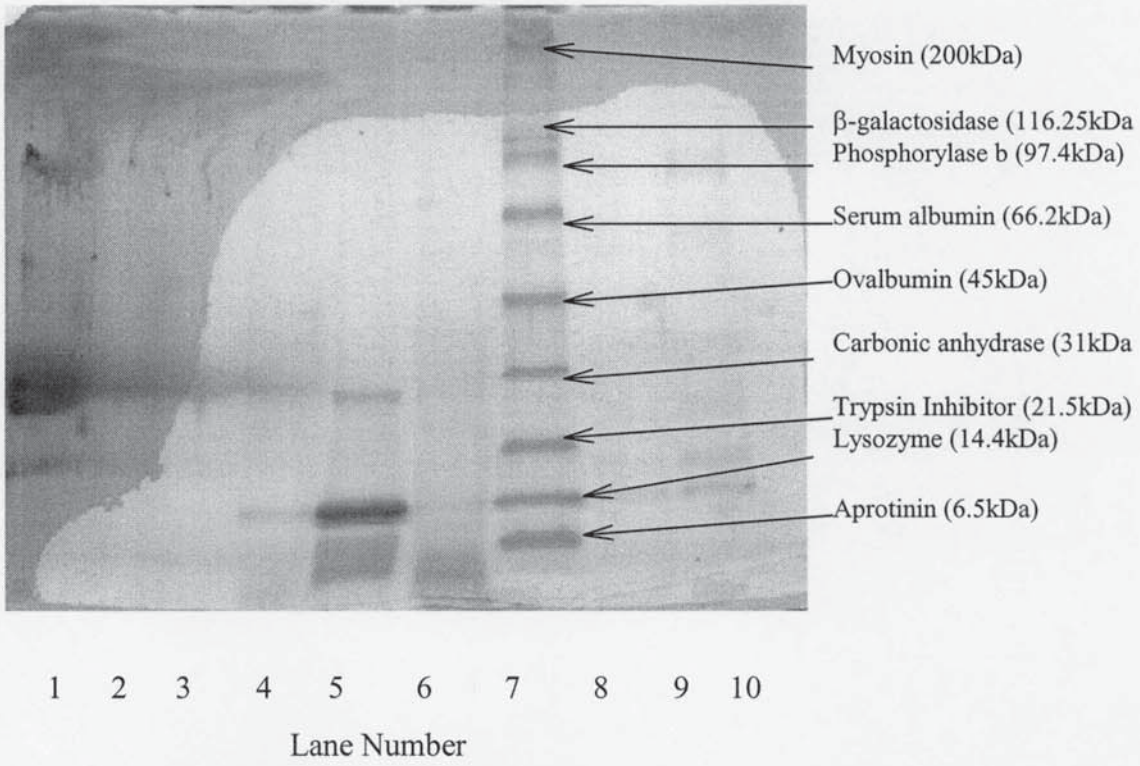


Figure 8.6 Representation of the protein pattern obtained by method B on Acuvue 1 day disposable lenses compared to that obtained via extraction on a number of different lens types and makes. Lane 1 = Dailies in vitro spoilt, Lane 2 = Acuvue in vitro spoilt, Lane 3 = Ciba 'Dailies' Janet 1 day wear, Lane 4 = Purevision Julie 1 month wear, Lane 5 = Acuvue Janet 1 day wear, Lane 6 = Hydron Sharen 3 year wear, Lane 7 = MW markers, Lane 8 = Janet 1 day wear in 200μl 2X (alternative extraction), Lane 9 = Janet 1 day wear in 150μl 2X (alternative extraction). Again background was due to contamination of staining vessels. The tear removal involving 150μl 2X has clear protein bands as do the extracts. The removal using 200μl of 2X has only one faint band.

8.5 Assessment of Method B

By direct comparison to samples produced via the usual extraction method on a number of different lens types (Figure 8.6) and to samples produced by the extraction method on matching lenses worn for matching durations (Figure 8.5), it can be seen that Method B for tear envelope removal results in the isolation of a number of clear protein bands from the lens. The pattern obtained is better than that given by Method A and the use of the 0.5ml eppendorf as a support reduces any handling of the lens which could alter the pattern seen and relieves the detrimental fragmentation/ destruction of the lens itself.

The pattern produced using this method can also be favourably compared to the results from extractions with number and clarity of bands being similar. In actual fact the lack of some bands that are seen clearly with extracts from the envelope pattern may indicate that these proteins are not normally encountered in the tear envelope but are in fact derived due to absorption and concentration by the lens and so the extraction pattern is not as 'true' a picture for what we are trying to study.

8.6 Discussion

The results obtained using these two methods for removal of the residual tear left attached to a contact lens upon removal from the eye (also termed the tear envelope) conclusively show that such methods can yield data with regards to the tear pattern of contact lens wearers. Distinctly stained bands of protein were produced using both methods, with the pattern produced using Method B being both highly reproducible and favourably comparable to those achieved with the harsher, more time-consuming extraction method.

