PROTEOMICS OF THE OVINE CATARACT

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy

At

Lincoln University

By

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Lincoln University

2008

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The lens of the eye needs to be completely transparent in order to allow all light entering the eye to reach the retina. This transparency is maintained by the highly ordered structure of the lens proteins the crystallins. Any disruption to the lens proteins can cause an opacity to develop which is known as cataract. During cortical cataract formation there is increased truncation of the lens crystallins. It is believed that overactivation of calcium-dependent cysteine proteases, the calpains, is responsible for the increased proteolysis of the crystallins seen during cataractogenesis. Within the ovine lens there are three calpains, calpain 1, 2 and the lens specific calpain Lp82. The aim of this thesis was to determine the changes in the lens proteins during ageing and cataractogenesis, and to establish the role of the calpains in these processes.

Calpain 1 and 2 were purified from ovine lung and Lp82 was purified from lamb lenses using chromatography. Activity and presence of the calpains was determined by using the BODIPY-FL casein assay, gel electrophoresis, Western blot and casein zymography. Changes in the lens proteins, specifically the crystallins, were visualised using two-dimensional electrophoresis (2DE). Lenses from fetal, 6 month old and 8 year old sheep were collected, as well as stage 0, 1, 3 and 6 cataractous ovine lenses. The proteins from the lenses were separated into the water soluble and urea soluble fractions and analysed by 2DE. Mass spectrometry was used to determine the masses and therefore modifications of the crystallins. Finally, the individual crystallins were separated using gel filtration chromatography and incubated with the purified calpains in the presence of calcium. The extent of the proteolysis was visualised using 2DE and truncation sites determined by mass spectrometry.

Purification of the calpains resulted in samples that were specific for each calpain and could be used in further experiments. 2DE analysis showed that there were changes to the crystallins during maturation of the lens. The α-crystallins become increasingly phosphorylated as the lens ages and a small amount becomes truncated. The β-crystallins were also modified during ageing by truncation and deamidation. When crystallins from cataractous lenses were compared using 2DE there were changes to both the α- and β-crystallins. The α-crystallins were found to be extensively truncated at their C-terminal tail. Four of the seven β-crystallins, βB1, βB3, βB2 and βA3, showed increased truncation of their N-terminal extensions during cataract formation.

All three calpains truncated αA and αB-crystallin at their C-terminal ends after incubation. Calpain 2 and Lp82 each produced unique αA-crystallin truncations. All three calpains truncated βB1 and βA3 and calpain 2 also truncated βB3.

When the truncations from the calpain incubations were compared to those seen during cataract formation, many of the truncations were found to be similar. Both the unique truncations from calpain 2 and Lp82 were found in cataractous lenses, with the Lp82 more obvious in the 2DE. The β-crystallin truncations found after incubation with the calpains were similar to those found during cataractogenesis.

In conclusion this study documents the changes to the ovine lens during maturation and cataractogenesis and indicates a role for the calpain family in the increased proteolysis observed in the ovine cataract.

Keywords: Cataract, calpain 1, calpain 2, Lp82, α-crystallin, β-crystallin, lens, ageing, 2DE, sheep, Fast protein liquid chromatography,

Acknowledgments

There are many people who have contributed to this thesis over the last few years. There are too many to mention in this document so if you don't get a mention I apologise.

Firstly I would like to thank my supervisor Dr Jim Morton, your guidance and expertise has been invaluable. Without your support this thesis would never have made it off the ground. Also thank you for securing a FRST Enterprise scholarship for me. I especially would like to thank you for being so accommodating with my rugby refereeing, without your support and allowing me to be flexible in my PhD I would never have been able to achieve what I have in refereeing. Thanks to my associate supervisor Prof. Roy Bickerstaffe for your critique of my thesis in its final stages and your support throughout.

I would like to thank Dr Lucinda Robertson, who has been a fantastic mentor, without your enthusiasm and positive attitude I would never have got here. Thanks for running some of my samples and the huge amount of time you dedicated in Oregon to getting some proteins to run. Also thank you for being such an awesome travel buddy, we have had some great trips around the world and I couldn't ask for a better travel companion.

Thanks to the crew in the cataract research group at Lincoln University. Karl for being there when a whinge, gossip or general chinwag was required. Dr Hannah Lee for letting me share her office for 2 years, thanks. Thanks to all the other students that have come and gone in our labs Jacqui Wood, Anna Wright, Josh McDermott, Jie Lie and Gareth Wilson. You guys rocked and made my life at Lincoln University rather enjoyable with constant baking, morning teas and always being up for pie day friday.

Thanks to the staff at JML, Nigel Jay for looking after our precious sheep and Martin Ridgeway for sacrificing so many sheep and getting me lungs and eyes when needed.

Thanks to Abi and Colin down at Ashley Dene for collecting all those lambs for me in spring. To everyone else at Lincoln that helped me at some stage during this thesis cheers, it was a pleasure working you.

To those who were so accommodating to me whilst overseas, thanks. Dr Larry David at OHSU your enthusiasm is boundless, thanks for putting me up when I visited your lab and showing me the complex world of mass spectrometry. Mike Riviere thanks for the help whilst in Portland.

A huge thanks to those who provided financial support during my studies: The Foundation for Research, Science and Technology (FRST) Enterprise Scholarhip (2004-2007), Douglas Pharmaceuticals, the New Zealand Society for Biochemistry and Molecular Biology, AMI and the Canterbury Rugby Referees Association. Without your cash I would have never been able to contemplate a PhD.

To my family thanks for always being there for me. Allowing me to stay in the house, baking etc you made my life so easy, especially the last few months. Nothing was ever a problem and you were very patient with me pursuing a PhD. I would like to thank all my flatmates from the last 4 or so years. The girls in Clarence St, the crew from Martin Ave times two and briefly the boys from Hagley Ave. I appreciate the support from all of you and putting up with the trials and tribulations of living with a PhD student. Thanks to all my friends that have “encouraged” me over the years, cheers guys. To Lucy, without your support over the last year this thing would never have got done, thank you so much. Finally, thanks to everyone, you have been awesome.

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Abbreviations

αA	Alpha crystallin subunit A
αB	Alpha crystallin subunit B
βA_	Beta crystallin acidic subunit
βB_	Beta crystallin basic subunit
βME	Beta mercaptoethanol
µL	Microlitre
µM	Micromolar
1DE	One-Dimensional Polyacrylamide electrophoresis
2DE	Two-Dimensional Polyacrylamide electrophoresis
Asp	Aspartic Acid
Asn	Asparagine
BCA	Bicinchoninic acid assay
BODIPY-FL	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid
C-terminal	carboxy terminal
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate
Cys	Cysteine
DEAE	Diethylaminoethyl
DTT	Dithiothreitol
E64	Trans-epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester
EDTA	Ethylenediaminetetra-acetic acid disodium salt
EGTA	Ethylene glycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid
FU	Fluorescence Units
g	gram
g	gravity
GF	Gel filtration
His	Histidine
hr	hour
IPG	Immoblised pH Gradient
kDa	kilodalton

LC-MS	Liquid Chromatography Mass Spectrometry
Lys	Lysine
M	Molar
MALDI-MS	Matrix Assisted Laser Desorbtion Ionization-Mass Spectrometry
min	Minute
mL	Millilitre
mS	Millisiemens
MS	Mass spectrometry
N-terminal	Amino terminal
PL	Phospholipid
PS	Phenyl Sepharose
PVDF	Polyvinylidene flouride
RR	Reactive Red
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
sHSP	small Heat Shock Protein
TEMED	N, N,N'N'-Tetra-methylethylenediamine
TTBS	Tris buffered saline with Tween
TBS	Tris buffered saline
USF	Urea soluble fraction
v	Volts
Vh	Volt hours
WSF	Water soluble fraction

Chapter 1
Literature Review

1.1 Introduction

The primary role of the lens in the eye is to transmit and focus light onto the retina which contains the light sensing cells of the eye. For this to occur, the lens needs to be completely transparent. Any change to the transparency of the lens leads to scattering of light entering the eye and subsequent loss of vision. This opacification of the lens is known as a cataract. The opacification can become so severe that no light enters the eye resulting in blindness. The causes of cataracts have been extensively studied throughout the world. However the aetiology of the diseases is not entirely understood.

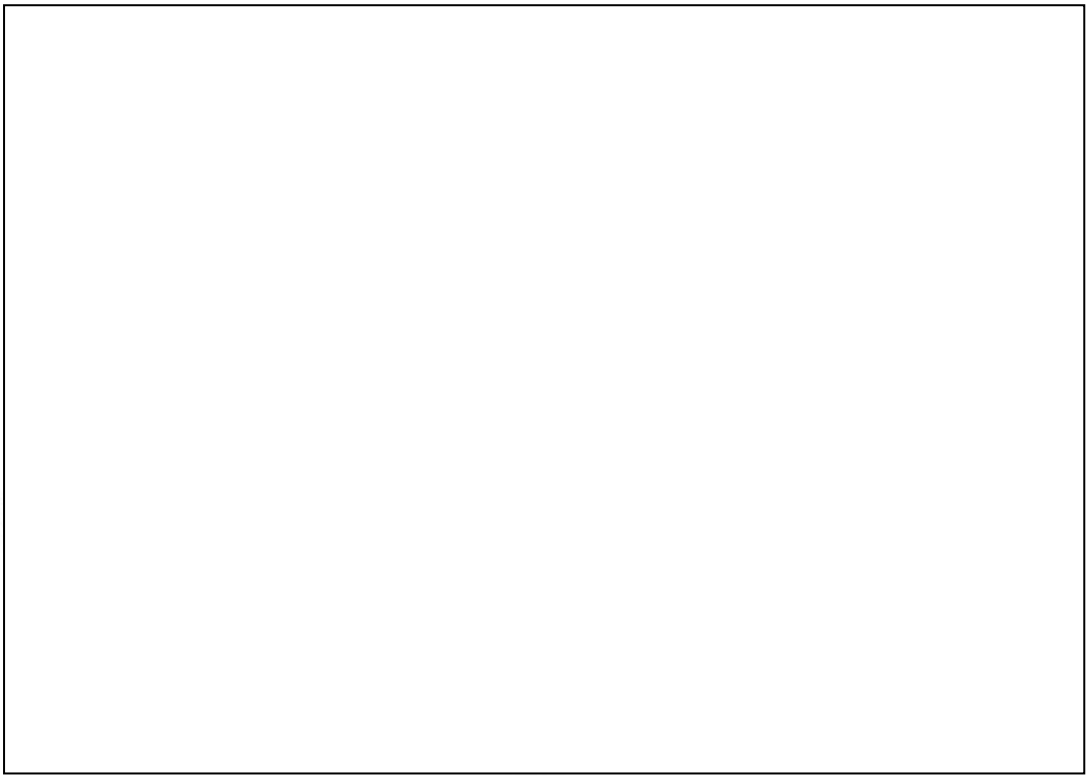

Figure 1-1 Cross Section of an eye

1.2 The lens

The lens is a transparent avascular tissue located in the anterior section of the eye behind both the pupil and iris (Figure 1-1). The lens is encased in a capsule. Attached to the capsule at the equatorial region are zonular fibres. These fibres connect the lens to the ciliary muscle and hold the lens in place. The zonular fibres and ciliary muscle are also involved in helping the lens to focus the light entering the eye.

The posterior surface of the capsule is in contact with the vitreous humor and the anterior surface with the aqueous humor. These provide the nutrients required for lens growth and development.

The role of the lens is to focus light onto the retina. This requires the lens to be transparent and have a high refractive index. The transparency of the lens is achieved by the highly ordered structure of soluble proteins in the lens called the crystallins. The arrangement of the crystallins as well as their high concentration helps contribute to the high refractive index of the lens.

The lens consists of two types of cells, epithelial cells and elongated fibre cells. The epithelial cells are found in a single layer along the anterior surface of the lens (Figure 1-2). The epithelial cells and immature fibre cells are the only fully metabolically active cells in the lens, and are the only cells that can synthesise protein. The fibre cells are found on the posterior surface and the internal region of the lens and provide the bulk of the lens cells.

The lens has a unique growth and development pattern. Epithelial cells migrate from the germinative zone of the anterior surface to the equatorial elongation region where they differentiate into fibre cells. During this differentiation the epithelial cells elongate, their intracellular organelles are lost and they start synthesising the fibre cell specific crystallins and other fibre cell specific proteins. The removal of intracellular organelles is essential for the clarity of the lens as even small organelles will scatter light. As more fibre cells are formed they layer on top of existing fibre cells, producing a structure which looks like an onion. This process means that the cells in

the nucleus of the lens are the oldest and those closest to the equator are the youngest. As fibre cells are not turned over, the cells and their contents in the centre of the lens are as old as the lens and organism itself. The fibre cells in the nucleus of the lens contain a high concentration of tightly compacted crystallins. This leads to the high refractive index found in the centre of the lens. Nuclear cell morphology is similar to that of cortical cells but they are more tightly packed and smaller (Davson, 1980).

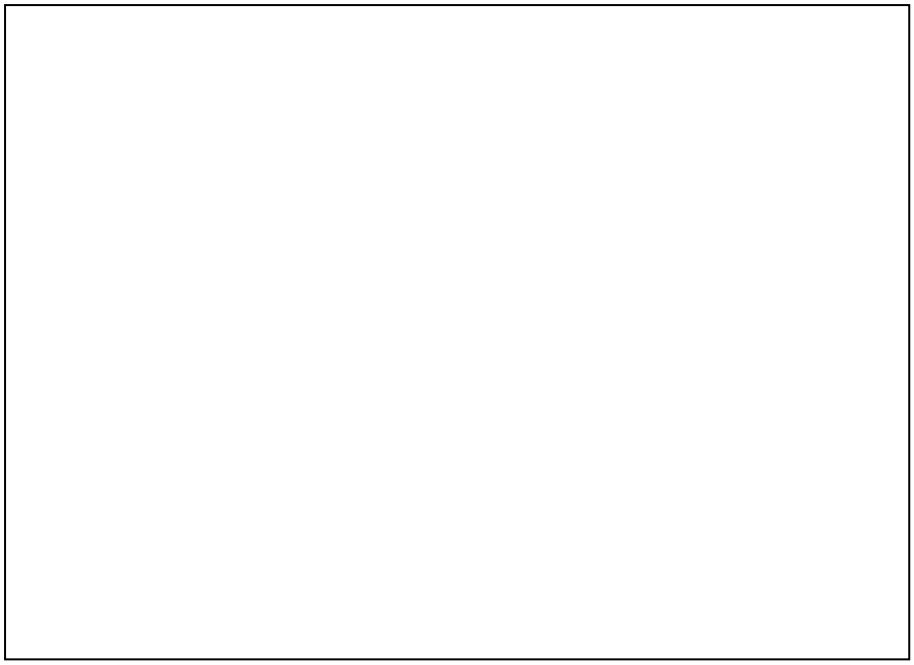

Figure 1-2 Drawing of cross section of a lens, adapted from Gupta *et al.*, (2005). The capsule surrounds the lens and houses the lens. The epithelial cells migrate from the centre of the anterior surface towards the equatorial region. There they differentiate into cortical fibre cells and eventually become nuclear fiber cells (note individual nuclear fibre cells are not shown in this diagram).

On the anterior and posterior surface of the lens there are visible Y-shaped lines known as suture lines. These lines are the junction points where fibre cells from opposite sides of the lens meet.

For the lens to focus light from both distant and near objects it needs to be able to change its shape which either increases or decreases the refractive power of the lens. This change in shape is known as accommodation and involves the ciliary muscles and zonular fibres. When the eye focuses on a near object the ciliary muscle around the lens contract towards the lens, this causes the zonular fibres to relax. Relaxation of the zonular fibres results in the lens becoming more spherical. When the ciliary

muscles contract they pull on the zonular fibres taut which causes the lens to flatten. This occurs when focusing on distant objects (Bray *et al.*, 1999).

1.3 Crystallins

Protein makes up to 30-35% of the lens wet weight and of these proteins 90% are crystallins (Hoenenwarter *et al.,* 2006). The unique packing of the crystallins provides both the clarity and the refractive index of the lens. The crystallins can be separated into 3 families: the α, β and γ crystallins. The α-crystallins are a family and the β and γ crystallins are closely related and belong to the same super family of proteins.

1.3.1 α-Crystallin

Crystallins are the most common protein in the lens and of the crystallins, α-crystallin is the most abundant. It is a multimeric protein made up of two types of subunit, αA-crystallin and αB-crystallin. They are coded for by different genes on different chromosomes but share approximately 60% homology in protein sequence (Graw, 1997). αA crystallin is a lens-specific protein, whereas αB-crystallin is found in a variety of tissues outside the lens; including the brain, heart, muscle and kidney (Iwaki *et al.*, 1990). In sheep these subunits have masses of 19875Da for αA and 20148Da for αB (Robertson *et al.*, 2005). The larger αB-crystallin is more basic than the smaller αA. The ovine α-crystallin subunits share 43% sequence homology to each other and there are multiple areas that contain conserved residues, including the C-terminal region. Comparison of α-crystallin sequences has shown that the genes have evolved slowly, a change of 3% per 100 million years compared with haemoglobin which changes at a rate of 20% per 100 million years (de Jong and Hendricks, 1986). Sequences for ovine αA and αB have been determined (Figure 1-3), they share 99.42% and 98.29% sequence homology with bovine α-crystallins respectively, and 93.46% and 97.14% sequence homology with human αA and αB crystallins respectively (Robertson *et al.,* 2008).

αA-Crystallin

```
Ovine    MDIAIQHPWFKRTLGPFYPSRLFDQFFGEGLFEYDLLPFLSSTISPYYRQ    50
Bovine   MDIAIQHPWFKRTLGPFYPSRLFDQFFGEGLFEYDLLPFLSSTISPYYRQ    50
Human    MDVTIQHPWFKRTLGPFYPSRLFDQFFGEGLFEYDLLPFLSSTISPYYRQ    50

Ovine    SLFRTVLDSGISEVRSDRDKFVIFLDVKHFSPEDLTVKVQEDFVEIHGKH    100
Bovine   SLFRTVLDSGISEVRSDRDKFVIFLDVKHFSPEDLTVKVQEDFVEIHGKH    100
Human    SLFRTVLDSGISEVRSDRDKFVIFLDVKHFSPEDLTVKVQDDFVEIHGKH    100

Ovine    NERQDDHGYISREFHRRYRLPSNVDQSALSCSLSADGMLTFSGPKVPSGV    150
Bovine   NERQDDHGYISREFHRRYRLPSNVDQSALSCSLSADGMLTFSGPKIPSGV    150
Human    NERQDDHGYISREFHRRYRLPSNVDQSALSCSLSADGMLTFCGPKIQTGL    150

Ovine    DAGHSERAIPVSREEKPSSAPSS                               173
Bovine   DAGHSERAIPVSREEKPSSAPSS                               173
Human    DATHAERAIPVSREEKPTSAPSS                               173
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αB-Crystallin

```
Ovine    MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPASTSLSPFYLR    50
Bovine   MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPASTSLSPFYLR    50
Human    MDIAIHHPWIRRPFFPFHSPSRLFDQFFGEHLLESDLFPTSTSLSPFYLR    50

Ovine    PPSFLRAPSWIDTGLSEVRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEV    100
Bovine   PPSFLRAPSWIDTGLSEMRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEV    100
Human    PPSFLRAPSWFDTGLSEMRLEKDRFSVNLDVKHFSPEELKVKVLGDVIEV    100

Ovine    HGKHEERQDEHGFISREFHRKYRIPADVDPLTITSSLSSDGVLTMNGPRK    150
Bovine   HGKHEERQDEHGFISREFHRKYRIPADVDPLAITSSLSSDGVLTVNGPRK    150
Human    HGKHEERQDEHGFISREFHRKYRIPADVDPLTITSSLSSDGVLTVNGPRK    150

Ovine    QASGPERTIPITREEKPAVTAAPKK                             175
Bovine   QASGPERTIPITREEKPAVTAAPKK                             175
Human    QVSGPERTIPITREEKPAVTAAPKK                             175
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Figure 1-3 Protein Sequences for ovine, bovine and human αA and αB crystallin. Sequence homologies to ovine αA are 99.42% and 93.46% for bovine and human αA respectively. Ovine αB sequence homology is 98.29% and 97.14% for bovine and human αB respectively. Sequences were acquired from Genbank (http://www.ncbi.nlm.nih.gov/genbank). Genbank Accession numbers, αA ovine: AY819022, bovine: NM_174289 and human: NM_000394, αB ovine: AY819023, bovine: NM_174290, and human: NM_001885.