One very important point is that these techniques were only beginning to be evaluated. Whilst they show a great deal of initial promise, further work with regards to optimising the amount of 2X buffer used in the initial step, the evaluation of different centrifugation conditions, etc. are needed to maximise the utility of the techniques in further studies. In addition, more repetition of these experiments is necessary to increase the faith that can be placed in the results produced. Work is ongoing within the group on these areas.

The potential of these methods is most applicable to studies of tear protein patterns following overnight lens wear and through the course of normal daily wear. Subsequent use of the techniques on lenses that have been removed immediately upon waking may yield a clearer picture of a nocturnal tear compared to tear sampling upon eye opening via methods such as microcapillary or Schirmer Strip collection.

The main reasons for this is that there would be a minimal delay between eye opening and sample collection and also because removal of something already present in the eye should have much less impact than the introduction of something new. In addition the removal of the lens is a natural event whilst the introduction of a capillary or strip usually involves both some unavoidable stimulation of the ocular surface and for the patient to look in a particular direction. All of these delays and unnatural events could lead to changes in tear patterns.

In terms of studies throughout the day, a lens could be collected after a defined period of wear to allow the eye to accommodate the new lens and the establishment of the regular lens wearers tear pattern following the sometimes mildly traumatic event of lens insertion. Following collection of a lens upon waking, another lens could be collected after three hours of use and a new lens inserted. This could be repeated throughout the day and a number of tear envelope samples collected. Periods of wear needed before a normal lens wearer pattern was established could also be investigated by moving these time boundaries.

All in all, although further work is needed, the foundations of an extremely useful and relatively simple technique for removal and analysis of the tears of contact lens wearers have been established. The benefits of such a technique in producing a truer representation of the tear pattern of lens wearers cannot be underestimated and the advantages over an extraction method are clear.

Chapter 9

Discussion and Future Work

9.1 Discussion

The tear film is a unique and extremely complex fluid which harbors enormous potential for the diagnosis of diseases of both the ocular environment and the body as a whole. Comparatively easy and painless to collect compared to blood samples, the relationship between the distribution of proteins found in tears to that in the plasma make tear sampling an ideal medium for study and diagnosis of a number of clinical conditions. In addition to the tear fluid, the contact lens provides another potential source of samples. This can be seen to be of particular relevance and importance when considering the number of serious diseases in which increased susceptibility has come to be associated with lens wear[29, 91, 93, 95, 97, 99, 102, 104, 110].

Tear samples and lens extracts (and especially tears which have been removed from a worn lens by less stringent methods than extraction regimes) may prove to be a ground breaking tool in the struggle for early diagnosis and treatment of lens associated disorders of the ocular environment. In order for this to be the case, a suitable method must also be determined for analysis of these samples. Before such methods can be used routinely and with confidence, techniques for sample collection and analysis must be optimised to enable the clearest and most detailed picture possible to be drawn and be shown to be highly reproducible in terms of the data they generate.

Today there are a large number of techniques that are suitable for the analysis of tear protein patterns and concentrations. Amongst the potential techniques are HPLC, ELISA and electrophoresis. Each of these techniques has its own inherent strengths and weaknesses and the aim of this thesis was to evaluate a number of these techniques in order that one or two might be selected for optimisation and then applied to the study of tear samples and lens extracts. Overall the hope was to produce a method that could be used to generate a complete picture of the pattern of proteins in a particular sample and then be further exploited in such a way that it would be possible to determine the nature of a particular protein in this pattern.

Name of Technique	Strengths	Weaknesses
HPLC (high performance liquid chromatography)	▪ Small sample volume required ▪ Short run times mean rapid sample turnover ▪ Identity and concentration of protein can be determined from one trace	▪ Peaks of species with similar molecular weights can overlap leading to loss of data (e.g. lactoferrin and albumin) ▪ Difficult technique to learn/set up for the novice user
ELISA (enzyme-linked immunosorbent assay)	▪ Very sensitive ▪ Allows the presence of a particular antigen to be measured along with the concentration of that antigen in the sample	▪ Only gives a quantitative result – the nature of the species giving a positive result cannot be determined (it could be a complete protein or just a subunit) ▪ Restricted by commercially available antibodies
2-D Electrophoresis	▪ Small sample volume needed (2-5µl) ▪ Proteins separated according to two parameters (isoelectric point and molecular weight) ▪ High sensitivity can be obtained depending on the stain used	▪ It is not possible to analyse two samples on the same gel and so direct comparison is difficult ▪ No further sample processing is possible (e.g. antibody analysis) ▪ Molecular weight of proteins cannot be calculated due to lack of marker proteins for this method
1-D Electrophoresis and Western Blotting	▪ Up to ten samples can be run side by side on the one gel and therefore under identical conditions ▪ Western blotting allows samples to be further processed (antibody analysis or sequencing) ▪ Both the overall pattern of proteins in a sample and the identity of the proteins can be measured	▪ Restricted by commercially available antibodies (e.g. lipocalins) ▪ Two step process is time consuming taking at least two days to achieve results ▪ Cross reactivity of species to antibodies can cause false positive results