The secondary structure of the α-crystallins have not been fully elucidated due to the inability to crystallise the α-crystallin subunits. Comparison of sequences amongst

other proteins has shown that both αA-crystallin and αB-crystallin are homologous with members of the small heat shock protein (sHSP) family (Ingolia and Craig, 1982). This led to the discovery of a common 80 amino acid motif amongst α-crystallin and the sHSPs termed the α-crystallin domain (Figure 1-4) (Caspers *et al.*, 1995). Predictions from circular dichroism and infrared measurements are that α-crystallin contains predominantly β-sheet and a small amount of α-helix (Thomson and Augusteyn, 1989). Although the tertiary structure is unknown it can be predicted from other members of the sHSP family and in particular the heat shock protein, hsp 16.9 (Figure 1-4), which has an ordered N-terminal extension (green) and the α-crystallin domain (red). The α-crystallin domain contains a C-terminal extension, β-sheet sandwich in the middle and an interdomain loop at the top (van Montford *et al.*, 2001).

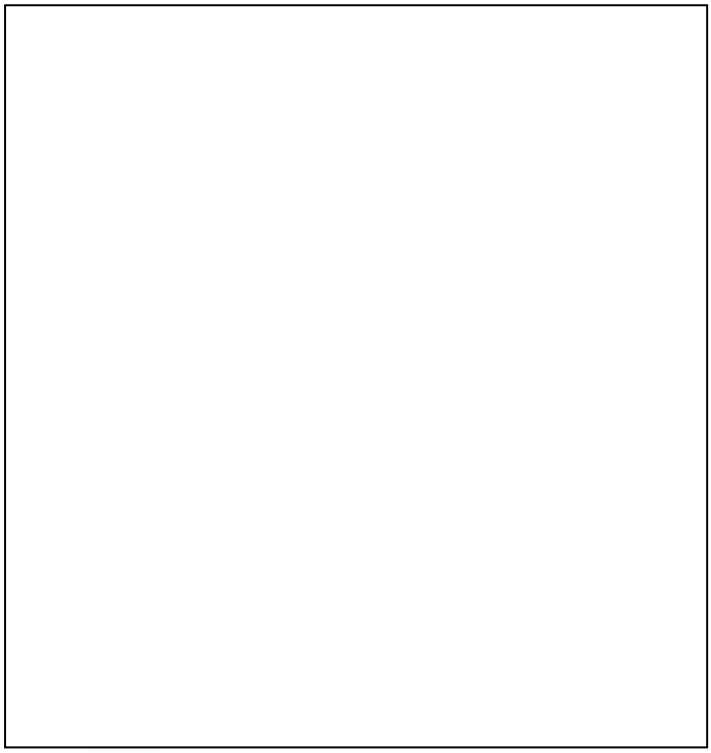

Figure 1-4 Structure of hsp 16.9. The ordered N-terminal region is in green. The α crystallin domain is in red containing the C-terminal region, seven stranded β-sandwich and an interdomain loop (top). Adapted from van Montford *et al.*, (2001)

In human lenses the ratio of αA to αB crystallin varies with age. In the fetal lens the ratio is 2:1 which decreases to 3:2 in the adult lens (Ma *et al.*, 1998). Although the ratio of the two types of α-crystallin are known, the exact molecular weight of α-crystallin molecules in the lens is unknown. This is due to the polydispersity of α-

crystallin in the lens, and there is also a large variation in the size of α-crystallin isolated from the lens. Some papers report a size of approx 200kDa (Thomson and Augusteyn, 1988) while others report the size of α-crystallin in the mega Dalton range (Spector *et al.*, 1971). This large variation in the molecular size of α-crystallin is due in part to the differences in isolation techniques and conditions. For example, isolation of α-crystallin at body temperature reveals an average molecular weight of 320kDa whereas isolation at 5ºC result in average molecular weights of 800kDa (Augusteyn, 2004). Another cause for the various sizes of α-crystallin molecules is that the size of the protein aggregates increase as the lens ages. Therefore α-crystallin in the nucleus of the lens is larger than that in the cortex and epithelium (Augusteyn, 2004). This is thought to be due to the increased packing of protein and removal of water that is seen in the nucleus. This is required for creating the refractive index gradient. Research continues to determine the lowest molecular weight subunit consisting of αA and αB that make up the larger proteins seen across the lens.

A major breakthrough in α-crystallin research occurred in 1992 when it was discovered that α-crystallin can act as a chaperone (Horwitz, 1992). Horwitz showed that purified bovine α-crystallin could prevent the thermal aggregation of some enzymes as well as β/γ-crystallins. Since this time a large amount of α-crystallin research has studied the chaperone effect and its role in the lens.

In order for the lens to remain transparent the crystallins must remain stable and in solution. As there is no protein turnover to remove and replace denatured crystallins another system must be in place to prevent their aggregation. Otherwise these aggregates would lead to cataract formation. It was thought initially that α-crystallin may bind β and γ-crystallin as they became denatured and prevent aggregation and precipitation of the crystallins and hence prevent cataracts forming (Horwitz, 1992). As α-crystallin comprises approximately 50% of the lens protein it would be logical to assume that α-crystallins could act as a sink to bind and hold in solution denatured proteins in the lens and so prevent their aggregation. Once this ‘sink’ of α-crystallin is used up proteins would start to aggregate and cataracts would form (Horwitz, 1992). There is substantial evidence that α-crystallin binds to both β and γ-crystallins and prevents their aggregation as well as that of other lens proteins (Horwitz, 2003). Once

bound to α-crystallin the denatured protein is not refolded and released like the action of other sHSP (Bloemendal *et al.*, 2004). In most cells α-crystallin directs the bound proteins to either proteolysis machinery or to other chaperones for refolding and release (Bloemendal *et al.*, 2004). In the lens this is thought not to occur as the fibre cells are not in a metabolically active state and that the α-crystallins store the substrates in a partially folded state.

The exact mechanism of chaperone action by α-crystallin is unknown. It is known that the quaternary structure of α-crystallin is highly dynamic with a temperature dependent subunit exchange (Bova *et al.*, 1997). In α-crystallin oligomers the hydrophobic areas of individual subunits are buried. However the hydrophobic areas are exposed during subunit exchange, which is the reversible exchange of subunits between oligomers. This allows for substrates to bind to the α-crystallin subunits (Andley, 2007). Subunit exchange is increased at physiological temperatures which results in more hydrophobic faces being exposed to potential target proteins. Interestingly increased temperatures result in a shift towards smaller oligomers which increases the number of hydrophobic sites available (Andley, 2007). This would enhance the chaperone ability of α-crystallin, which would be required as proteins start to denature at higher temperatures.

Many studies have been conducted to determine the functional sequences in α-crystallins that are required for chaperone activity. Using protein pin array Ghosh *et al.,* (2005), showed a number of sequences within the α-crystallin core domain had the ability to bind normal and heated (denatured) proteins. They used β-crystallins, γD crystallin, alcohol dehydrogenase and citrate synthase as target proteins. The consensus from all the experiments resulted in 7 common chaperone sequences that interacted with at least 3 or more of the proteins. Two sequences were found in the N-terminus, 4 were in the α-crystallin core domain and one was in the C-terminal extension. Interestingly 5 of 7 of these sequences are also involved in subunit-subunit interactions within αB-crystallin oligomers (Ghosh and Clark, 2005).

1.3.2 β- and γ-Crystallins

The β and γ crystallins are structurally and evolutionarily related and are part of the β/γ-crystallin super family. The common element amongst the β/γ super family is that the protein structures are all built on a Greek Key motif. The differences between them are that the β-crystallins are oligomeric and have N-terminal extensions, whilst the γ-crystallins exist as monomers and lack an N-terminal extension.

There are two types of β-crystallin, acidic (βA1, βA2, βA3 and βA4) and basic (βB1, βB2 and βB3), with the basic proteins having a C-terminal extension (Bloemendal *et al.*, 2004). βA3 and βA1 proteins are coded from the same gene, where the transcription of βA3 starts upstream of βA1 and therefore the only difference between the proteins is the length of the N-terminal extension. As a consequence of this βA1 and βA3 are often represented as the same protein as discerning between them is difficult. β-crystallin expression is varied throughout development and they are fibre cell specific.

β-crystallin assemblies separate into two categories based on elution profiles from gel permeation chromatography: βH- which represents the hexamers/octamers and βL which represents dimers. There is a gradient of β-crystallin assemblies across the lens from βL in the cortex to βH in the nucleus. This is due to the increased crystallin packing required in the centre of the lens for refractive index.

There are 7 γ-crystallins found in mammalian lenses, γA-F and γS. γA-F are closely related and are coded for within a linked gene cluster with a similar sequence. The other γ-crystallin is γS which is coded for on another chromosome and has a different structure and sequence to the other 6 γ-crystallins. The γ-crystallins are monomeric and are the smallest crystallins with molecular weights of around 20kDa. They are also the most basic of the crystallins (Graw, 1997). The γ-crystallins are fibre cell specific. They are expressed early in lens development and their expression decreases postnatally, as a consequence of this they are found in higher levels in the nucleus compared to the cortex. The proportion of different γ-crystallins in the lens varies

widely according to the species studied. Those species that require a 'hard' lens (e.g. rodents) contain all 7 γ-crystallins whereas those species requiring 'soft' lenses (e.g. humans) only contain γC and γD in high concentrations.

1.3.3 Post-translational modifications

Crystallins are not replaced throughout the lifetime of a lens. Therefore they are extensively post-translationally modified to continue their development within the lens. There are 4 major methods of modifying crystallins: deamidation, phosphorylation, disulfide bond formation and truncation. Early studies suggested that post-translational modification was a marker for lens ageing and increased as the lens matured. However modifications in human lens have been observed in lenses as young as 3 years old and the modifications seen in a 17 year old lens are very similar to that in a 54 year old lens (Lampi *et al.*, 1998).

Deamidation is a common post-translational modification where asparagine is converted to aspartate. It is the most common post-translational modification of lens crystallins (Wilmarth *et al.,* 2006, Hains and Truscott, 2007a). Chemically, deamidation is the conversion of the $-NH_2$ group of asparagine to –OH. This gives the amino acid a mass increase of 1Da and a decrease in the isoelectric point. The mass change of only 1 made deamidation difficult to detect in early mass spectrometry experiments as the mass change was within the error associated with the technique. However with more powerful mass spectrometers and software, deamidation can now be readily detected and appears to be an important modification of the crystallins. Currently the exact role of deamidation in proteins is uncertain but it is thought the process may be a precursor to proteolytic cleavage. αA, βB1, βB2, βA3, βA4, and γS are the main crystallins that undergo deamidation in the adult human lens (Lampi *et al.*, 1998).

Phosphorylation of proteins occurs in many cellular processes and is evident in the lens. Phosphorylation occurs on serine, threonine, histidine and tyrosine residues. In the lens the major proteins phosphorylated are αA and αB-crystallin (Hoehenwarter *et al.*, 2006). To a lesser extent some β-crystallins become phosphorylated namely,

βA1/A3, βA4, βB1, βB2 and βB3 (Hoehenwarter *et al.*, 2006). The exact role of phosphorylation in crystallin maturation is unknown. Research suggests that α-crystallins chaperone ability is modified with phosphorylation (Horwitz, 2003, Aquilina *et al.*, 2004, Ecroyd *et al.*, 2007).

Disulfide bond formation is the bonding of two thiol moieties from cysteine amino acids. The disulfide bonds can be within protein molecules to stabilise tertiary protein structures or can be between different proteins. γS crystallins contain disulfide bonds which can bind monomers to form a dimer, whose function is unknown (Bloemendal *et al.*, 2004).

Almost every crystallin in the lens undergoes truncation during its lifetime. In particular the α-crystallins are truncated at the C-terminal, and the β-crystallins are truncated at both their N- and C- terminal ends. Many of the truncations of α-crystallins have been shown to be calpain specific in mouse (Ueda *et al.*, 2002a). This study showed that both calpain 2 and Lp82 are responsible for the truncations seen during ageing on the C-terminal arm of α-crystallin (For more see 1.4.1).

1.4 Cataract

Cataracts are described as opacification of the lens of the eye that partially or totally scatters and/or absorbs the light entering the eye resulting in loss of some, if not all of vision. Cataracts are the leading cause of blindness accounting for 50% of the total blindness in the world (Hammond, 2001). There are numerous types of cataracts, that can be classified by (i) their location in the lens e.g. nuclear, cortical, posterior, anterior, or (ii) their colour, e.g. white through to yellow to brown/black or (iii) cause e.g. age related, diabetic cataracts (Hammond, 2001). Many cataracts are mixed and have both corical and nuclear invovlment. Nuclear cataracts are the most common age related cataract and accounted for 60% of cataracts in the Beaver Dam Eye study whilst cortical cataracts made of 30% of the cataracts (Klein et al., 2002).

Age is the most common factor contributing to cataractogenesis. The Beaver Dam Eye Study showed that 45% of 55-64 year olds and 88% of those aged over 75 have cataracts (Klein et al., 2002).

This thesis will concentrate on changes in crystallins as a factor in the formation of cataracts and in particular the truncation of the crystallins. As mentioned above crystallins can undergo a number of post-translational modifications including truncation, deamidation, phosphorylation and disulfide bond formation. All of these modifications to crystallins have been implicated in cataractogenesis.

1.4.1 α-Crystallin

Phosphorylated α-crystallin has been found in both normal and cataractous human lenses (Lund *et al.*, 1996). Interestingly in a recent study α-crystallin was shown to be phosphorylated in normal lenses but not in the urea soluble fraction of age matched cataractous lenses (Hains and Truscott, 2007a). The authors postulated that this is because the phosphorylated α-crystallins were involved in other reactions and were therefore not detectable in the urea soluble proteins from a cataractous lens. As yet the exact role of phosphorylating crystallins is not known. There is evidence that phosphorylation of α-crystallin has an effect on its chaperone abilities. Some studies suggest phosphorylation decreased chaperone activity (Kamei *et al.*, 2001) whilst others have concluded that there was an increase in activity (van Boekel *et al.*, 1996). This makes it difficult to establish how the phosphorylation of crystallins affects cataract formation.

Truncation of the lens crystallins during the formation of cataracts is well documented. Recently, Hoehenwater et al., (2006) conducted a review of the eye lens proteomics and documented all known changes to the crystallins in normal ageing and cataract in humans, mice, rats and cows.

Crystallin	Total Truncations	Normal Lens	Cataract Lens
αA	20	19/20	11/20 (1)
αB	15	15/15	5/15 (0)

Table 1-1 Summary of α-crystallin truncations from Hoehenwarter *et al.*, (2006). Table shows the total truncations from human, mouse, rat and bovine species, of the total truncations the table indicates those that are found in normal lenses of any age and those found in cataractous lenses. Numbers in brackets indicates number of truncations unique to the cataract lens.

As of 2006 there have been 20 reported truncations of αA-crystallin in human, bovine, mouse and rat lenses (Table 1-1), of these 20 truncations 19 have been found in normal lenses and 11 found in cataractous lenses. This overlap suggests that αA-crystallins from cataractous lenses are not that different to those found in normal lenses. What is interesting about the truncations found in the cataract lens is that all 11 truncations are found at the C-terminal end of αA-crystallin (amino acids 118-173) and that only 1 truncation of αA-crystallin is unique to cataract. All the others are found at some stage in the ageing lens. This table and summation does not quantify the relative amounts of the proteins in normal and cataractous lenses only to confirm the presence of the species. Therefore some modifications reported may only be found in minor amounts within the lens.

According to Hoehenwarter *et al.,* (2006) there are 15 αB truncations found in the lens (Table 1-1). All 15 of these are found in the normal lens and 5 are found in cataractous lenses. The results were similar to αA-crystallin in that most of the truncations are found in the C-terminal end of αB-crystallin and none of the truncations are unique to cataracts.

1.4.2 Chaperone function of α-crystallin

The chaperone function of α-crystallin, where it binds denatured proteins and prevents them from becoming insoluble, is vital to maintaining lens transparency. Loss of this activity is suggested to play a role in the formation of cataract. During ageing of the lens there is a decrease in the chaperone activity of α-crystallin, with fetal bovine lens able to provide better protection to β-crystallins denatured by heat than aged bovine α-crystallin (Horwitz *et al.*, 1992). α-Crystallins' chaperone function also varies across

the lens, with the cortical region, containing the youngest α-crystallin, providing better chaperone protection than the nuclear region which contains the oldest α-crystallin within the lens (Augusteyn *et al.,* 2002).

Many modifications to α-crystallin within the lens could be attributed to this change in chaperone function both with age and across the lens, including glycation, deamidation, phosphorylation and proteolysis. For the purpose of this study only proteolysis and phosphorylation will be examined in further detail.

As described above there is an increase in phosphorylation of the α-crystallins with ageing. There are conflicting results on the effect that phosphorylation has on chaperone function. Some studies show that phosphorylation has no effect on the chaperone function of α-crystallin (Wang *et al.,* 1995, Augusteyn *et al.,* 2002) whilst others report a change in chaperone function with either an increase (Ecroyd *et al.,* 2007, Ahmad *et al.,* 2008) or decrease (Kamei *et al.,* 2001, Aquilina *et al.,* 2004) in activity. However it is important to note some of the studies that showed changes in chaperone function used a phosphorylation mimicking α-crystallin (Ecroyd *et al.,* 2007, Ahmad *et al.,* 2008). Phosphorylation mimicking involves the production of recombinant α-crystallin incorporating charged residues at the places of normal phosphorylation, instead of using native α-crystallin with a phosphorous moiety on the affected residue. This places a limitation on the research's application within the lens, whereas the studies that showed no change used native phosphorylated α-crystallin. As a result of this, additional research is required to determine the role phosphorylation has in the lens and what effect phosphorylating α-crystallin has on the chaperone function that is so vital to lens transparency.

Within the structure of both α-crystallins is the unstructured and flexible C-terminal tail, from residues 140-173/5 of αA/B-crystallin, which is essential for chaperone function. Many studies involving truncation and site-directed mutation of the C-terminal tail have shown the importance of this region for chaperone activity.