Table 9.1 Comparison of the strengths and weaknesses of commonly used techniques for protein analysis

When choosing the techniques to be evaluated, a number of parameters were considered:

- Sample volume required. This needed to be as small as possible due to the limited amount of tears which can be collected while still be confident that there has been no stimulation of reflex tearing.
- Turnover time. Samples needed to be processed quickly to enable a large number of samples to be analysed.
- Number of samples that could be compared in one run. The higher the number of samples that could be run at one time in one experiment, the greater the confidence in the comparison of these results and the lower the possible sources of variation.
- Flexibility. The ideal technique would be applicable to a wide range of different samples and allow a number of variables to be assessed.
- The method needed to be sensitive enough to detect low abundance species.
- High levels of specificity were required for conclusive isolation and identification of a particular protein in a highly complex mixture.
- The methodology needed to be highly reproducible to enable complete confidence in the results and any conclusions made.
- Ease of use. It is easier to eliminate potential sources of error if the number of steps involved is small.
- Quality of results generated both in terms of clarity and volume of information that could be produced.
- Safety

HPLC was the first technique to be investigated. This technique was of potential interest due to the relatively small samples needed along with a short overall run time and the potential to generate traces which showed the position and presence of the complete tear protein pattern. The nature of the pattern generated also enabled the relative concentrations of each protein to be determined in the mixture by comparing the size of the signal trace. With all these features being well established, the main need was to investigate the sensitivity and reproducibility of the method when applied to tear samples and the proteins of interest contained in the tear fluid.

As described in Chapter 3, initial sensitivity of the chosen HPLC method was evaluated by the use of samples which contained ten times the reported physiological concentrations of the proteins albumin, lysozyme, lactoferrin and IgG. The traces produced seemed to show that the system could be used for more dilute samples and so physiological concentrations were analysed. At first the results seemed encouraging but further exploration of mixtures of proteins showed that the results were not very reliable and the calculated standard deviations were outside acceptable limits. When the technique was used to analyse a tear sample, the same problem was observed along with problems cleaning the column and obtaining a baseline between sample runs.

After several months of work, the lack of reproducibility lead to the technique being put to one side in favour of some of the other methods of protein analysis available. HPLC is known for being a difficult method to set up initially, but one that can offer large amounts of useful information once established. Unfortunately, the time limits associated with Ph.D. research meant that it was necessary to move on to another area of investigation. The potential of HPLC remains undiminished in its use for tear analyses, but the number of variables that needed to be investigated in order to produce an optimised technique proved too restrictive for further investment of the time and resource available.

The next technique to be tested that fulfilled a large number of the necessary criteria for an ideal method of sample analysis was two-dimensional electrophoresis. The technique was again optimal in terms of the complexity of the data that could be generated, the small sample volume needed and the number of samples which could be processed. In addition, when coupled with silver staining the reported sensitivity was well within the required range and the results produced allow proteins to be identified due to both their molecular weight and isoelectric point being displayed. Compared to one-dimensional electrophoresis this was a clear advantage as one-dimensional methods can only show the molecular weight of a species.

The main difficulty encountered with this technique was the method used to calculate the molecular weight of the proteins that could be seen on a gel once it had been stained. The isoelectric points were relatively easy to determine due to the linear nature of the pH gradient across the gel and so the distance traveled is directly related to the isoelectric point value. The calculation of a protein's molecular weight, on the other hand, is based on knowledge that the movement of proteins in the second dimension in a polyacrylamide gel is related to the molecular weight in a log ratio. In order to calculate molecular weight of each spot molecular weight markers are used which separate as a 'ladder' of bands. The markers are then used to plot a calibration curve from which the molecular weight of unknown bands can be determined. At this time no markers of this type exist for two-dimensional methods which are based on tube gels for the first dimension. As a direct result of this problem although the pattern of normal tears could be recorded and around ten different spots could be easily identified the actual nature of these spots could not be determined.

In addition to this particular problem, a number of other sources of difficulty were encountered. Some of these problems included the fact that the technique involved a large number of complicated steps such as tube gel extrusion and the silver staining method, the toxicity of a number of the reagents and the inherent fragility of the tube gels themselves, which lead to difficulties in generating good reproducible results. Overall, although this method produced a good quality picture of the tear protein pattern using an acceptably small sample volume, the complexities of the protocol again meant that the necessary flexibility and reproducibility were not achieved.

The limited time available made it inevitable that this technique was put to one side, with a view to returning to it once the other potential techniques had been investigated as to their suitability for the proposed application. It was regarded that this two-dimensional electrophoresis might still become the technique of choice if none of the others proved suitable and further time could be assigned to it.