In 1993 Takemoto *et. al.,* showed that removal of the C-terminal region of αA-crystallin by tryptic digestion decreased the ability to protect alcohol dehydrogenase from heat induced aggregation. Further work by the same researcher identified that

the loss of residues 158-173 of αA resulted in decreased chaperone function (Takemoto, 1994). A similar study involving the use of calpain 2 to remove 11-12 residues from the C-terminus of α-crystallin, showed that the truncated α-crystallin had reduced chaperone activity (Kelley *et al.,* 1993). When αA-crystallin was cloned with the last 17 residues deleted there was a reduction in the chaperone function of the mutant (Andley *et al.,* 1996). Removal of between 5 and 11 residues reduced the chaperone function of αA-crystallin and as more resides were removed the effect on chaperone function was more pronounced (Rajan *et al.,* 2006, Aziz *et al.,* 2007). Interestingly, the removal of the C-terminal serine of αA results in a protein with increased chaperone function (Aziz *et al.,* 2007). Many studies on α-crystallin use site-directed mutagenesis to determine the role of specific regions, motifs and residues on chaperone function. Mutation of the final two C-terminal serines in αB-crystallin reduces its chaperone function (Plater *et al.,* 1996). Insertion of charged residues into the C-terminal tail of αA-crystallin has no effect on chaperone function but the insertion of hydrophobic resides greatly reduces chaperone function (Smulder *et al.,* 1996).

Recent work has focused on the role of the C-terminal extensions of αA and αB-crystallin, in forming homo- and heteroaggregates (Kallur *et al.,* 2007). This study researched the effect of the most common truncations in αA ($\alpha A_{1-172,}$ αA_{1-168}, αA_{1-162}) on their ability to form homoaggregetes with wildtype αA and heterodimers with wildtype αB. It showed that removal of 1 or 5 residues from the C-terminal end of αA resulted in a two fold decrease in subunit interaction and that removal of 11 residues resulted in a six fold reduction in subunit interaction. The conclusion from this is that the formation of α-crystallin complexes is reduced with truncation of αA-crystallin C-terminal residues, leading to a reduction in the ability of α-crystallin to act as a molecular chaperone.

All of this research clearly shows a role for the C-terminal region of the α-crystallins in chaperone function and any disruption to this region can have severe consequences on chaperone function.

1.4.3 β-Crystallins

Truncation of crystallins is not limited to just α-crystallins, β-crystallins also undergo truncation during ageing and cataractogenesis. Table 1-2 shows the β-crystallins that undergo truncation and their incidence in the normal and cataract lens. The βA1/βA3 species has been reported to be truncated 5 times *in vivo* (David *et al., 1993,* Lampi *et al.,* 1997, Lampi *et al.,* 1998, Srivastava and Srivastava, 1999). Of these truncations, 3 are found in normal lenses only and 2 are unique to cataractous lenses. βA3 alone has one truncation which is found in both cataractous and normal lenses (Shih *et al.,* 1998, Ueda *et al.,* 2002b). Similarly βA4 only has one known truncation which is only found in cataractous lenses (David *et al.,* 1993). The largest crystallin, βB1 has been shown to have 13 truncations of which 9 are in the normal lens and 4 in the cataractous lens only (David *et al.,* 1993, David *et al.,* 1994, David *et al.,* 1996, Shih *et al.,* 1998, Ma *et al.,* 1998, Wilmarth *et al.,* 2004). The β-crystallin results indicate that truncation occurs during normal maturation and ageing of the lens. However "incorrect" truncation is found in the cataractous lenses indicating that uncontrolled truncation of the β-crystallins may be one of the contributing factors to cataractogenesis.

Crystallin	Total Truncations	Normal Lens	Cataract Lens
βA1/βA3	5	3/5	2/5 (2)
βA3	1	1/1	1/1 (0)
βA4	1	0/1	1/1 (1)
βB1	13	9/13	4/13 (4)
βB3	3	2/3	2/3 (1)
βB2	2	2/2	0/2 (0)

Table 1-2 Summary of β-crystallin truncations from Hoehenwarter *et al.,* (2006). Table shows the total truncations from human, mouse, rat and bovine species. This table indicates those that are found in normal lenses of any age and those found in cataractous lenses. Numbers in brackets indicates number of truncations unique to the cataract lens.

1.5 The Calpain Proteolytic System

The calpains, previously known as calcium-dependent neutral proteases, are a family of calcium-dependent cysteine proteases. There are two major ubiquitously expressed calpains, calpain 1 (also known as μ-calpain) and calpain 2 (also known as m-calpain), as well as other ubiquitous and tissue specific calpains.

1.5.1 Calpain isoforms

There are genes for 14 different calpains in the human genome. The major calpains are calpain 1 and calpain 2 which are distributed ubiquitously. The calpain family can be split into two groups the typical calpains and the atypical calpains (Table 1-3) (Suzuki *et al.,* 2004). The domain structure of the large subunit of the typical calpains is similar to that of the large subunit found in calpains 1 and 2. The typical calpains are calpains 1, 2, 3, 8, 9, 11, 12, 13 and 14. The atypical calpains do not have the calmodulin like EF hands in domain IV of the large subunit and some lack domain IV altogether. Atypical calpains also cannot form a dimer with the small 30kDa subunit. The atypical calpains are 5, 6, 7, 10 and 15. It is important to note that the small subunit is also known as calpain 4.

Calpain	Other Names	Tissue Distribution	Typical	Reference
Calpain 1	μ-calpain	Ubiquitous	Yes	(Cong *et al.,* 1989)
Calpain 2	m-calpain	Ubiquitous	Yes	(Cong *et al.,* 1989)
Calpain 3a	p94	Muscle	Yes	(Sorimachi *et al.,* 1989)
Calpain 8	nCL-2	Stomach	Yes	(Sorimachi *et al.,* 1993)
Calpain 9	nCL-4	Digestive Tract	Yes	(Lee *et al.,* 1998)
Calpain 11	-	Testis	Yes	(Dear *et al.,* 1999)
Calpain 12	-	Hair follicle	Yes	(Dear *et al.,* 2000)
Calpain 13	-	Ubiquitous	Yes	(Dear and Boehm, 2001)
Calpain 14	-	-	Yes	(Dear and Boehm, 2001)
Calpain 5	hTRA-3, nCL-3	Ubiquitous	No	(Dear *et al.,* 1997)
Calpain 6	CANPX	Muscle	No	(Dear *et al.,* 1997)
Calpain 7	PalBH	Ubiquitous	No	(Futai *et al.,* 2001)
Calpain 10	-	Ubiquitous	No	(Horikawa *et al.,* 2000)
Calpain 15	SOLH	Ubiquitous	No	(Kamei *et al.*, 1998)

Table 1-3 Family of Calpains and their distribution within tissues.

An important member of the calpain family of proteases is calpain 3, which has a number of splice variants, including a lens specific isoform, Lp82 (Ma *et al.,* 1998a).

An important component of the calpain proteolytic system is the endogenous calpain inhibitor, calpastatin (Dayton *et al.,* 1976). Calpastatin is the only endogenous protein inhibitor for calpain and is specific to calpain enzymes. It has been shown not to react with other cysteine proteases i.e. papain, cathepsin B, or bromlin. It also fails to react with other types of protease e.g. trypsin, chymotrypsin, pepsin (as reviewed by Goll *et al.,* 2003).

Although there is a single gene for calpastatin there are up to 9 different isoforms, found in various tissues in the body. This is due to the use of different promoters and/or alternative splicing mechanisms (as reviewed by Goll *et al.,* 2003). Calpastatin

is a multi headed inhibitor and is capable of inhibiting more than one calpain molecule at a time. Calpastatin consists of 4 domains (I-IV), which can inhibit one calpain molecule each and a N-terminal region, named L, which has no calpain inhibitory properties (Maki *et al.,* 1987, Takano *et al.,* 1988, Hanna *et al.,* 2007). Each of the inhibitory domains I-IV can be further divided into 3 conserved subdomains A, B and C (Maki *et al.,* 1988).

1.5.2 Calpain structure

The classical calpains 1 and 2 are the most researched and best characterised of the calpains. They are both heterodimers, with a large subunit (80kDa), which contains the catalytic triad, and a small subunit (28kDa), which is involved in the regulation of calpain. Calpain 2 has a flat oval disc shape with dimensions of 115Å long, 70Å wide and up to 50Å thick (Reverter *et al.*, 2001a). Originally a four domain structure was proposed based on the amino acid sequence analysis for the large subunit and a two domain structure for the small subunit (Ohno *et al.,* 1986, Imajoh *et al.,* 1988). This six domain structure was confirmed when the crystal structures for rat (Hosfield *et al.*, 1999) and human calpain 2 were determined (Strobl *et al.*, 2000) (Figure 1-5).

Domain I (dI) is an α-helix that sits in a cavity of domain IV and plays a role in anchoring both domains together. Domain II (dII) contains the catalytic site and has been split into two subdomains dIIa and dIIb (Reverter *et al.,* 2001b). DIIa contains the papain-like 'active site helix', which harbours the active site residue Cys105. The subdomain IIb has a barrel like shape similar to that of papain. The other two residues form the catalytic triad, His262 and Asn286, reside in dIIb. The substrate binding cleft is between the two subdomains dIIa and dIIb, however this cleft has a wide gap of 15Å. This large gap disrupts the catalytic triad which makes it incompatible with binding and cleavage of the substrate (Reverter *et al.*, 2001a). Domain III (dIII) is folded in a β-sandwich and shows similar topology to C2 domains, which are found in a large number of proteins and in particular those involved in intracellular signalling e.g. protein kinase C, phospholipase C, etc. DIII has been shown to bind both Ca^{2+} and phospholipids (PL) and is involved in the activation of calpain 2 (Strobl *et al.*, 2000) . Of particular interest is the extended exposed acidic loop in dIII (Figure 1-5). Within

this 11 residue loop are 10 acidic residues, these residues interact electrostatically with the positive charges on the surface of subdomain dIIb. Domain IV (dIV) is a calmodulin-like domain, consisting of 8 α helices. These α-helices contain 2 pairs of 2 EF hand structures. dIV also contains a potential fifth EF hand, when paired with domain VI. EF hands are protein secondary structures that bind Ca^{2+} ions, and consist of a long α-helix with two perpendicular smaller α-helixes at each end. The final domain of calpain is domain VI (dVI) in the small subunit. dVI has similar structure to dIV of the large subunit, dVI contains 2 pairs of 2EF hand structures and could possibly form a fifth EF hand when associated with dIV (Reverter *et al.*, 2001b).

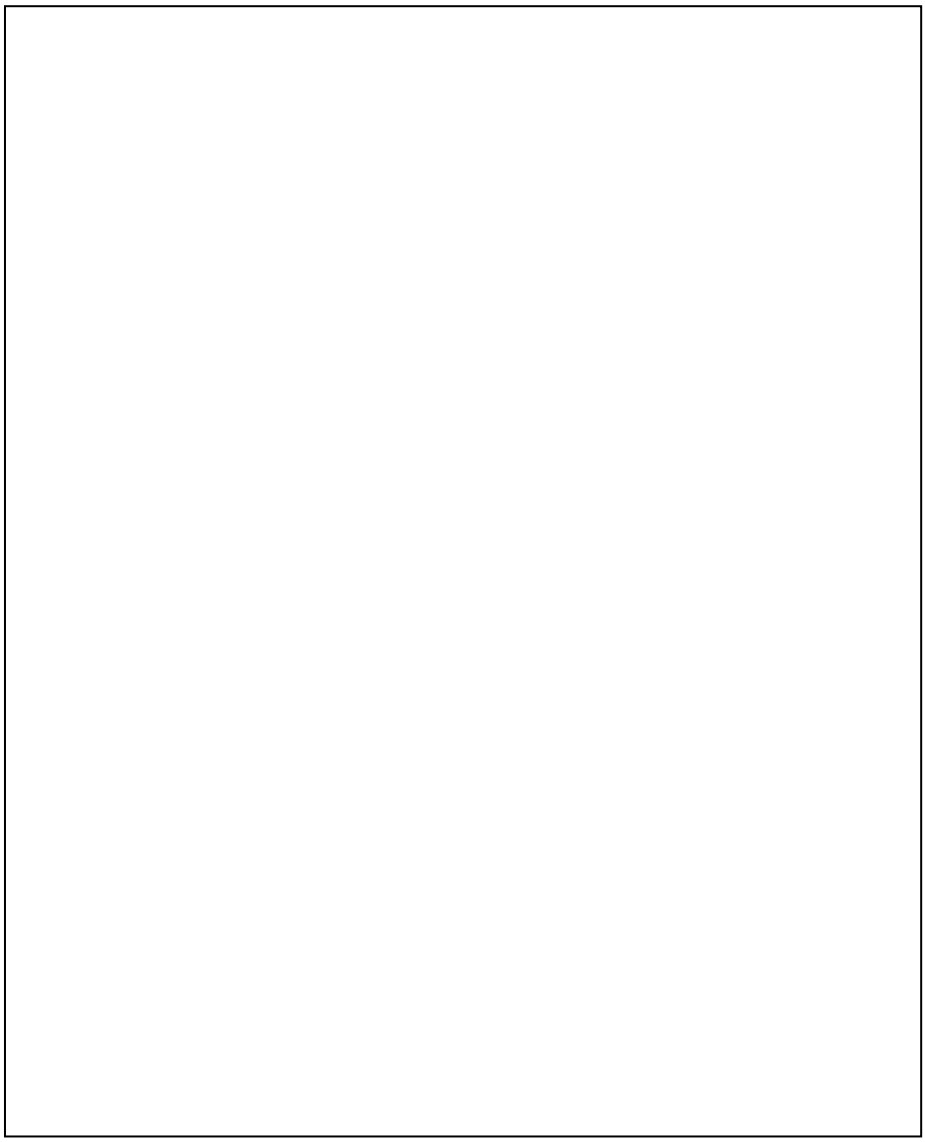

Figure 1-5 Ribbon Structure of human calpain 2 (Reverter *et al.*, 2001b). The active site is between domain IIa (yellow) and domain IIb (red). The catalytic triad residues are shown Cys105 in subdomain IIa and His262 and Asn286 in subdomain IIb.

1.5.3 Activation of calpain

Activation of the calpain molecules has been an area of extensive research and a number of theories about this mechanism have been proposed. One of the unique properties of the calpain family is an absolute dependence on calcium for activation. As mentioned above the catalytic triad is located between dIIa and dIIb, however when calcium is not bound the distance between the catalytic residues of Cys105 and His262 and Asn286, is over 15Å (Reverter et al., 2001b). This is too wide to allow substrate binding and catalytic cleavage of the substrate so in its native state (ie with no calcium bound) calpain is inactive. Therefore activation of calpain upon calcium binding requires a conformational change to the molecule to allow the catalytic residues to move close enough for successful binding and cleavage of substrates. The calcium concentrations required for half activation of calpain 1 and calpain 2 *in vitro* are 3-50μm and 400-800μM respectively (Goll *et al.*, 2003). These concentrations are considerably higher than *in vivo* intracellular Ca^{2+} concentrations of <1μM (Goll *et al.*, 1992).

There are three calcium binding sites within the calpain 2 molecule, (i) the EF hands in the calmodulin-like domains of dIV and dVI (Blanchard *et al.*, 1997), (ii) the acidic loop region of the C2-like dIII (Strobl *et al.*, 2000) and (iii) within dII (Moldveanu *et al.*, 2002).

Calcium binding to calpain is followed by rapid autolysis. This involves the autolytic removal of N-terminal amino acids from both the large and small subunits (Elce *et al.*, 1997). This reduces the mass of calpain 1 large subunit from 80kDa to 76kDa, the mass of calpain 2 large subunit from 80kDa to 78kDa and the mass of the small subunit from 28kDa to 18kDa (Edmunds *et al.*, 1991). This autolysis reduces the Ca^{2+} concentration required for activation of calpain. The Ca^{2+} concentration for half maximal proteolytic activity of calpain 1 reduces from 3-50μM to 0.5-2μM and that of calpain 2 from 400-800μm to 50-150μM (Goll *et al.*, 2003).

As mentioned above the dI region of the calpain molecule is a molecular anchor sitting within a hydrophobic pocket of dVI. This not only binds the large and small

subunits together but also constrains the movement of dI. Proteolytic cleavage of dI removes this anchor. There is evidence that this results in dissociation of the large 80kDa subunit from the small 30kDa subunit (Michetti *et al.,* 1997, Nakagawa *et al.,* 2001). However separation of the subunits is not necessary for the activation of calpain (Elce *et al.*, 1997). The removal of the anchor allows the remaining dI to have more freedom of movement which eases the tension of dIIa and allows it to move towards dIIb. This movement closes the gap between the two subdomains (Jia *et al.*, 2001). However the movement acquired by dIIa after removal of dI is not enough to close the catalytic triad for proteolytic cleavage (Jia *et al.*, 2001). Therefore dIIb also needs to move into the proteolytic cleft to align the catalytic triad, but it is held in place by hydrostatic interactions with dIII (Reverter *et al.*, 2001a).

Calcium can also bind to dIII. This binding can play a role in the activation of the calpain molecule. The part of dIII that plays a role in the activation of calpain is the acidic loop (Figure 1-5). The acidic loop contains 10 negatively charged acidic residues which interact with the positively charged residues in dIIb and so prevent dIIb from moving to reduce the gap in the catalytic triad. It has been suggested that calcium binding to the acidic loop would reduce its electrostatic charge and therefore electrostatic interaction with dIIb. This would allow dIIb the freedom to rotate over dIIa and to form a functional catalytic domain (Strobl *et al.*, 2000).

Another major site of Ca^{2+} binding in the calpain molecule is to the EF hands in domains IV and VI. It was initially suspected that these two domains and in particular the EF hands within them, were the key to understanding the requirement of calpain for Ca^{2+}. This view changed when the crystal structure of calpain was elucidated. It was discovered that the structural changes induced by calcium binding to the EF hands was too limited to have a major effect on the enzyme (Reverter *et al.*, 2001a). However calcium binding is required for activation of the enzyme but no specific EF hand is required (Dutt *et al.*, 2000). One effect calcium has on its binding to dVI is the disruption of the dILys7-dVIAsp154 salt bridge that holds dI and dIV together. This frees dI and allows it to go under autolysis as described above (Reverter *et al.*, 2001a).

The final area of calcium binding in the calpain molecule is within dII. It has been shown that one calcium ions binds each of the subdomains IIa and IIb. These calcium

ions help in the formation of the catalytic cleft by allowing rotation of the domains to form an active calpain molecule (Moldveanu *et al.*, 2002).

All of the above mechanisms require calcium for the activation of calpain. There is evidence for other factors involved in the activation of the calpain system, in particular PL have been shown to be involved with calpain activation (Pontremoli *et al.,* 1985, Arthur and Crawford, 1996). Domain III is a C2-like domain, and like C2 domains in other enzymes (Phospholipase C and protein kinase C, for example) binds PL (Tompa *et al.*, 2001). The inclusion of PL in buffers has been shown to decrease the calcium concentration required for autolysis of calpain *in vitro* (Coolican and Hathaway, 1984). Extensive studies by Arthur and Crawford (1996) showed that phosphotidyl -inositol; -choline; - glycerol; - serine; - ethanolamine and phosphatidic acid all reduce the Ca^{2+} concentration required for autolysis and hence the Ca^{2+} concentration for activation. It was postulated in the above paper that the PL was bound to the N-terminus of the small subunit of calpain. However once the crystal structure of calpain 2 was determined it was shown that it was dIII that bound PL (Tompa *et al.*, 2001). Binding of PL to dIII was shown to promote Ca^{2+} binding several fold (Tompa *et al.*, 2001) and consequently, PL could play a role in reducing the gap between *in vivo* and *in vitro* Ca^{2+} concentration for the activation of calpain.

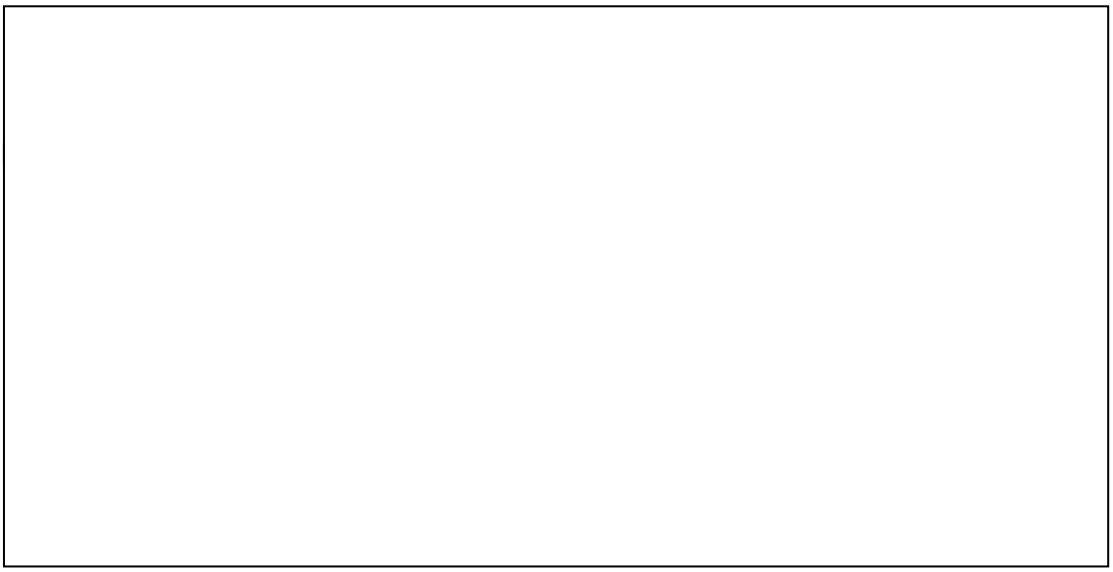

Figure 1-6 Calpain 2 activation with the presence of calcium and phospholipid. Each domain is represented by a shaded sphere as in Figure 1-5. Adapted from Reverter *et al.,* (2001a).

In summary activation of calpain by calcium and PL is a complex process and not one that is fully understood. For full activation of calpain there needs to be up to 10 Ca^{2+} ions bound to the calpain. As shown in Figure 1-6 calcium binding to the EF hands in dVI disrupts the salt bridge between dVI and dI which frees dI for autolysis. Calcium also binds to the negative acidic loop region of dIII which relieves the hydrostatic tension between dIII and dIIb. This allows movement of dIIb to form an active catalytic domain with dIIa. PL also bind dIII and reduces the hydrostatic interaction between the two domains. Calcium binding within the catalytic domain also helps in catalytic domain fusion.

Binding of calpastatin to calpains was found to be dependent on the presence of Ca^{2+} (Imajoh and Suzuki, 1985) and that subdomain A bound to dIV (Ma *et al.*, 1994), and subdomain C bound to dVI (Takano *et al.*, 1995). At present the binding site for subdomain B has not been elucidated but it is suspected to bind in domain III (Wendt *et al.*, 2004).

1.5.4 Calpains in the lens

There has been extensive research on calpains in the lens. Calpain activity has been found in human (Shih *et al.*, 2001), sheep (Robertson *et al.*, 2005), cow (Yoshida *et al.*, 1985), mouse (Fukiage *et al.*, 1997), rat (David and Shearer, 1986), rabbit (Fukiage *et al.*, 1997) and guinea pig (Fukiage *et al.*, 1998) lenses. At present five calpains have been discovered in the lens, these being calpain 2 (Yoshida *et al.,* 1984, Yoshida *et al.,* 1985), calpain 1 (Roy *et al.*, 1983), Lp82 (Ma *et al.*, 1998b), Lp85 (Ma *et al.*, 2000b) and calpain 10 (Ma *et al.*, 2001). Of these calpains calpain 2 is the most abundant isoform. Calpain 2 is found in the epithelium, cortex and nuclear region of the lens with highest activity being in the epithelium, then cortex and the lowest activity being found in the nucleus (Yoshida *et al.*, 1985).

The lens specific calpain Lp82 was discovered in rats by Ma *et al.,* (1998a). It is a splice variant of the muscle specific calpain 3 (p94), resulting from deletion of the IS1 and IS2 regions of calpain 3 and a different exon 1 (Ma *et al.*, 1998a). So far Lp82 has been isolated in mice (Ma *et al.,* 1999), rats (Ma *et al.,* 1998b) and cattle (Ueda *et al.,*

2001). In rats Lp82 was found in all regions of the lens with the highest activity in the nucleus then the cortex and lowest levels in the epithelium (Ma *et al.*, 1998b), which is the reverse of calpain 2 which has the relative activity of epithelium>cortex>nucleus. Biochemical analysis of bovine Lp82 found that it migrated to 82kDa on SDS-PAGE and that the calcium concentration for half maximal concentration of Lp82 was 20μM (Ueda *et al.*, 2001). This is lower than that of calpain 2, which suggests that it may play more of a role than calpain 2 in the lens at lower calcium concentrations. The optimal pH for activation of Lp82 was found to be 7.2, which is lower than the 7.5 optimum found for calpain 2 from the lens (Ueda *et al.*, 2001).

During purification of Lp82 another lens specific calpain was discovered named Lp85. It co-eluted with Lp82 on a DEAE Sepharose column (Ma *et al.,* 2000b). After analysis of the sequence Lp85 was discovered to have the same sequence as Lp82 with a 28 amino acid insertion in the calcium binding domain IV. This resulted in a protein that migrates to 85kDa on SDS PAGE. Lp82 and Lp85 have very similar characteristics. Both require the same calcium concentration, 20uM, for 50% activation and have the same optimal pH of 7.2 (Ma *et al.,* 2000b). They have the same distribution profile across the lens with relative levels being nucleus>cortex>epithelium. One difference between Lp82 and Lp85 is that Lp85 shows distribution around the peri-nuclear region of the nucleated cortical fibres (Ma *et al.*, 2000a) conversely Lp82 was found to be in the cytoplasm of the nuclear and cortical fibre cells (Ma *et al.*, 2000a). Thus it was proposed that the insertion sequence promotes the association of Lp85 with the nuclear membrane. Importantly Lp85 at present has only been found in young rodent lenses.

It is also important to note that neither Lp82 nor Lp85 are expressed in human lenses due to a stop codon in the sequence (Fougerousse *et al.,* 2000). This is due to a difference in human intron 18, where it is 2 bases longer. This produces a frame shift and consequently the formation of a stop codon 4 nucleotides into exon 4 (Fougerousse *et al.,* 2000). However a Lp82-like cleavage site on α-crystallin has been found in human lenses (Takemoto, 1995b). Interestingly calpain 3 isoforms have been found at a genetic level in human lens epthelial cells hUp84 and hUp49 and it

has been suggested these isoforms play a role in human cataract formation similar to that of Lp82 (Kawabata *et al.,* 2003).

1.6 Calpains, Crystallins and Cataracts

Many studies have looked at the concentration of metal ions in normal and cataractous lenses and in particular Ca^{2+}, with a number showing an increase in cystolic Ca^{2+} in association with opacification of the lens (Duncan and Bushell, 1975, Hightower *et al.,* 1987, Sanderson *et al.,* 2000, Robertson *et al.,* 2005). Some of these authors concluded that this increase in free Ca^{2+} could result in the activation of the calcium-dependent cysteine proteases; the calpains. Interestingly only cortical and not nuclear cataracts are associated with an increase in calcium concentration (Duncan and Bushell, 1975, Sanderson *et al.,* 2000).

The calpain family of proteases have an absolute requirement for Ca^{2+} for activation. Several human diseases are the result of overactivation of calpains responding to increased levels of intracellular Ca^{2+}, and include muscular dystrophies, myocardial infarctions and strokes (Goll *et al.,* 2003, Zatz and Starling, 2005). Cataracts are another disease characterised by a loss of Ca^{2+} homeostasis and uncontrolled calpain activity. Calpains have been implicated in various animal models of cataract which include the selenite (David *et al.,* 1993), diamide (Azuma and Shearer, 1992), galactose (Azuma *et al.,* 1995) and Shumiya (Inomata et al., 1997b) rat cataracts.

1.6.1 Calpain 2

Calpain 2's role in the lens has been extensively researched since its discovery in the lens in 1984 (Yoshida *et al.*, 1984). Early studies showed that the crystallins are substrates for calpain 2 (David and Shearer, 1984). Since then more evidence has supported a role for calpain 2 in cataractogenesis. Calpain 2 has been shown to proteolyse both α and β-crystallins but to have no effect on the γ-crystallins (David and Shearer, 1986). Calpain 2 activity on crystallins and in cataract lenses has been demonstrated in various animals and animal models of cataractogenesis, including rat

(Fukiage *et al.,* 1997, Ueda *et al.,* 2002b), mouse (Fukiage *et al.,* 1997, Ueda *et al.,* 2002a), guinea pigs (Fukiage *et al.,* 1997), cows (Ueda *et al.*, 2001), monkeys (Nakajima *et al.*, 2001) and sheep (Robertson *et al.*, 2005).

Lens culture systems are a valuable tool for assessing cataract formation and the role of calpains in a controlled *in vitro* system. Many of the lens culture systems use calcium ionophores as the initiators for cataract formation (Marcantonio and Duncan, 1991, Sanderson *et al.,* 1996, Lee *et al.,* 2008). These ionophores elevate cystolic Ca^{2+} levels by transporting Ca^{2+} across the cellular membrane. When the Ca^{2+} ionophore, ionomycin, is added to lens culture systems the lenses develop opacification, which is not observed when ionomycin was added to the lens cultures without Ca^{2+} (Sanderson *et al.,* 1996, Lee *et al.,* 2008). In these studies it was shown that in the lenses that lose transparency, the cytoskeletal proteins vimentin and spectrin were proteolysed. These cytoskeletal proteins are known calpain substrates. To further test the role of calpains in the ionomycin model of cataracts, known calpain inhibitors were added to the culture system. The addition of the calpain inhibitors attenuated the formation of the opacity associated with ionomycin (Sanderson *et al.,* 1996, Lee *et al.,* 2008).

The above experiments were carried out using rat and ovine lenses, similar studies using human lenses to assess calpain's role in the formation of human cortical cataracts have been performed (Sanderson *et al.*, 2000). Opacification was seen in the human cultured lenses after the addition of ionomycin, and the opacification was prevented when the calcium had been chelated with EGTA. Again there was proteolysis of the cytoskeletal protein vimentin and also a loss of the crystallins from the water soluble fraction of the lens (Sanderson *et al.*, 2000). This evidence shows a role for calpain 2 in both human and other mammalian lens cataract models.

In the sheep model of cataracts Robertson *et al.,* (2005) showed that the levels of calpain 2 decreased as the cataract development progressed. This indicated that calpain 2 had been autolysed which is consistent with the activation of calpain 2 having an involvement in cataractogenesis.

1.6.2 Lp82

Since the lens specific calpain Lp82 was discovered it has been suggested to play a role in the proteolysis observed during cataractogenesis (Ma *et al.,* 1999). Lp82 activity has been reported in rats and mice (Nakamura *et al.*, 2000), cows (Ueda *et al.*, 2001) and sheep (Robertson *et al.*, 2005). So far only rodent Lp82 has been shown to be active during cataract formation (Nakamura *et al.*, 2000). In the young rat lens there are equal quantities of both Lp82 and calpain 2 and, consequently, both are suggested to play a role in lens maturation and cataract formation (Shearer *et al.*, 1998). However in the young mouse it was discovered that Lp82 is the dominant form of calpain (Ma *et al.*, 1999). The authors suggested that the majority of the proteolysis seen during cataract formation in young mouse lenses could be attributed to Lp82 rather than calpain 2.

1.6.3 Calpains and α-crystallin

Both calpain 2 and Lp82 from bovine (Yoshida *et al.,* 1986, Ueda *et al.,* 2001), rat (Ueda *et al.,* 2002b) and mice (Nakamura *et al.,* 2000, Fukiage *et al.,* 1997) can truncate αA- and αB-crystallin. What however is interesting is that they have two different specific places where they cleave αA crystallin, calpain 2: $\alpha A_{1\text{-}163}$ and Lp82: $\alpha A_{1\text{-}168}$ (Yoshida *et al.,* 1986, Ueda *et al.,* 2001). Bovine (Yoshida *et al.,* 1986) and rat (Ueda *et al.,* 2002b) calpain 2 truncate αA-crystallin in a number of places but there are two main truncated forms of αA-crystallin found after the exogenous incubation of αA with calpain 2 in the presence of Ca^{2+}. The first was $\alpha A_{1\text{-}162}$ which is missing 11 amino acids from its C-terminus and the second was $\alpha A_{1\text{-}163}$ missing 10 amino acids from its C-terminus (Figure 1-7) (Yoshida *et al.,* 1986, Ueda *et al.,* 2001). But crucially calpain 2 did not truncate αA crystallin at $\alpha A_{1\text{-}168}$ (Yoshida *et al.,* 1986, Nakamura *et al.,* 2000, Ueda *et al.,* 2001, Inomata *et al.,* 2002).

Lp82 from bovine and rat lenses has only one main truncation product when incubated with exogenous αA-crystallin, that is $\alpha A_{1\text{-}168}$ (Ueda *et al.,* 2001, Ueda *et al.,* 2002b) where five amino acids are missing from the C-terminus of αA crystallin

(Figure 1-7). It has also been found that the $\alpha A_{1\text{-}163}$ truncation was produced by Lp82 but not the $A_{1\text{-}162}$ truncation.

These two different and specific truncation sites for calpain 2 ($\alpha A_{1\text{-}162}$ missing 11 amino acids) and Lp82 ($\alpha A_{1\text{-}168}$ missing 5 amino acids) allow assessment of which calpain is more dominant in the lens by measuring the relative abundance of these two species during lens maturation and cataractogenesis. In the selenite cataract the major αA truncation product seen after 16 days was the Lp82 truncation product $\alpha A_{1\text{-}168}$, with a small amount of $\alpha A_{1\text{-}163}$ being found as well (Ueda *et al.*, 2002b). However $\alpha A_{1\text{-}162}$, the calpain 2 truncation product, was not found in the cataract. This showed that the proteolysis seen during selenite cataract formation was due to Lp82 and not calpain 2. It is important to note that the selenite cataract is a nuclear cataract.

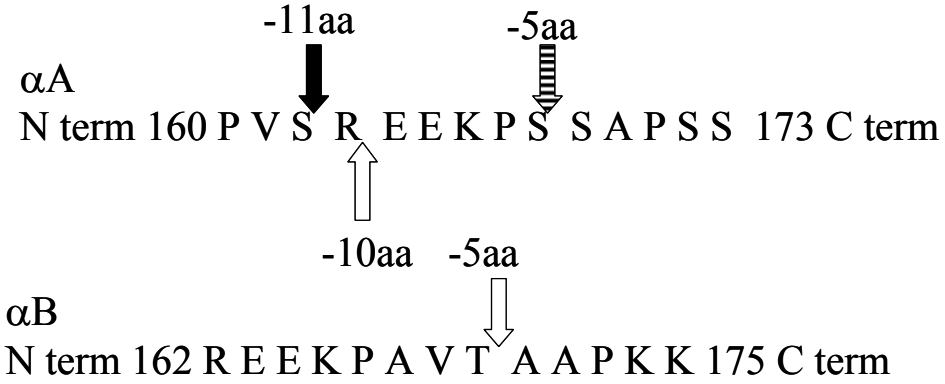

Figure 1-7 αA and αB crystallin C-terminal amino acid sequence. Arrows represent cleavage points for calpain 2 (Block arrow), Lp82 (striped arrow) and both calpain 2 and Lp82 (white arrow).

Both calpain 2 and Lp82 from bovine (Yoshida *et al.*, 1986, Ueda *et al.*, 2001) and rat (Ueda *et al.*, 2002b) truncate αB crystallin when incubated *in vitro*. Unlike αA-crystallin both calpain 2 and Lp82 truncate αB-crystallin at the same place, $\alpha B_{1\text{-}170}$, cleaving 5 amino acids from the C-terminus (Figure 1-7) (Ueda *et al.*, 2001, Ueda *et al.*, 2002b). This αB-crystallin species missing 5 amino acids from its C-terminus is found in cataractous lenses from the selenite rat (Ueda *et al.*, 2002b), Shimuya rat (Inomata et al., 1997a) and ICR/f rat (Takeuchi *et al.*, 2004). As this truncation is produced by both calpain 2 and Lp82 it is not possible to determine which calpain is responsible for the modification seen during cataract formation.

The lens specific Lp82 is not found in human lenses due to there being a stop codon in the sequence (Fougerousse *et al.,* 2000). However C-terminally truncated α-crystallin with cleavage sites identical to those associated with bovine, rat and mouse Lp82 cleavage of α-crystallins ($\alpha A_{1\text{-}168}$) have been found in human lenses (Takemoto, 1995b, Lund *et al.,* 1996) and cataracts (Baruch *et al.*, 2001).

The α-crystallins are related to the small heat shock proteins and can act as molecular chaperones within the lens environment (Horwitz, 1992). The C-terminal extension of α-crystallin is essential for chaperone activity. It is involved in subunit to subunit interaction as well as subunit-substrate interaction. In 1994 Takemoto showed that loss of amino acids from 158-173 of αA-crystallin resulted in loss of chaperone activity. Therefore calpain induced truncation of α-crystallin would reduce the chaperone function of α-crystallin.

1.6.4 Calpains and β-crystallins

Although β-crystallins are the second most abundant protein in the lens, little research has focussed on the effect of calpain 2 on the β-crystallins. In 1992 David *et al.,* showed in rats that calpain 2 can proteolyse β-crystallin and produce similar fragments, when analysed by electrophoresis, as those found in the selenite cataract. The study also showed that the β-crystallins that were truncated became insoluble in solution. Insolubilisation of β-crystallins is one of the hallmarks of cataract. Insoluble β-crystallins tend to aggregate in the lens which leads to light scattering. Further to this research it was found that the β-crystallins were truncated at their N-terminus, by calpain 2 (David and Shearer, 1993) and that β-crystallin insolublised by calpain 2 *in vitro* was truncated at the same sites as that found on β-crystallin in cataractous lenses. Sequence analysis showed that calpain 2 cleaved between 5 and 49 amino acids from the N-termini of βB3, βB1, βA4, and βA1/A3, and these identical truncated species were found in the cataractous lenses (David *et al.,* 1993). Only a small amount of work has examined the role of Lp82 (the lens specific calpain) in the truncation of the β-crystallins. When Lp82 is incubated with βA3 crystallin, it cleaves βA3 at the same position as calpain 2 (Ueda *et al.,* 2001), suggesting that Lp82 may also have a role in β-crystallin truncation. This evidence suggests that calpains cleaves

β-crystallins *in vivo* and that these truncated products become insolublised in the lens and contribute to the formation of cataracts.