As mentioned previously, one dimensional electrophoresis was initially not considered to be as applicable as a two dimensional electrophoretic separation because of the clear advantage that generating data about two characteristics of a particular protein/protein spot represents. However the ability to run up to ten different samples simultaneously and thus to compare the protein patterns of these samples on the one gel was a unique advantage that the two dimensional system could not match. Although up to six samples could be separated at once in the first dimension of a two dimensional analysis, only two tube gels could be run at any one time in the second, and these would use two different slab gels. When making any direct comparison of two-dimensional results this potential source of variation had to be considered. In contrast, the ten samples loaded into different lanes of a particular one-dimensional gel could be compared with the knowledge that they had all been run under identical conditions with identical buffers and an identical gel recipe.

In addition to this clear advantage, it was discovered that one dimensional electrophoresis was also a suitable method of analysis in terms of a number of the other parameters outlined earlier in this discussion. The technique was straightforward and easy to troubleshoot, had a rapid turnover of samples, required samples of as little as 5μl and had a number of variables which could be explored in order to obtain an optimal view of a sample. It also had a reasonable reported level of sensitivity. At first the focus of the work done using one-dimensional electrophoresis was centered on achieving a set of parameters that would give the best possible results. Amongst the conditions that were investigated were the benefits of using stacking gels, variations in the running conditions, the gel recipe used, the contribution of gradient gels and the strengths and weaknesses of specific staining methods.

After much experimentation and adjustment of the available variable parameters a set of conditions were arrived at which gave the clearest picture of the tear protein pattern. Although these conditions may not have been the most sensitive or the most efficient in terms of time and effort, the results obtained were considered the best achieved.

The relevant conditions were:

- **No stacking gels**
- **Run at 30 mA**
- **Stain with Coomassie Blue**
- **Use gradient gels (either 7.5-15% or 7.5-20%)**
- **Treat samples with an equal volume of 2X treatment buffer**
- **Scan gels as soon as possible after run**
- **Depth of running buffer in lower chamber kept as low as possible**

The signature pattern of normal tear proteins had now been achieved and the conditions for obtaining the best possible view of this pattern via one-dimensional electrophoresis had been determined. The next step was to answer the requirement for conclusive identification of the nature of the observed bands, which was earlier described as the 'specificity' of the technique.

The use of molecular weight markers in experiments involving one-dimensional electrophoresis did allow at least one property of the protein bands seen to be positively determined, namely their molecular weights. The molecular weight of a protein is one of its characteristics and the measurement of the molecular weight of each band coupled with existing knowledge of the proteins commonly found in tears allowed the identity of most of the bands seen in an optimised pattern to be postulated (as seen in Chapter 5). The confidence that could be placed in the calculation of these values was investigated by comparing the calculated molecular weight of a number of standard protein solutions with the reported molecular weight of the protein. The results achieved showed that the calculated values were all within a reasonable margin of the reported values with the widest variation being around ten percent. This showed that the determined molecular weights of the unknown tear protein bands could be used with confidence when estimating the identity of these bands.

Although such an estimate of the identity of the bands which made up the general tear protein pattern could be made, it could not be conclusive without further evidence. As a result of this it was seen that in order to achieve the desired degree of specific staining/visualisation of the proteins a separate step would be needed. At this point it was found that the established electrophoretic method could be utilised in such a way that it could provide true specificity. In this way one-dimensional electrophoresis fulfilled more of the necessary criteria for a method of analysis in terms of its flexibility and achievable specificity. The method by which electrophoresis could be used to achieve specificity was that of Western blotting of the gels followed by antibody based staining of the transferred proteins.

Western blotting involves the transfer of proteins from within a gel to the surface of a membrane such as nitrocellulose or PVDF using an electric current. This transfer makes the proteins accessible to a number of staining methods that would not work while they are enclosed by the gels used for electrophoretic separation. One such staining method involves the use of antibodies that bind to a particular protein of interest (immunostaining). These antibodies are conjugated (or linked) to other moieties which either act themselves as reporters of a positive result, such as luminescent or fluorescent subunits, or which can be used to trigger some reaction which acts as a positive signal, for example horse radish peroxidase. The beauty of these techniques is that antibodies are specific. They only bind to one particular protein in a mixture or to a subunit of this protein and this means that a positive result obtained using immunostaining can be considered to be conclusive proof of the identity of a previously unknown protein band/species.

The major drawback of the technique is that it is limited by the commercial availability of antibodies raised against the proteins you want to study. This is of particular inconvenience when considering antibodies raised for the tear lipocalins which to date are not widely available to buy and so these proteins cannot be analysed using this technique.

Another drawback is that careful consideration must be given into which antibodies should be used. With the large number of unknown proteins in the tears, it will be a big undertaking to use this technique to positively identify them as only one protein can be assessed per blot. A thorough understanding of the nature of the tears is essential to facilitate a worthwhile positive result and avoid any losses of time and resources that would be associated with testing for a large number of proteins whose presence is only postulated.

Initial evaluation of immunostaining with some of the well-known tear proteins such as albumin and lactoferrin showed that levels of sensitivity and specificity were particularly high giving confidence in the data generated. It was at this stage that the suitability of electrophoresis for the analyses we wanted to carry out became particularly clear. The synergy of the results derived from the Western blotting method with the patterns achieved with simple electrophoresis and Coomassie Blue staining provide a superb overall picture of the tear proteins.

The strengths of each technique are the exact match for the weaknesses seen in the other and the ability to run two gels at once means that one can be Coomassie stained whilst the other is used for blotting and subsequent antibody reactions. This means that the two gels are loaded with identical samples which are run under identical conditions and so the results recorded for the samples of one gel using one method can be confidently applied to those in the other. One dimensional electrophoresis gives a view of the pattern of proteins in a particular tear or extract sample and allows a comparison between these patterns which could be linked to a particular condition or disorder. However it can not be used to determine the identity of the absent or abnormally abundant protein that has caused the observed difference or indeed the nature of any of the bands that comprise the pattern. Western blotting and immunostaining by comparison can clearly be used to show whether a particular protein is present in a sample or not. It can't be used to monitor any differences that present themselves in the overall protein pattern that are associated with the presence of this protein.

In conclusion, using the techniques of one-dimensional electrophoresis and immunostaining we can collect this data from a particular set of up to ten samples. When these results are combined we get the supremely thorough and detailed picture of the proteins found in tear samples and lens extracts that is the ultimate goal of the research.

One unforeseen problem associated with immunostaining of the tear proteins was encountered. This was related to the study of the immunoglobulins using this particular technique. The common subunits that are shared by all immunoglobulins caused cross reactivity of samples and false positive results were produced. In a particularly clear example of this problem, when samples of two standard protein solutions which contained IgA and IgG respectively were immunostained for IgA using a commercially available antibody, both samples were stained with equal intensity.

Because of the roles that these proteins play in the body's immune responses and the importance of these responses to studies of lens wear problems, it can be seen that this is a major cause for concern in our ongoing research. A potential answer to this problem will be further discussed in the following section 'Scope for Future Work'.

Having discovered the potential of combining data generated by the two methods to construct a wonderfully complex and detailed story of what was happening in our samples, it was decided that the techniques were ideally suited to an investigation of a protein whose presence in tears is not widely reported. The name of this protein is kininogen, and while not widely reported to date, it has the potential to become very important in our attempts to gain a better understanding of the tear film and its relationship with contact lens use. Kininogen is a precursor to a member of a family of proteins that are known to be involved in both inflammatory and immune pathways/cascades called the kinins as well as playing a number of roles in activity in the blood coagulation cascade in its 'precursor' form. It is clear from this fact why its presence in a particular sample would be of interest to us, and also the potential of both kininogen and its breakdown products as indicators of a problem in the tear film.

What we found was that kininogen could be consistently measured in 'normal' tears (which to our knowledge is the first time this has been shown using this particular technique) as well as being at a significant, recordable level in contact lens extracts. More importantly there was a clear difference between the positive results obtained with these two types of sample and this was where the combination of techniques proved particularly useful. The molecular weights of the bands identified as being kininogen were calculated and it was seen that the kininogen band in tears was equivalent to the intact form of high molecular weight kininogen at 120kDa whilst the bands seen in contact lens extracts seemed to correspond to a breakdown product or subunit with a molecular weight of around 35kDA.

It remains to be conclusively determined what caused this difference in the samples, but the most likely story is either that lens wear has caused the conversion of high molecular weight kininogen to its active form called bradykinin or that the extraction method has caused a change to the protein. As discussed in the following section, this particular area merits further investigation to establish the actual source of the difference seen. This is because of the impact this information could have on our understanding of one of the ways in which lenses influence the tear film.

Due to the very likely possibility of the lens extraction method having a significant effect on the protein pattern obtained it was decided it would be beneficial to evaluate a method for removal of the tear envelope from a freshly worn lens. What we were looking to develop was a method wherein the tears of a lens wearer could be removed from the surface of the lens. These could be analysed in terms of what was missing from the established protein pattern seen with tears of non lens wearers rather than analysing the extracts in an attempt to obtain a pattern of what had been removed by the lens.

The protein pattern derived from a lens extract cannot be seen to be truly representative of what has been taken from the tears because of the harsh conditions required to extract these proteins which could cause them to split into their subunits. In addition a false picture where some proteins are much more abundant in the extract than others could result solely from the fact that these proteins are easier to remove from the lens or are taken up preferentially by the lens from the tears. So, it can be seen that the extract cannot be trusted to give a reliable view of what the contact lens is doing to the tear film proteins and this is why a method of tear removal is needed.