1.7 Heritable Ovine Cataract

There are rodent models of cataract using mice (Kuck, 1990, Ai *et al.,* 2000) and rats (David and Shearer, 1984, Shumiya, 1995). These models of cataract are from small rodents which, although they have functional eyes with a similar structure to human eyes, have a lens that is spherical in shape compared with the elliptical shape of the human lens. The rodents also focus light by a different mechanism to humans. To focus light humans change the shape of the lens (for more see 1.2 The Lens), whereas rodents don't change the shape of the lens but shift the entire lens backwards and forwards, to focus light on the retina.

So although the rodents are a model for human lenses they have their limitations as far as replicating the human lens. A larger mammal model of cataract would provide more valuable information for treatment of human cataracts.

In 1982 Brooks *et al*., described a Romney sheep with inherited cortical cataracts. The cataract was inherited in an autosomal dominant fashion. The opacity starts to develop by 1-2 months of age and reaches maturity by 10-12 months of age (Brooks *et al.,* 1982/1983, Robertson *et al.,* 2005). An independent scoring system was developed in which lenses were graded from 0 (no cataract) to 6 (mature cataract) (Figure 1-8). The initial description of the ovine cataract reported an increase in calcium levels in the cataractous lens. It has been shown that as the cataract progresses intracellular lens calcium levels increase (Brooks *et al.,* 1982/1983, Robertson *et al.,* 2005). This led to the suggestion that calpain may be activated in the ovine cataract lens by increased calcium levels.

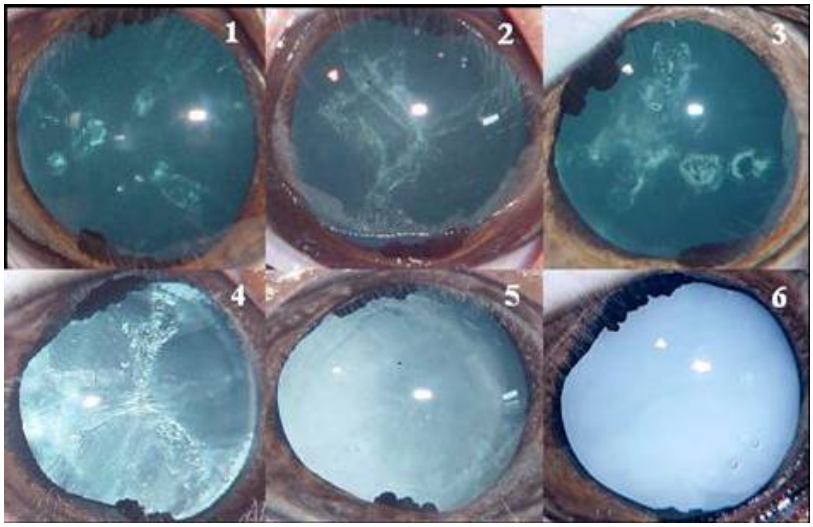

Figure 1-8 Progression of the ovine cataract and scoring system. 1) Small opacities at either posterior or anterior suture lines, 2) Small opacities at both suture lines, 3) Opacities at sutures lines and mild cortical involvement, 4) Moderate to severe cortical involvement, 5) Immature cataract involving the whole lens, 6) mature cataract. Adapted from Robertson *et al.,* (2005).

Calpain 2, calpain 1 and Lp82 have all been found in the ovine lens, and interestingly the level of active calpain 2 decreases as the cataract progresses, which is in indication that the calpain 2 has been activated and subsequently autolysed (Robertson *et al.,* 2005). In the same study a calpain inhibitor SJA6017 was applied to the eye of sheep with inherited cataracts and the inhibitor was shown to slow the progression of the cataract over the first month of treatment.

Study of the crystallins in the ovine lens reveals the usual complement found in most other mammalian species, αA, αB, βA1, βA1/βA3, βA2, βB1, βB2, βB3, γS and γA-F (Robertson *et al.,* 2008). In the ovine cataract some of these crystallin are truncated, in particular, αA, αB, βB1, βB2 and βB3. The modifications to α-crystallins are the same as those found in other cataract models and are known calpain 2 and Lp82 truncation sites (Robertson *et al.,* 2008). The cytoskeletal proteins spectrin and vimentin were both truncated during ovine cataract formation (Robertson *et al.,* 2005).

All this evidence indicates a role for the calpain family of proteases in the heritable ovine cataract.

Chapter 2
Experimental Rationale

A variety of biochemical changes occur in the lens during cataractogenesis. Specifically the lens crystallins are modified by deamidation, phosphorylation and truncation. The aim of this research is to document the changes that occur within the ovine lens during maturation and cataractogenesis. The research will focus primarily on the crystallins and their truncation. It will also aim to establish the role that the calpain family of proteases play in truncation of the crystallins. The three calpains that have been found in the ovine lens, calpain 1, 2 and Lp82, will be studied.

Hypothesis: (i) the crystallin truncations seen in cataractogenesis are unique to this disease and different to the truncation seen with normal maturation of the lens.

(ii) That incubation of calpains 1, 2 and Lp82 with the crystallins will result in truncation of the crystallins in a similar manner to that seen in cataractogenesis.

Chapter 3 Calpain Purification

The aim of this chapter was to purify the calpains that are found in the lens. This was achieved using chromatography techniques. Combinations of hydrophobic, ion exchange and gel filtration chromatography were used. The ubiquitous calpains 1 and 2 were purified from ovine lung and the lens specific Lp82 was purified from lamb lenses. These purified calpains were used in further experiments (Chapter 5)

Chapter 4 Ovine Crystallins

The changes to the lens crystallins during maturation of the lens need to be determined before the changes that occur during cataractogenesis could be determined. Therefore lens proteins from fetal, 6 month old and 8 year old sheep were visualised by 2DE and the changes determined using mass spectrometry. Lenses from cataract stages 0, 1, 3 and 6 were obtained to determine the crystallin changes that

occur during cataractogenesis. The changes in the crystallins were visualised by 2DE and the modifications of the crystallins determined by mass spectrometry.

Chapter 5 Calpains and Crystallins

To study whether the calpains could truncate lens proteins, the purified calpains from Chapter 3 were incubated with lens proteins. Calpains 1, 2 and Lp82 were all incubated individually with α- and β-crystallins. The truncations produced by these incubations were visualised by 2DE and the exact truncation sites were determined using mass spectrometry techniques. Calpains 2 and Lp82 were also incubated with the lens cytoskeletal proteins. The truncated proteins from incubation with the calpains were compared to the truncated proteins seen during cataractogenesis

Chapter 6 General Conclusion and Future Directions

In this chapter the results from each chapter are highlighted and related to the hypotheses generated at the start of this thesis. Future directions are also discussed to carry on this research.

Chapter 3
Calpain Purification

3.1 Introduction

The calpains are a family of calcium-dependent cysteine proteases. They make up a vital part of the cellular proteolytic machinery. Currently there are 14 different isoforms of calpain, 7 of these are ubiquitously expressed and include the two major calpains, calpain 1 (μ-calpain) and 2 (m-calpain). The remaining 7 are tissue specific calpains and are found in muscle, lens, stomach, digestive tract, testis and hair follicles (Goll *et al.,* 2003).

All the calpains have an absolute requirement for Ca^{2+} to become active but have different concentration requirements for Ca^{2+}. For example, calpain 1 requires 3-50μm and calpain 2 400-800μM Ca^{2+} for half maximal activation *in vitro* (Goll *et al.,* 2003). These values are substantially higher than the Ca^{2+} concentration found *in vivo* which is <1μM (Goll *et al.,* 1992), therefore other events must occur *in vivo* that reduce the amount of Ca^{2+} required for activation to the physiological levels of Ca^{2+} found in cells. Two events that may accomplish this are autolysis and phospholipid binding. Autolysis is a common feature of calpain activation and involves the autolytic removal of N-terminal amino acids from both the large and small subunits (Elce *et al.,* 1997). Autolysis reduces the calcium requirement of calpain 1 to 0.5-2 μM and that of calpain 2 to 50-150μM (Goll *et al.,* 2003). Binding of calpains to phospholipids in the cellular membrane has also been shown to reduce their calcium activation requirement *in vitro* (Pontremoli *et al.,* 1985, Arthur and Crawford, 1996).

Within most mammalian lenses there are 4 isoforms of calpain, the 2 major ubiquitous calpains 1 and 2, the lens specific calpain Lp82 and calpain 10. At present calpain activity in the lens has been found in a variety of animal species, including humans (David *et al.,* 1989), sheep (Robertson *et al.,* 2005), cow (Yoshida *et al.,* 1985), mouse (Fukiage *et al.*, 1997), rat (David and Shearer, 1986), rabbit (Fukiage *et al.*,

1997) and guinea pig (Fukiage *et al.*, 1998). Calpain 2 is the dominant isoform in the sheep lens (Robertson *et al.,* 2005), and has been shown to have roles in lens maturation and cataractogenesis in rodent and bovine lenses (David and Shearer, 1984, Ueda, 2001, Ueda *et al.,* 2002b).

There is a lens specific calpain, Lp82. This was discovered in 1998 by Ma *et al.,* and has been described in rats (Ma *et al.,* 1998a), mice (Ma *et al.,* 1999) cattle (Ueda *et al.,* 2001) and sheep (Robertson *et al.,* 2005). Importantly, however the human Lp82 gene contains a stop codon in the middle of the sequence and therefore is not translated in human lenses (Fougerousse *et al.,* 2000). Recently other calpain 3 splice variants have been found in human and monkey lenses (Nakajima *et al.,* 2001, Kawabata *et al.,* 2003).

This chapter deals with the three main calpains found in the sheep lens, calpain 1 and 2 and Lp82. The calpains were purified from lung tissue (calpains 1 and 2) or the lens (Lp82), and their biochemical properties were determined. This experiment is important for determining the role that the calpains play in lens maturation and cataractogenesis in the ovine cataract model.

3.2 Methods

The different calpains were purified using protein liquid chromatography. Different combinations of ion exchange, hydrophobic and size exclusion chromatography media were used in the purifications. All columns were run using a BioLogic DuoFlow Chromatography System (BioRad, Ca., USA).

3.2.1 Calpain 1 and 2 purification

Three hundred grams of fresh ovine lung was homogenised in 3ml/g of ice cold Buffer A (20mM MOPS pH 7.5, 5mM EDTA, 5mM EGTA, 5mM βME) containing protease inhibitors (2.5μM E64, 0.2mM phenylmethylsulponylflouride and 0.1mM trypsin inhibitor) using 3 bursts of 30 seconds in a Waring blender (Waring, Ct.,

USA). The resultant homogenate was centrifuged at 13 800 x *g* for 1 hr at 4ºC in a Beckman J2-MI Centrifuge using a JA14 rotor (Beckman, Ca., USA). The supernatant was filtered through damp cheese cloth to remove any solid particles and the pellet was discarded. The volume of the supernatant was measured and $(NH_4)_2SO_4$ was added slowly over 2 hr to 50% saturation (29.54g/100mls of supernatant), at 4°C. This suspension was then centrifuged at 13 800 x *g* for 1 hr at 4ºC in a Beckman J2-MI Centrifuge using a JA14 rotor (Beckman, Ca., USA). The supernatant was discarded and the pellet containing the calpains was resuspended in Buffer A. The suspension was centrifuged at 13 800 x *g* for 1 hr at 4ºC in a Beckman J2-MI Centrifuge using a JA14 rotor (Beckman, Ca., USA) to remove any particles of undissolved $(NH_4)_2SO_4$. The supernatant was filtered, collected and loaded onto a Phenyl Sepharose (Pharmacia, Uppsala, Sweden) XK26 column (30cm x 2.6cm), equilibrated in Buffer A + 0.5M NaCl at a flow rate of 2ml/min. Five hundred millilitres of Buffer A +0.5M NaCl was pumped through the column at a flow rate of 4ml/min to remove any unbound proteins. The calpains were eluted with 100% Buffer A at a flow rate of 4ml/min and 140 x 8ml fractions were collected. BODIPY-FL casein assay (see 3.2.3.1) was used to determine which fractions contained the calpains and the relevant fractions containing activity were then pooled.

The pooled calpain was loaded onto a diethylaminoethyl (DEAE) ion-exchange column (Pharmacia, Uppsala, Sweden) XK26 (35cm x 2.6cm) equilibrated in Buffer A. Two hundred millilitres of Buffer A then 200mls of 10% Buffer A +1M NaCl, was washed through to remove any unbound or weakly bound proteins. The calpains were eluted and separated with a 0.1-0.4M NaCl linear gradient of Buffer A +1M NaCl. 150 x 8mL fractions were collected. Calpain activity was assayed using the BODIPY-FL casein assay and the fractions containing calpain 1 and calpain 2 were pooled separately.

3.2.1.1 Calpain 2 purification continued

Calpain 2 was further purified on Reactive Red Agarose (Pharmacia, Uppsala, Sweden) XK1.6 (13cm x 1.6cm) column. Sodium Chloride was added to 50mL of calpain 2 from the DEAE column to increase the concentration to 0.5M NaCl. The

calpain 2 was loaded on to the Reactive Red column at a flow rate of 1-2mL/min. Thirty millilitres of Buffer A + 0.5M NaCl was washed through to remove any unbound proteins and calpain 2 was eluted with 100% H_2O. Again calpain activity was determined by BODIPY-FL casein assay and the relevant active fractions were pooled.

For the final purification step the pooled calpain 2 was loaded onto a 1ml Mono Q® column (Pharmacia, Uppsala, Sweden) equilibrated in Buffer A. The calpain was eluted with a linear gradient of 0-1M NaCl in Buffer A. Calpain activity was determined using BODIY-FL casein assay. For Ca^{2+} dependence studies the final step was performed using Buffer A without EDTA or EGTA.

3.2.1.2 Calpain 1 purification continued

Calpain 1 was further purified on Reactive Red Agarose, 50mL of calpain 1 from DEAE ion-exchange was added to a Reactive Red column equilibrated in Buffer A +0.5M NaCl. Buffer A + 0.5M NaCl was washed through at 0.5mL/min until the absorbance (280nm) was level and then the calpain 1 was eluted with Buffer A + 1M NaCl. Calpain activity was determined by BODIPY-FL casein assay and relevant calpain 1 containing fractions were pooled.

To further purify and concentrate the calpain 1, 30mL of calpain 1 from the Reactive Red was added to a 5ml HiTrap® Phenyl Sepharose Column (Pharmacia, Uppsala, Sweden) equilibrated in Buffer A + 0.5M NaCl. Unbound proteins were removed with Buffer A +0.5M NaCl at 0.5ml/min. Proteins were eluted with a linear gradient of 0-100% H_2O, over 20mL and 100% H_2O for 5mL.

3.2.2 Lp82 purification

Young lamb eyes were obtained post-mortem from Ashley Dene farm (Lincoln University, New Zealand). The lenses was removed, weighed and each whole lens was homogenised in 3µL/mg of lens weight Buffer A containing Mini Complete

Inhibitor cocktail (Roche, Mannheim, Germany), one tablet per 10mL. The homogenate was centrifuged at 20 400 x *g* for 45min at 4ºC to remove the insoluble lens protein (Beckman J2-MI Centrifuge, Beckman, USA). The supernatant (total soluble protein) was applied to a DEAE Ion Exchange column equilibrated in Buffer A, and the calpains were eluted using a 500mL linear gradient of 0-500mM NaCl. All fractions were assayed for protease activity using the BODIPY-Fl localisation assay (3.2.3.1) and the different calpain isoforms were determined using a combination of western blot and casein zymography. Fractions containing Lp82 and Calpain 2 were pooled separately. The Lp82 fraction was then concentrated using ultrafiltration (Amicon Ultracel-10k, Millipore, Ma., USA) at 2050 x g for 45min at 4ºC. The concentrated Lp82 containing fraction was then run through a Superose 6 Gel Filtration (Pharmacia, Uppsala, Sweeden) (30cm x 1cm) column and Lp82 containing fractions were determined, pooled and concentrated by ultrafiltration as above. For the final step Lp82 was diluted 1:3 with Buffer A + 2M NaCl and applied to a Reactive Red Agarose Column equilibrated in Buffer A + 0.5M NaCl. Lp82 was eluted using dH_2O.

3.2.3 BODIPY-FL casein assay

To determine calpain activity the BODIPY-FL casein assay was used. BODIPY-FL was purchased from Molecular Probes, Invitrogen, USA and the BODIPY-FL casein stock reagent was prepared as described by Thompson *et al.,* (2000).

3.2.3.1 Calpain localisation

The calpain localisation assay was used to determine which fractions from the FPLC contained calpain activity. Fifty microlitres of each fraction was combined with 100µL of BODIPY-FL casein substrate (2mM NaN_3, 50µl BODIPY-FL casein stock/10ml final volume, 10mM $CaCl_2$, 0.1% β-ME in 20mM Tris-HCl, pH 7.8). The assay was performed in black microtiter plates and the fluorescence immediately read every minute for 10 minutes in a BMG Fluorescence plate reader (Fluostar, BMG Labtechnologies GMbH. Offenburg, Germany) with an excitation wavelength of

485nm and emission wavelength of 538nm. Calpain activity was determined as an increase of fluorescence over 10 minutes.

3.2.3.2 Calpain specific activity

To determine specific activity during the purification procedure the above method was adapted. In the calpain specific activity assay 50µl of pooled sample was combined with 50µL of Buffer A (without EDTA or EGTA) and 100µl of BODIPY-FL casein substrate. The calcium blanks contained 100µl of Buffer A (without EDTA or EGTA) and 100µl of BOPIDY-FL casein substrate. EDTA blanks contained 50µl of 100mM EDTA (pH 7.5), 50µl of pooled sample and 100µL of BOPIDY-FL casein substrate.

The following calculations were used to determine the specific activity of the calpain after each purification step. Specific activity was measured in units of fluorescence released per 10 minutes per mg of protein.

1. Fluorescence change per 10 minutes: [(fluorescence at 10 minutes)-(fluorescence at zero minutes)] – [average (calcium+ EDTA blanks)]

2. Total calpain activity: Fluorescence (1) x 20 (correction for 50µl sample used to take it up to 1ml) x volume of pooled sample volume.

3. Specific activity U/mg: Total calpain activity (2) ÷ total protein in sample (mg) as determined by BCA assay.

3.2.3.3 Calpastatin assay

To assess whether the endogenous calpain inhibitor calpastatin was present in any of the samples the calpastatin assay was utilised. Ten microlitres of purified calpain 2 was incubated with 90µL of sample and 100µL of BODIPY-FL casein and the change in fluorescence measured. Calpastatin presence was shown as a decrease in the fluorescence recorded compared with the control which contained 90µL of Buffer A.

3.2.3.4 BCA protein assay

Protein concentration in each sample was determined using Bicinchoninic Acid reagent (Pierce, Rockford, Il., USA) according to manufactures instructions. 10µl samples were assayed in triplicate and compared to a standard curve generated using dilutions of 2mg/ml Albumin Standard (Pierce, Rockford, Illinois, USA), to provide concentrations ranging from 0.0625mg/ml to 1mg/ml.

3.2.4 SDS-PAGE, Western blot and casein zymography

To determine the relative purity and confirm the presence of each calpain a combination of Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), western blotting and casein zymography was used.