The methods assessed for tear envelope removal were based on centrifugation. Both involved immediately placing lenses that had been taken from the eye into volumes of sample treatment buffer. Removal of the mixture of tears and sample buffer was then achieved by initially drawing off all the liquid that was easily removed using a pipette and then placing the lens of interest in some form of supporting holder. Hard to remove liquid was subsequently drawn from the lens surface by centrifugation which was seen to yield a significant additional, if somewhat small, volume of sample.

Initial results using these techniques were encouraging in that they showed a comparable number of bands to those seen with a lens extraction but with a different pattern. In addition the technique is a great deal less time consuming and it provides more confidence that what is shown has not been significantly altered by the process of sample production. More work is needed to determine just how valuable this technique may be in studying the effect of lenses on the tears. Some aspects of how this technique could be further investigated are covered in the following section titled Scope for Future Work.

Overall, this work has produced several key areas of importance/value:

- Evaluations of both HPLC and tube gel based two dimensional electrophoresis were carried out and they were found to be unsuitable for our needs
- Optimisation of one dimensional electrophoresis, Western blotting and antibody based staining methods to produce a detailed, reproducible picture of the both the pattern of proteins in tears and lens extracts and the identification of some of the components of the pattern was achieved
- The discovery that our optimised one dimensional protocol when coupled with Western blotting produced a method which fulfilled almost all of the criteria considered necessary to carry out an investigation of the tear film was made
- Conclusive, positive (and reproducible) proof of the presence of kininogen in normal tears using these methods is of particular value and excitement as is the clear difference between the positive results for tears and extracts. Of lesser magnitude, but still important, is confirmation that kininogen can indeed be found in lens extracts.
- Finally, a new technique for tear removal from lenses which is much less destructive than existing methods of lens extraction is very exciting, but needs a large amount of further evaluation and optimisation.

9.2 Future Work

1. *Further investigation of existing and novel methods for protein analysis.* So far, four possible methods were investigated for their suitability to our studies of the tear proteins. These are HPLC, two-dimensional electrophoresis, one-dimensional electrophoresis and Western blotting. As discussed a combination of two of these methods appears to provide a perfect answer to our needs. However, many other techniques exist for the analysis of samples containing proteins and new methods are constantly being developed. In addition the methods already explored are evolving as manufacturers work towards better, easier to use, safer products. Methods such as Thin Layer Chromatography, ELISA and amino acid analysis/protein sequencing all have the potential to provide additional data that would help to clarify the events associated with lens wear and ocular disorders. Of particular note is the development of technology for two-dimensional analyses. Tube gels are no longer widely used, or indeed commercially supported, as a result of the problems described earlier in this thesis and new methods based on less fragile first dimension media are the norm. This makes this particular method both viable and attractive once again. Overall, it is clear that alternative methods should not be ignored purely because it would seem that we have our ideal technique already, particularly when one of these alternative methods may be able to give either better results or similar results in a shorter time with less effort.

2. *Evaluation of antibodies raised against the unique subunits of the immunoglobulins.* The problems of cross-reactivity of the immunoglobulins when immunostaining has been outlined previously, with antibodies targeted against IgA staining IgG control samples with equal intensity. This is thought to be caused by the shared subunits of this family of proteins being recognised as antigens by the IgA antibody. What is needed is some method whereby the five immunoglobulins can be identified from one another in a particular sample with no fear of false positives. This is essential to our work due to the different origins of the individual immunoglobulins and the unique roles of these proteins in the immune and allergy pathways. A simple and easy to implement answer exists to this problem, but it was one which was not possible within this study. Antibodies are

available that target the unique subunits of immunoglobulins, such as the gamma chain of IgG, and because these subunits are not shared by the immunoglobulins there should be no cross reactivity seen. Work using these antibodies is being explored within our group and it is hoped that they will indeed soon provide a method whereby the contribution of a particular immunoglobulin to the tear protein pattern, and thereby its role in particular ocular conditions, can be determined.

3. *Determination of the nature and cause of the difference between the kininogen positives seen for tears and lens extracts.* A clear difference was seen when tear samples and lens extracts were positively immunostained for kininogen. The tear sample appeared to contain intact high molecular weight kininogen whilst the extracts contained some breakdown product with a molecular weight of around 35kDa. The real question is what actually caused this observed difference. The two most likely answers are that either the kininogen was broken into a number of subunits by the extraction method or that lens wear had triggered some response which had lead to kininogen being converted to its active form bradykinin. Again, determining which is the true case has a large impact in our attempts to understand the impact of lens wear on the ocular environment. In order to determine the nature of the 35kDa band a method would have to be developed wherein active bradykinin could be distinguished from any probably inert breakdown product. The most obvious problem of achieving this using our existing methods would be a similar one to that encountered with the immunoglobulins and described above. Because the processing of kininogen produces bradykinin it would share common areas which would cause cross reactivity with antibodies used in immunostaining. The answer might lie in a completely different direction. Instead of analysing the samples using our existing antibody based methods it might be necessary to assess the enzymatic activity of the samples. Bradykinin should be able to trigger a number of reactions involved in inflammatory pathways whilst a breakdown product of extraction would not. This activity could be measured either by production of some quantifiable by product or the triggering of a reaction that could be manipulated to produce a visible signal. A great deal of work would be needed to develop an assay that would allow the nature of this unknown species to

be determined in this way based on its enzymatic activity. Such a method would, however, provide valuable insight into this exciting protein family and a possible method whereby lens wear could trigger both immune and allergic responses.