3.2.4.1 SDS-PAGE

SDS-PAGE was performed using NuPAGE® 4-12% Bis-Tris precast gels (Invitrogen, California, USA) using the NuPAGE® Surelock system. Twenty micrograms of protein from each purification step was combined with six times SDS-PAGE loading buffer (350mM Tris-HCL, pH 6.8; 10%SDS, 30% glycerol, 5% βME, 0.012% Bromophenol Blue). The samples were denatured by heating at 90ºC for 5 min. Samples were loaded into individual wells and the gel run at 200V for 50 min with NuPAGE® MOPS running buffer.

After electrophoresis the gel was removed and washed 3 times for 5 min with 5mM HCl. After the washes the gels were stained with GelCode® Blue Stain Reagent (Pierce, USA) overnight. The gels were destained with 5mM HCl. All gels were scanned using a flat bed scanner (CanoScan D2400 U, Canon) at 600dpi and saved as JPEG files.

3.2.4.2 Western blot

Gels were run as for SDS-PAGE (see 3.2.4.1). Following SDS-PAGE, the protein was electroblotted onto PVDF (polyvinylidene fluoride) transfer membrane (BioRad, Ca., USA), over 1 hr at 100V in cold transfer buffer (25mM Tris, pH 8.3; 192mM Glycine). After transfer the membrane was blocked with 5% non-fat dry milk powder in TTBS (20mM Tris-HCl, pH 7.5; 500mM NaCl, 0.05% Tween 20) for 1 hr at room temperature. The PVDF membrane was then washed 3 times 5 min with TTBS. Incubation with primary antibodies was performed in TTBS with 1% non-fat dry milk powder, with primary antibodies prepared according to Table 3-1, for 1 hr at room temperature. The membrane was washed 3 times 5 min in TTBS to remove any unbound primary antibody. Suitable secondary antibodies were incubated for 1 hr at room temperature according to Table 3-1 in TTBS with 1% non-fat dry milk powder. The membrane was washed 2 times 5 min in TTBS and once in TBS (TTBS without 0.05% Tween 20). The blots were developed with an alkaline phosphatase Conjugate Substrate Kit (BioRad, Ca., USA), according to the manufacturers instructions.

Calpain	Concentration	Host Species	Source
Bovine Calpain 1 80kDa subunit	1:2000	Mouse	Affinity BioReagents Inc. Catalogue MA3-942
Bovine Calpain 2 80kDa subunit	1:2000	Mouse	Affinity BioReagents Inc. Catalogue MA3-940
Rat Lp82	1:1000	Rabbit	Supplied by H.Ma, OHSU, USA.

Table 3-1 Primary antibodies used for western blot analysis of the calpains.

3.2.4.3 Casein zymography

Casein zymography was used to visualise the activity of the individual calpains. A 10% non-denaturing gel was prepared (0.225M Tris-HCL, pH 7.5, 10% acrylamide (37.5:1), 0.05% casein, and set with 0.06% ammonium persulphate and 0.06% TEMED). A 4% non-denaturing stacking gel (0.125M Tris-HCL, pH 6.8, 4% acrylamide, 0.1% ammonium persulphate, 0.1% TEMED) was prepared on top of the resolving gel. The gel was pre-run for 15 min at 125V at 4ºC in zymography running

buffer (25mM Tris-HCL, pH 8.3, 192mM Glycine, 1mM EGTA, 1mM DTT). Samples were mixed with zymogram loading buffer (0.04M Tris-HCL, pH 6.8, 6.5% glycerol, 0.005% Bromophenol Blue) and loaded onto the pre-run gel. The gels were run at 125 V for 150 min in 4ºC zymogram running buffer. After electrophoresis the gels were placed in calcium incubation buffer (20mM Tris-HCl, pH 7.4, 20mM $CaCl_2$, 10mM DTT) overnight at room temperature to activate the calpains. After incubation the gels were rinsed in dH_2O for 15 minutes and then stained with Simply Blue™ Safe Stain (Invitrogen, CA., USA) and destained with dH_2O. Calpain activity appeared as transparent bands on a blue stained background.

3.3 Results

FPLC was used to purify calpain 1, calpain 2 (Figure 3-1) and the lens specific calpain Lp82. This resulted in the removal of unwanted proteins from the tissues and produced calpains that could be used in further experiments.

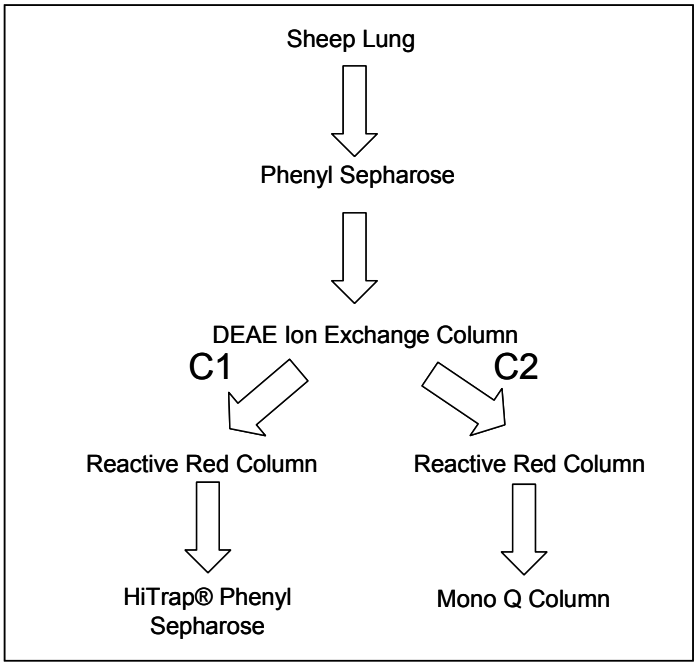

Figure 3-1 Flow Chart of Calpain 1 and 2 purification from sheep lung using FPLC. Each chromatography step is labelled. Calpain 1 (C1) and calpain 2 (C2) are separated after the DEAE Ion Exchange Column.

3.3.1 Calpain 1 and 2

After homogenisation and $(NH_4)_2SO_4$ precipitation, FPLC was used to further purify the calpains. The first step was to remove the endogenous calpain inhibitor calpastatin. This was achieved using Phenyl Sepharose hydrophobic interaction chromatography (Figure 3-2). High UV absorbance (Blue) was observed when loading the sample onto the column indicating that several proteins did not bind to the Phenyl Sepharose. Once the unbound proteins were washed through the column, the calpains were eluted with a decreasing salt concentration as shown with the sharply decreasing conductivity (Red) and increasing protease activity as indicated by the fluorescence units (Bars). The active fractions were pooled (Tags 1-2). The pooled sample contained both calpain 1 and calpain 2.

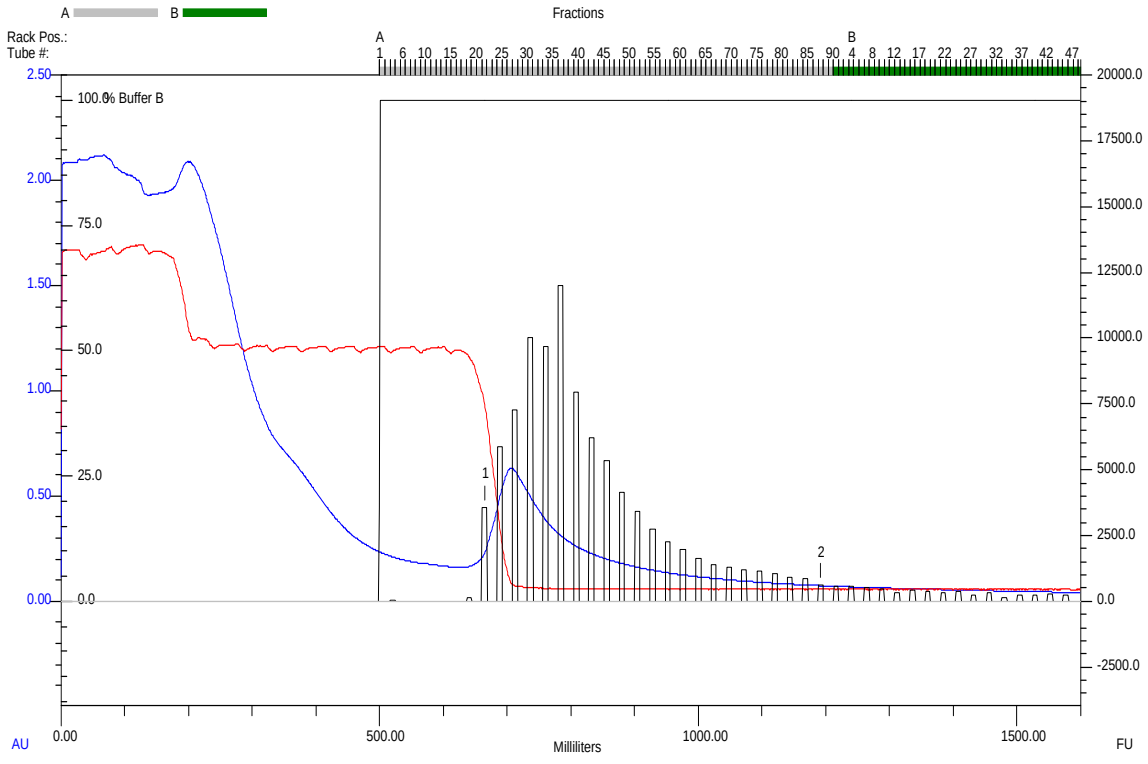

Figure 3-2 Chromatogram from Phenyl Sepharose purification of the calpains. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Bars represent Fluorescence Units (Calpain Activity). Fractions that were pooled were between Tags 1 and 2.

Calpastatin was separated from the calpains in this step and eluted with the unbound fraction. When the calpastatin assay was used the unbound fraction had no change in

fluorescence over ten minutes (0 FU) compared to the positive control which had an increase in fluorescence (290 FU).

DEAE ion exchange chromatography (Figure 3-3) was used to separate calpains 1 and 2 found in the pooled sample obtained from Phenyl Sepharose chromatography.

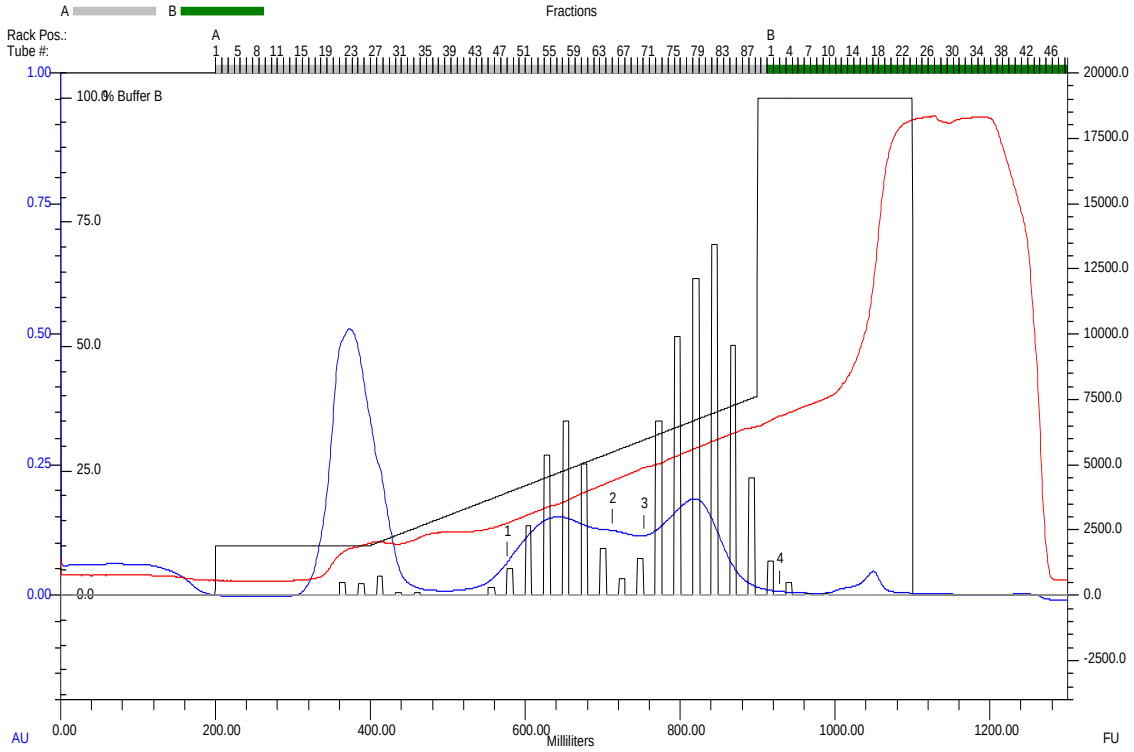

Figure 3-3 Chromatogram of DEAE Ion Exchange. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Bars represent Fluorescence Units (Calpain Activity). Tags indicate fractions that were pooled for Calpain activity, Tags 1-2 Calpain 1 and Tags 3-4 Calpain 2.

DEAE ion-exchange chromatography of the Phenyl Sepharose pooled fraction, resulted in 3 major protein peaks measured by absorbance (Blue) and BODIPY-Fl casein assay. The first peak was due to the initial conductivity increase and weakly bound proteins being eluted. The following two peaks of BODIPY-Fl casein activity contained calpains 1 and 2. Calpain 1 eluted between 120mM and 200mM NaCl, while calpain 2 eluted between 230mM and 340mM NaCl (Figure 3-3). The calpain 2 peak had higher activity than calpain 1, indicating a higher abundance of calpain 2 than calpain 1 in the lung. The two calpains were pooled separately as indicated by the tags (1-2 for calpain 1 and 3-4 for calpain 2). These samples were used for further purification.

3.3.1.1 Calpain 2 continued

To further purify the calpain 2 fraction a Reactive Red Agarose column was used. In initial experiments, a linear gradient was performed from 0.5-0M NaCl in Buffer A to elute the calpain 2. However the calpain 2 could not be eluted from the column, as it was bound tightly to the matrix. A solution with lower ionic strength was required. Therefore a gradient from Buffer A + 0.5M NaCl to dH_2O was used and it eluted calpain 2 from the Reactive Red (Figure 3-4). Elution with dH_2O resulted in a large peak of protein being eluted (Blue), which contained calpain 2. The active fractions were pooled (Tags 1-2).

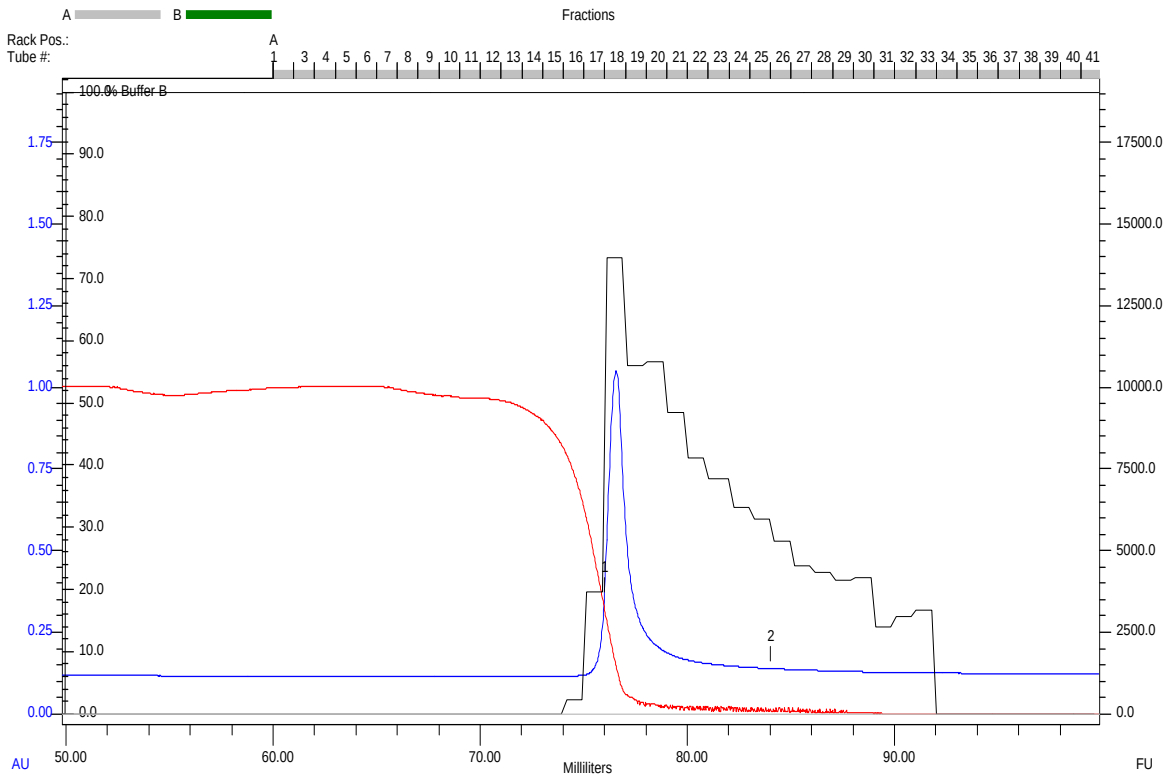

Figure 3-4 Chromatogram of Calpain 2 on Reactive Red Sepharose. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Bars represent Fluorescence Units (Calpain Activity). Tags 1-2 indicate area of calpain 2 that was pooled and used for further purification.

The pooled calpain 2 from the Reactive Red column was further purified using a Mono Q® ion exchange column (Pharmacia, Uppsala, Sweden). The calpain was eluted with an increasing salt gradient from 0-1M NaCl in Buffer A. This elution

produced two major protein peaks (Figure 3-5) that corresponded to two areas of protease activity. The second peak of protein and protease activity at 21-25 mL, contained two distinct peaks of UV absorbance. The second peak corresponded to a sharp increase in BODIPY FL -casein activity. This peak was pooled (Tags 1-2) and was subsequently used for all enzymatic proteolytic assays.

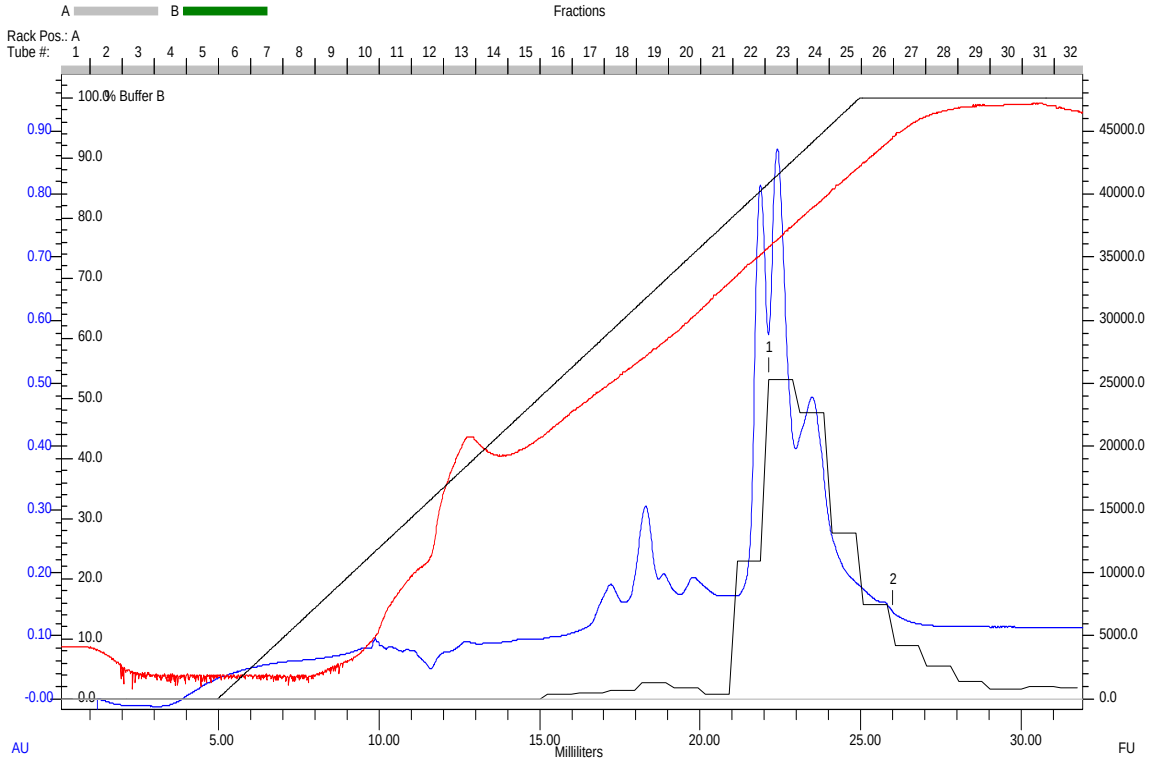

Figure 3-5 Chromatogram of final purification step from Mono Q column. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Bars represent Fluorescence Units (Calpain Activity). The final calpain 2 was pooled from between Tags 1-2.