4. *Identification of more of the eleven protein bands seen in the one-dimensional pattern.* To date, the combination of Coomassie Blue staining of one dimensional gels with immunoanalysis of Western blots has allowed us to conclusively identify only two or three of the eleven bands seen in our optimised picture of the tear protein pattern. For complete identification of the remaining bands a particular combination of experimentation and research of existing work is necessary. Only one protein can be immunostained per Western blot, but one answer to this might be to run duplicate aliquots of a particular tear sample, mark the position of each well and then cut the blot into strips, each of which could be analysed for a particular protein. The only obstacle to identifying the unknown bands lies in the large number of possibilities and this is where the need for research comes in. The most likely identities of the eleven bands could be derived from study of work done by other research groups. This would provide a starting point wherein antibodies for each of the top ten most commonly reported proteins were used to analyse a particular set of blot strips. If the result was negative for a particular band with all these antibodies, the next ten most commonly reported proteins could be tested for and so on until all eleven bands were conclusively identified. The other possibility is that a band might correspond to more than one protein due to similarities in molecular weight. The only way that this problem could be answered would be through either greater separation of the bands over the same distance of gel by modification of the gel recipes or through two-dimensional separations. Of course, it is possible that such a problem might not be encountered but if it was this would be the solution.

5. *Investigation of the variables involved in the tear removal methods.* The protocols developed for removal of the tear envelope had only started to be explored in the course of the research outlined here. Two key areas remained to be investigated in the methods described and other permutations of the methodology also need to be considered. The first area that needs to be studied is the volume of sample

treatment buffer that is used in the first step of the process wherein the lens is removed from the eye and immediately placed into a tube containing a certain amount of buffer. To date a small number of different volumes were tested in order to see which would give the best possible results, but a more complete and comprehensive range of this particular parameter remains to be evaluated. The other area that needs to be analysed is the centrifugation conditions. Variations in both the speed and duration of centrifugation were not explored but could prove valuable in the process of obtaining a complete picture of what is going on in the tear envelope. Other factors that could also contribute to successful removal and subsequent study of the tear envelope include the use of other support structures for the lens during centrifugation, some method of concentrating the resulting liquid and a definition of the period within which the lens must be placed into the sample treatment buffer before the sample is considered unusable/non-viable to our studies.

6. *Application of optimised techniques to clinical samples.* The laying of the foundations that would enable precious clinical samples to be studied with confidence was the initial driving force behind this research. The focus of this work to date was the development of optimised techniques for analysis of tears and other samples and then the use of these methods to produce a reproducible, detailed picture of the 'normal' protein pattern. The reason for doing this was to then utilise these methods in the study of the tears of patients with known ocular diseases/disorders. The aim of these analyses would be to discover some common difference between the pattern of proteins in the tears of sufferers of a particular condition when compared to non-diseased tears and then the identification of this difference. Once the identity of a protein that was either missing from, or unnaturally present in, the diseased tear pattern had been determined it could be used as a clue to the cause and possible treatment methodology of the condition. So far the techniques are ready and all that is needed are the clinical samples.

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Appendix I – Review of Recently Published Work

Due to the nature of PhD research, a significant gap has developed between the references available at the time this write-up began and those that are available as work begins on a final draft. In order to present as complete an account of the state of research into the tears, ocular disease and the roles of tear proteins/constituents in the functioning of the tears it was decided to add this Appendix to the existing thesis.

One example of newly published research that is closely related to what has been discussed in this thesis involves the use of flow cytometry to investigate inflammatory markers in patients with dry eye disorders[1, 2]. Using samples collected via impression cytology from patients who had been diagnosed as suffering from keratoconjunctivitis sicca (KCS) compared to normal subjects, it was found that elevated concentrations of various inflammatory markers could be seen in the dry eye patients. In particular it was recorded that three well-known mediators of inflammation (HLA DR, CD-40 and CD-40 ligand) were found at significantly higher concentrations in the samples collected from dry eye patients[1]. In addition it was found that these elevated levels could be further associated with those KCS subjects suffering from Sjögren's syndrome compared to non-Sjögren's syndrome KCS sufferers.

Another significant finding in this work was that treatment of these cells with the cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) lead to an increase of CD40 expression[2]. IFN-γ treatment also caused an increase in the levels of HLA DR. By combining these two sets of data it can be concluded that the cytokines (discussed in depth in Chapter 1 of this thesis) could be very important in the inflammatory aspect of dry eye. These recent findings make the various cytokines prime candidates for exploration using the techniques we have established and could be very significant in any future research into the causes and effects of dry eye disease, particularly where contact lens wear is involved.