The activity of the fractions collected after each step of the purification procedure was determined using the BODIPY-Fl Casein assay as described in 3.2.3.2.

Column	Activity/ml	Total Activity	Specific Activity	% Recovery	%Total Recovery
PS	19445	10694750	19445		
DEAE	25985	4937150	16241	46	46
RR	28752	3018925	17970	61	28
MONO Q	32812	820292	41015	27	8

Table 3-2 Specific activity from each step of the purification procedure for calpain 2. PS-Phenyl Sepharose (Figure 3-2), DEAE- DEAE Ion exchange (Figure 3-3), RR- Reactive Red agarose (Figure 3-4) and Mono Q- Mono Q (Figure 3-5). Activity/ml, Total Activity and Specific Activity are calculated as per 3.2.3.2. Percent Recovery is calculated as the percentage of total activity retained from previous step. Percent Total recovery is the percentage of total activity retained from the first Phenyl Sepharose column.

3.3.1.2 *Calpain 1 continued*

Following DEAE ion exchange separation, calpain 1 was further purified with Reactive Red Agarose. The interaction of calpain 1 with this matrix is unusual. The protein does not bind to Reactive Red but is retarded and moves through faster as the salt concentration increases. This can be seen in Figure 3-6, where the unbound fraction elutes as a large peak of absorbance at 20mL. The calpain 1 eluted when the conductivity increased (Red).

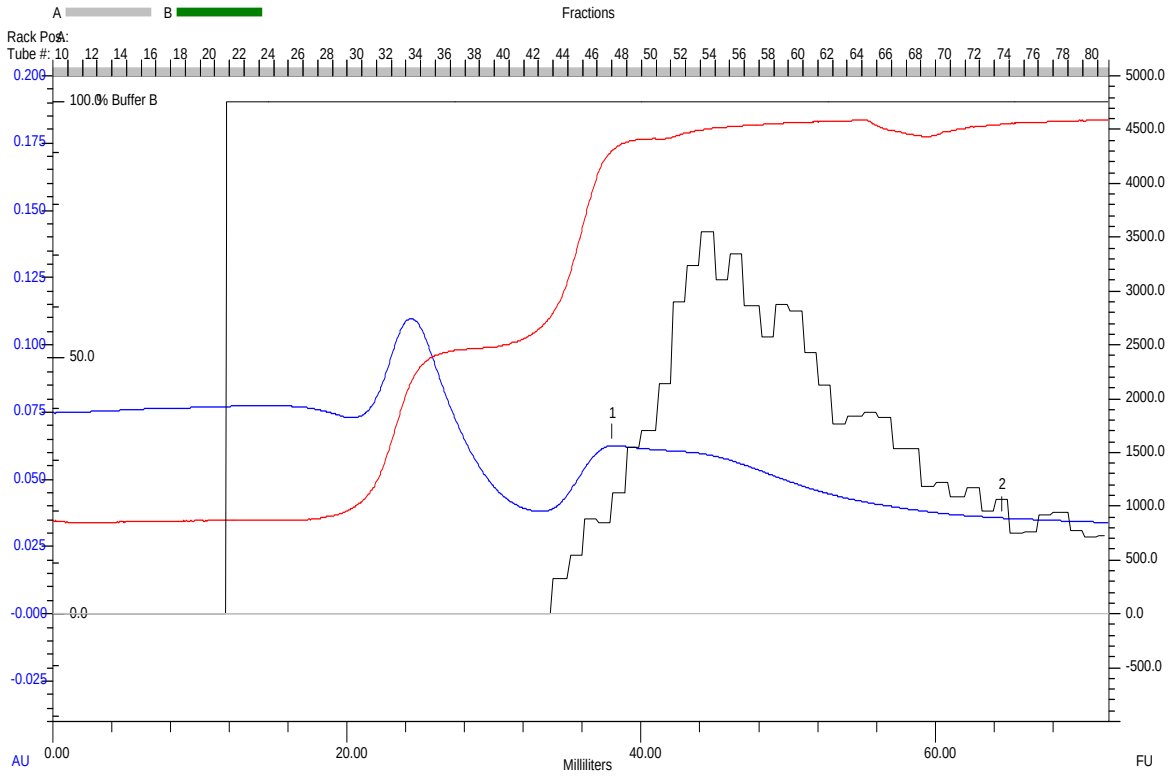

Figure 3-6 Chromatogram of Calpain 1 on Reactive Red Agarose. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Fluorescence Units, Bars represent Fluorescence Units (Calpain Activity). The final calpain 1 was pooled from between Tags 1-2.

After Reactive Red Agarose the pooled calpain 1 was further purified and concentrated on a HiTrap® Phenyl Sepharose Column (Pharmacia, USA). Calpain 1 bound to this column tightly and a decreasing salt gradient using dH_2O was used to elute the calpain (Figure 3-7).

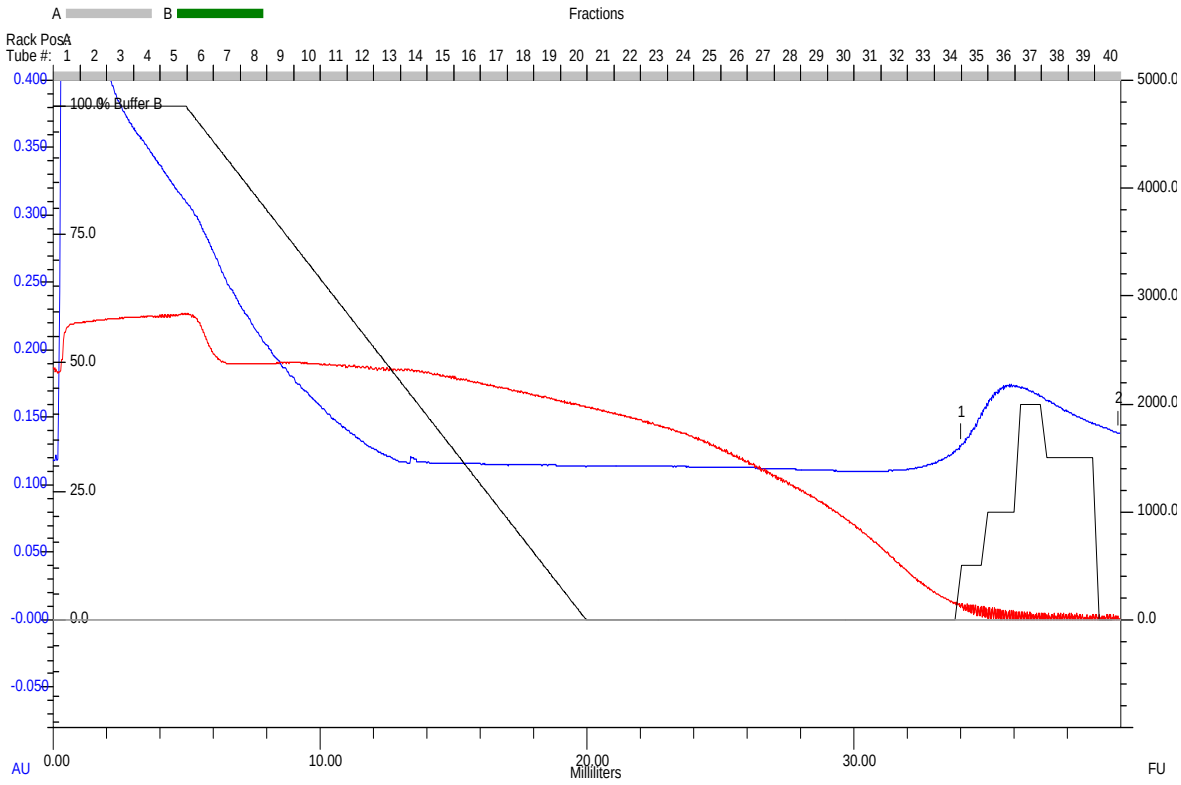

Figure 3-7 Calpain 1 on HiTrap® Phenyl Sepharose Column. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Bars represent Fluorescence Units (Calpain Activity). The final calpain 1 was pooled from between Tags 1-2.

3.3.2 Lp82 purification

Separation of total soluble lens protein during DEAE ion exchange chromatography resulted in the total lens proteins being separated into several protein peaks (Blue Figure 3-8). The BODIPY-FL casein assay showed two distinct calcium dependent proteolytic peaks. The first peak contained Lp82 and calpain 1 and the second and more active peak contained calpain 2 as determined by casein zymography (Figure 3-9) and western blot (Figure 3-10) analysis. Active fractions in each peak were combined.

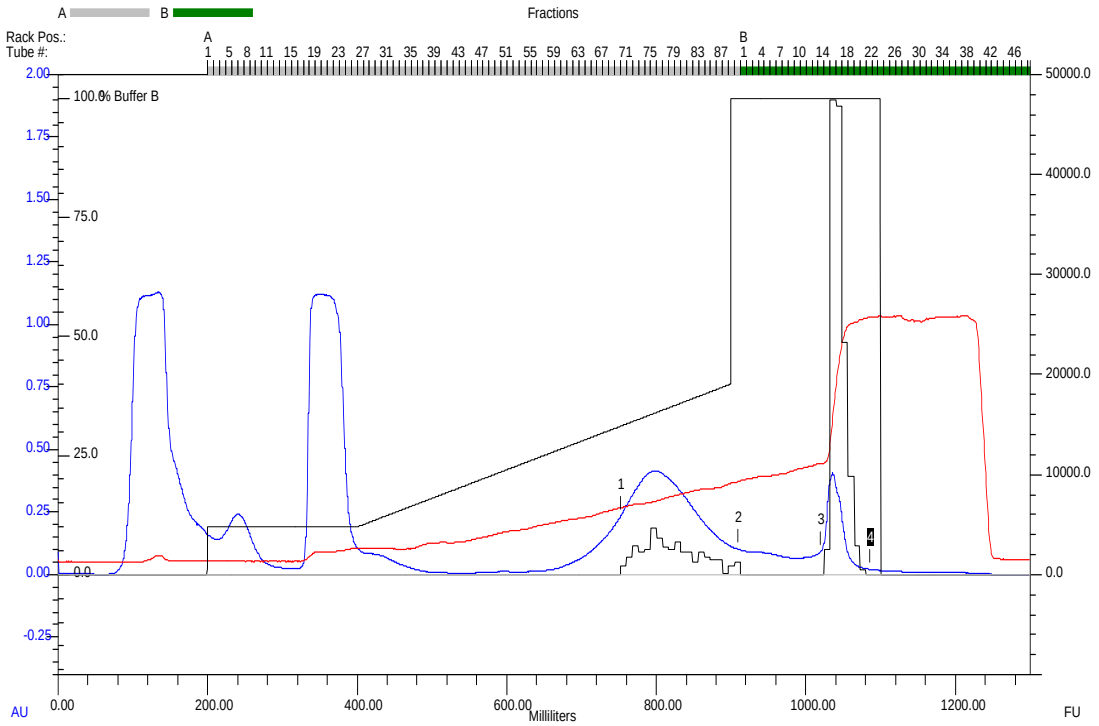

Figure 3-8 Chromatogram of DEAE Ion exchange. Total soluble lens proteins were loaded onto the column and calpains were eluted with an increasing NaCl gradient. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Bars represent Fluorescence Units (Calpain Activity). Tags 1-2 is Lp82 and calpain 1 and 3-4 is Calpain 2.

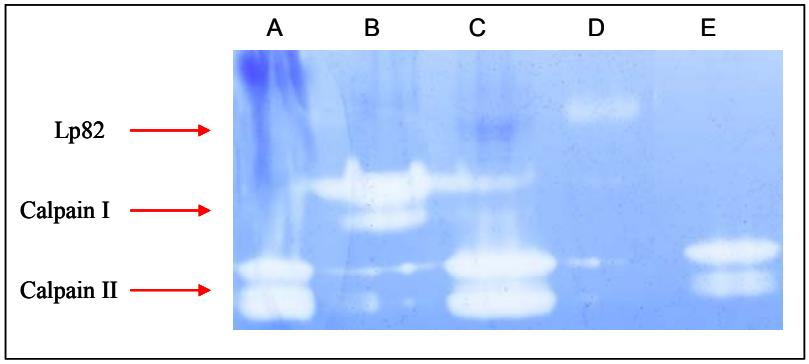

Figure 3-9 Casein zymography of Fractions from DEAE Chromatography of the Lens Homogenate. A) Total soluble lens proteins, B) Positive control of calpain 1, C) Positive control of Calpain 2, D) First peak from DEAE (Lp82 and Calpain 1), E) Second Peak from DEAE (calpain 2).

Zymography showed that the first peak of activity eluted from the DEAE column (Figure 3-8) was the lens specific Lp82, with a small amount of residual calpain 1 (Figure 3-9, Lane D). The second peak of activity was calpain 2 (Figure 3-9, Lane E). Western blot analysis confirmed this finding (Figure 3-10). A sample of the first pooled peak reacted with an Lp82 antibody and the second peak was reactive only to the calpain 2 antibody.

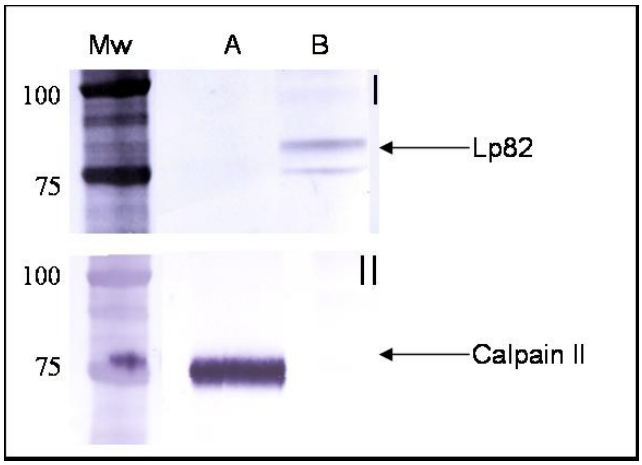

Figure 3-10 Western Blot analysis from DEAE Ion Exchange purification of Lens Homogenate (Figure 3-8) Lane A, Peak 2 of activity, Lane B Peak 1 of activity, Blot I is reactive to Lp82 antibody and Blot II reactive to Calpain 2 antibody. Mw molecular weight marker (kDa).

The Lp82 containing fractions were further purified using gel filtration chromatography. This produced two peaks of proteolytic activity (Figure 3-11). To determine which peak contained the Lp82, the active fractions were subjected to western blot analysis. The first peak of activity was not reactive to the Lp82 antibody (Figure 3-12, Lane 13-15). The second peak (Lanes 17-19) was reactive to the Lp82 antibody so these fraction were pooled for further purification. Both peaks were also assayed by western blot for calpain 1, which was detected in both peaks (Figure 3-13).

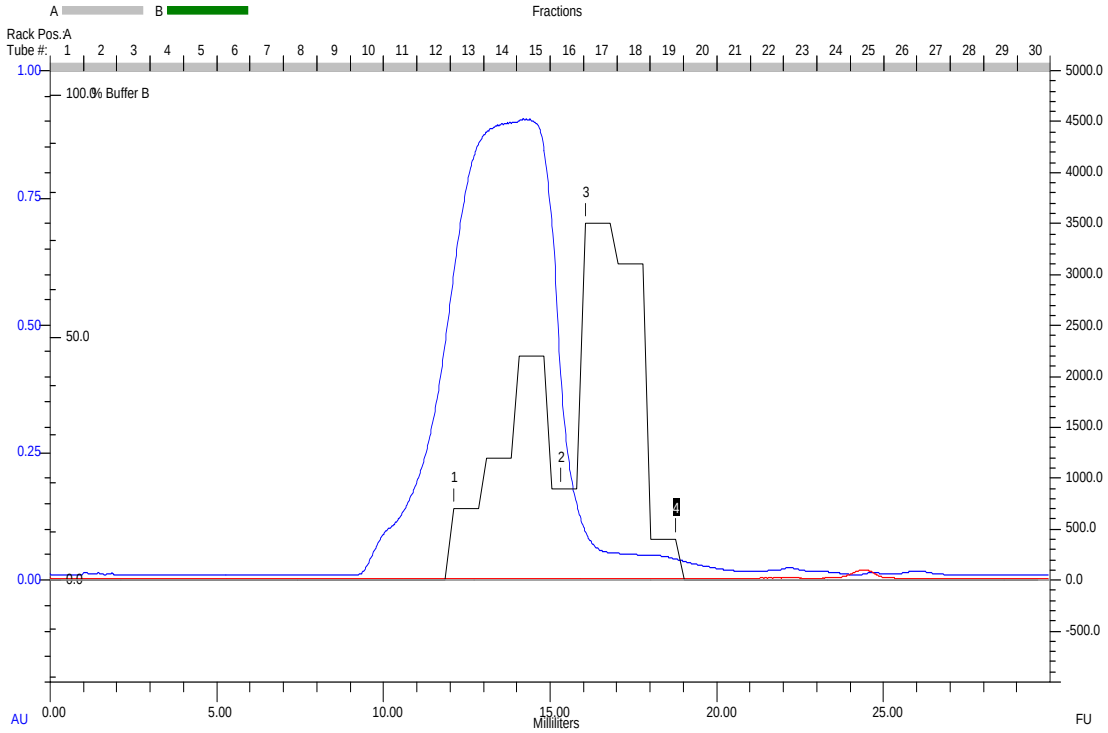

Figure 3-11 Gel filtration to further purify Lp82 from DEAE. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Bars represent Fluorescence Units (Calpain Activity). Tags 1-2 Peak I, Tags 3-4 Peak II .