A major area of research where there have been significant recent advances is the role of the sex hormones androgen and estrogen in the tear glands[3, 4, 5]. Although related research has been ongoing for a number of years, with various theories as to the roles of these hormones in dry eye syndromes[6,7] there have been several breakthroughs reported of late. These include the identification of androgen receptors in the human lacrimal glands and other human ocular tissues[3, 5], the presence of estrogen receptors in the meibomian glands[4] and the identification of androgen, estrogen and progesterone mRNAs in the eye[8].

The presence of both receptors for these hormones along with the hormones themselves in the lacrimal and meibomian tissues show that the sex hormones must have some role in the control of certain functions and responses of the tear glands. Although the exact mechanisms of hormonal regulation of tear secretion remain unclear, it can be seen that an increase or decrease in circulating levels of these hormones may be extremely significant in dry eye conditions.

In addition, there has also been a link found between androgen levels and the prevention of lacrimal gland degeneration that is seen in severe cases of dry eye. This destruction of glandular material is caused by programmed cell death but can be prevented by androgen treatment[9]. This is explained by the fact that androgens act as immunosuppressants in the lacrimal gland. They do this via reducing the levels of the inflammatory cytokines interleukin-1 beta (IL-1β) and TNF-α and by stimulating production and release of transforming growth factor beta (TGF-β) which is a cytokine with immunomodulating and anti-inflammatory properties.

In yet other work on the contribution of the cytokines to dry eye conditions it has been shown that the levels of both Il-1α and Il-1β are increased in the tears of meibomian gland disease and Sjögren's syndrome sufferers[10]. Due to a lack of increased levels of these agents in stimulated tears it can be postulated that these cytokines originated from the lacrimal glands. It remains to be determined if the presence of cytokines in the tear fluid is a result of the dry eye condition or a trigger, engendering aspects of the disease.

Also of interest is the recent identification of β-defensins in intraocular tissues[11]. These antimicrobial peptides have an antibacterial action that could be significant in the defense of the ocular environment. Although levels measured in this study were below those needed for successful bacteriocidal activity, it was shown that concentrations could be elevated through stimulation by inflammatory cytokines in response to infection. This shows that the β-defensins could be important in our understanding of the inflammatory pathway and ocular infection.

Finally, three other closely related topics have shown recent promise. These are the use of 1-D and 2-D electrophoresis in the identification of lacryglobin in the tears of patients suffering from various forms of cancer[12], the analysis of the tears of diabetics using 2-D electrophoresis[13] and a method for diagnosing mucin-deficient disorders by measuring the sialic acid content of the tears[14]. The convenience, accessibility and minimal trauma associated with taking tear samples compared to blood samples make tears an attractive target for disease diagnosis where applicable. The increase in such research is therefore no surprise. Although all three of these methods need further development, each looks promising in its' suitability for not only conclusive diagnosis but also early diagnosis of these potentially damaging conditions.

As can be seen from these brief summaries, there are many exciting areas yet to be explored in the field of tear proteins and their functions. The development of new proteomics tools and apparatus along with the optimisation of existing techniques through continued research mean that the volume, sensitivity and quality of the data that can be generated is constantly improving. Due to this continually evolving scientific environment it can be hoped that one day soon we will be able to conclusively determine the nature and role of each constituent of the complex mixture of proteins present in the tear film. It can be further envisaged that this knowledge will play a very significant part in efforts to eradicate or alleviate the current incidences of ocular disease such as those described in Chapter 1 of this thesis.

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Appendix II – List of Abbreviations

Ab	Antibody
Ag	Antigen
AP	Alkaline Phosphatase
APS	Ammonium Persulfate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
CIE	Counter Immunoelectrophoresis
CL-MGD	Contact lens-associated meibomian gland dysfunction
CL-MIK	Contact lens-induced microbial infiltrative keratitis
DAF	Decay Accelerating Factor
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked immunosorbent assay
HMWK	High Molecular Weight Kininogen
H.P.L.C.	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
I.D.	Internal Diameter
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IPG	Immobilised pH gradient
KCS	Keratoconjunctivitis sicca
LMWK	Low Molecular Weight Kininogen
MAC	Membrane Attack Complex
MCP	Membrane Co-factor Protein
MGD	Meibomian gland dysfunction
MW	Molecular Weight
NBT	Nitro blue tetrazolium
NSDE	Non-Sjögren dry eye
P.A.G.E.	PolyAcrylamide Gel Electrophoresis

pI	Isoelectric point
PMFA	Protein Moving Faster Than Albumin
PVDF	Polyvinylidine difluoride
SC	Secretory Component
S.D.S	Sodium Dodecyl Sulphate
SSDE	Sjögrens' Syndrome Dry Eye
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β	Transforming Growth Factor-beta
TNF-α	Tumour Necrosis Factor-alpha
UV	Ultraviolet