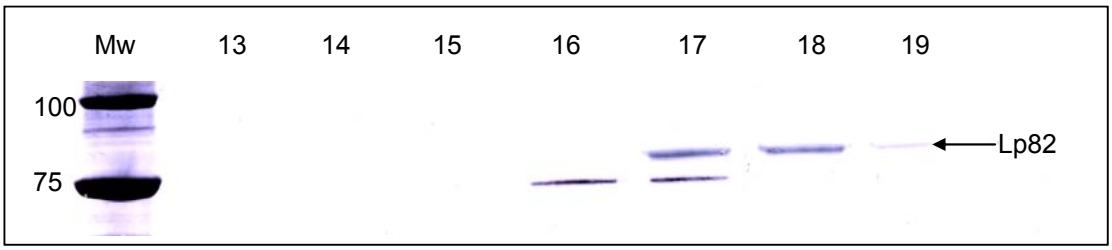

Figure 3-12 Western Blot analysis of the active fractions from the Gel Filtration (Figure 3-11). Lane numbers correspond to the fractions from the gel filtration chromatogram. Lp82 is as indicated. Mw Molecular weight marker (kDa).

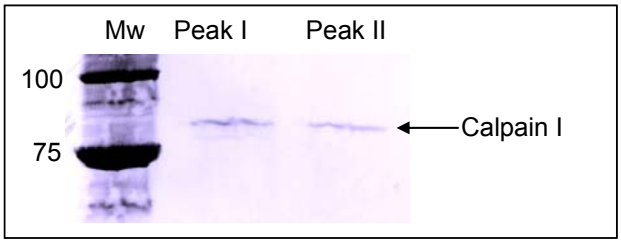

Figure 3-13 Western Blot analysis of the two peaks of proteolytic activity from the Gel Filtration (Figure 3-11). Peak I, First peak of activity Tubes 12-15 and Peak II tubes 17-20 (Figure 3-11). Calpain 1 is as labelled. Mw) Molecular weight marker (kDa).

The final pooled Lp82 fraction was purified using a Reactive Red Agarose column. Initially Buffer A was used to elute the Lp82 from the Reactive Red, but Lp82 failed to elute from the column. H_20 was used as the elution buffer to remove the Lp82. This produced 2 peaks of proteolytic activity as determined by the BODIPY-FL Casein system. The first peak did not bind to the Reactive Red matrix and was removed with the unbound fraction. None of the fractions in this peak were reactive to the Lp82 antibody. However, this peak was reactive to the calpain 1 antibody (Figure 3-15). The second peak of proteolytic activity, occurring after elution with H_2O, only showed reaction to the Lp82 antibody and not to the calpain 1 antibody. The fractions under the peak were pooled for use in subsequent analysis.

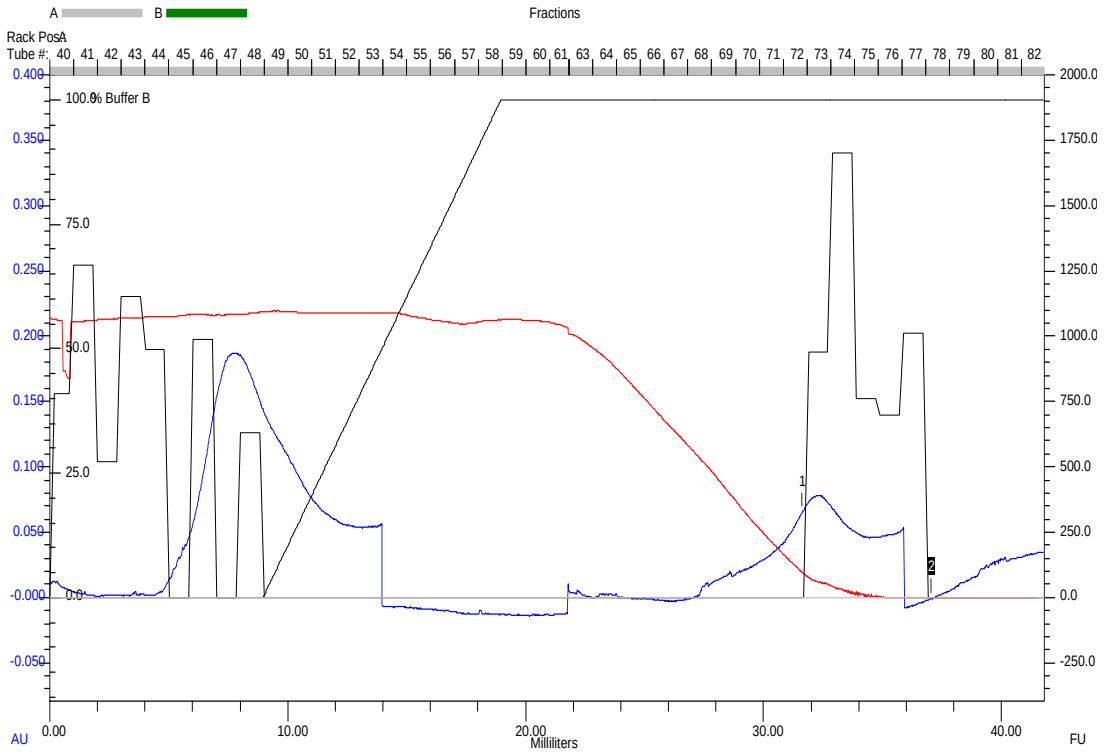

Figure 3-14 Reactive red purification to further purify Lp82 from gel filtration. Lines indicate UV absorbance @ 280nm (Blue), conductivity mS (Red), Bars represent Fluorescence Units (Calpain Activity). Lp82 was pooled from Tags 1-2.

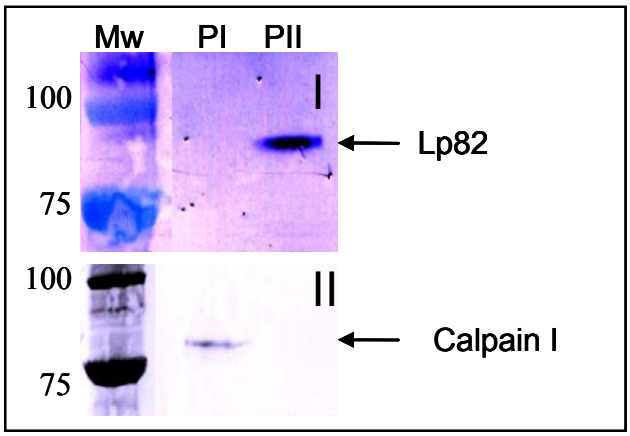

Figure 3-15 Western Blot analysis of the two proteolytically active peaks from the Reactive Red column (Figure 3-14). PI =Peak one, PII = Peak two. Blot I used Lp82 antibody and Lp82 is labelled, Blot II used calpain 1 antibody and calpain 1 is labelled.

The BODIPY-FL Casein assay was used to determine the specific activity after each of the purification steps (Table 3-3).

	Activity/ml	Total Activity	Specific Activity	% Recovery	% Total Recovery
Lens	2380	21420	31		
DEAE	590	141600	236	661	661
GF	1400	25200	359	18	118
RR	2200	8800	1157	35	41

Table 3-3 Specific activity from each step of the purification procedure for Lp82. Lens- Lens homogenate, DEAE- DEAE Ion exchange (Figure 3-8), GF-Gel filtration (Figure 3-11), RR-Reactive Red agarose (Figure 3-14). Activity/ml, total activity specific activity were calculated (as per 3.2.3.2). % Recovery is calculated as the percentage of total activity preserved from previous step. % Total recovery was the amount of activity retained from the lens homogenate.

3.3.3 SDS-PAGE, Western blot and zymography

To assess the effectiveness of each purification step, a combination of protein biochemistry techniques were used. Namely, SDS-PAGE, western blotting and casein zymography.

3.3.3.1 Calpain 2

The relative purity of the calpain 2 at each step of the purification process was assessed by SDS-PAGE (Figure 3-16). At each purification step a number of protein species were removed. During the DEAE Ion exchange (Lane E) step calpain 2 appeared to be eluting with a significantly larger protein (100kDa). This larger protein was removed during the Reactive Red step which resulted in a collected sample containing only the calpain 2 large subunit (Figure 3-16 upper arrow). The Mono Q step then concentrated the protein within the sample which allowed visualisation of the large subunit (76kDa) and small subunit (28kDa) of calpain 2 (Figure 3-16 lower arrow).

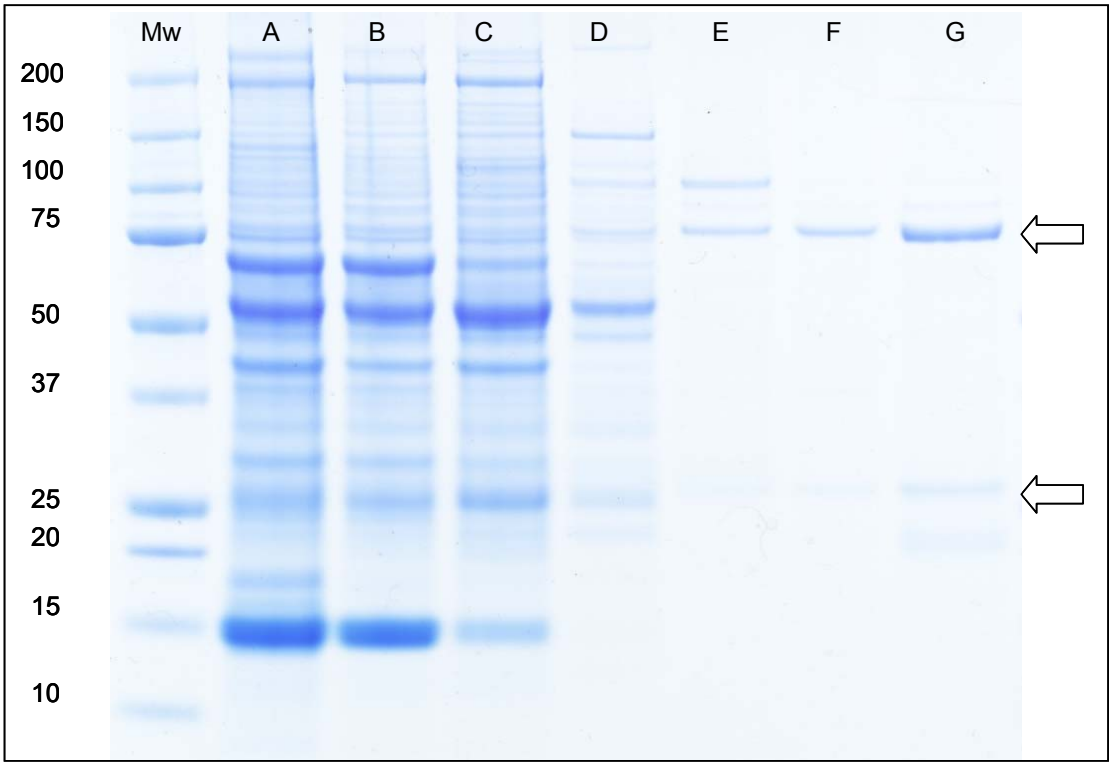

Figure 3-16 4-12% SDS-PAGE of the calpain 2 purification procedure. Lane, Mw) Molecular Weight Marker, Weights in kDa are to the left of the lane, A) Lung Homogenate, B) Total Soluble Lung Proteins, C) After NH_4SO_4 precipitation, D) After Phenyl Sepharose, E) After DEAE Ion Exchange, F) After Reactive Red, G) After Mono Q. The calpain subunits are indicated by arrows, the upper arrow indicating the large subunit and the lower arrow indicating the small subunit.

To confirm the presence of calpain 2 in samples following the purification steps, western blot analysis (Figure 3-17) and casein zymography were used (Figure 3-18). Western blot of the large subunit of calpain 2 clearly shows a protein species at

76kDa. This was found in all the fractions obtained after the various purification steps. The intensity of the band increased as the purification proceeded.

Figure 3-17 Western Blot analysis of calpain 2 purification steps. Lane, Mw) Molecular Weight Marker Weights in kDa are to the left of the lane, A) Lung Homogenate, B) Total Soluble Lung Proteins, C) After NH_4SO_4 precipitation, D) After Phenyl Sepharose, E) After DEAE Ion Exchange, F) After Reactive Red, G) After Mono Q.

Casein zymography (Figure 3-18) was also used to identify the calpains and to show that they were active. Calpain 1 can be identified as the higher band of activity and calpain 2 as the lower double band of activity. Both calpain 1 and 2 were present up to the Phenyl Sepharose purification step (Lane D Figure 3-18). DEAE ion exchange chromatography separated the two calpains as illustrated by the lack of the upper calpain 1 proteolytic band in lane E. The double band associated with calpain 2 represents the intact (upper band) and autolysed (lower band) forms of calpain 2.

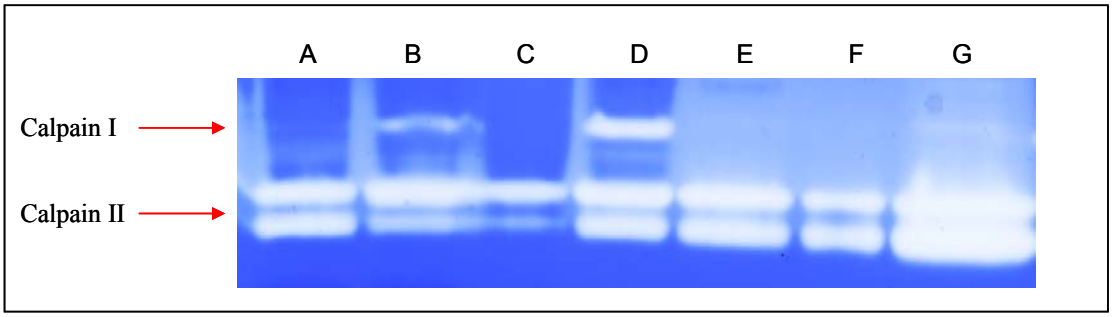

Figure 3-18 Casein Zymography of samples from calpain 2 purification steps. Lane, A) Lung Homogenate, B) Total Soluble Lung Proteins, C) After NH_4SO_4 precipitation, D) After Phenyl Sepharose, E) After DEAE Ion Exchange, F) After Reactive Red, G) After Mono Q. Calpains 1 and 2 are as indicated.

3.3.3.2 Calpain 1

The first four steps of the purification process removed several proteins from the calpain 1 containing fractions (Figure 3-19, Lane A-D). The DEAE fraction that separated the calpains 1 and 2 still contained some extraneous proteins (Figure 3-19 Lane E). The Reactive Red step removed most of these undesirable proteins except for one large protein (150kDa) (Lane F). The final step on HiTrap® Phenyl Sepharose also failed to remove this large protein. Nevertheless, the large subunit (79kDa) (upper arrow) and the small regulatory subunit (lower arrow) are visible in the concentrated sample.

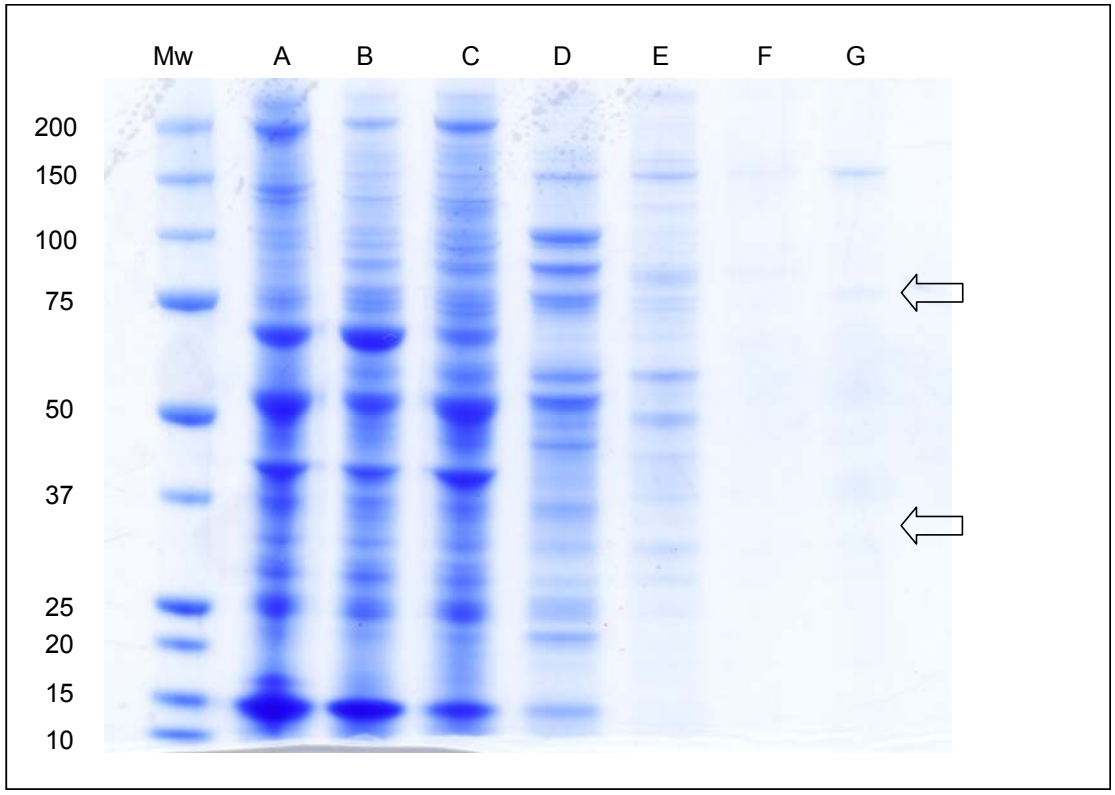

Figure 3-19 4-12% SDS-PAGE of the calpain 1 purification procedure. Lane, Mw) Molecular Weight Marker, Weights in kDa are to the left of the lane, A) Lung Homogenate, B) Total Soluble Lung Proteins, C) After NH_4SO_4 precipitation, D) After Phenyl Sepharose, E) After DEAE Ion Exchange, F) After Reactive Red, G) HiTrap Phenyl Sepharose. The calpain subunits are indicated by arrows, the upper arrow indicating the large subunit and the lower arrow indicating the small subunit

Western blot analysis was used to confirm the presence of calpain 1 in all the fractions collected at the various stages of the purification process (Figure 3-20). Western blot showed there was an increase in the intensity of the calpain 1 band as the purification procedure progressed and confirmed that concentrated calpain 1 was present in the final sample.

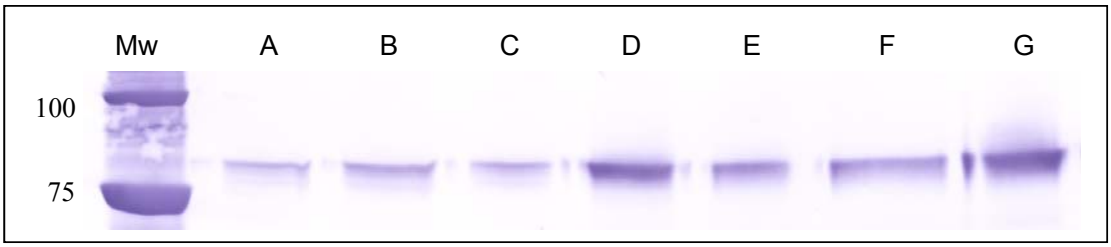

Figure 3-20 Western Blot analysis of calpain 1 purification steps. Lane, Mw) Molecular Weight Marker Weights, in kDa are to the left of the lane, A) Lung Homogenate, B) Total Soluble Lung Proteins, C) After NH_4SO_4 precipitation, D) After Phenyl Sepharose, E) After DEAE Ion Exchange, F) After Reactive Red, G) HiTrap Phenyl Sepharose.

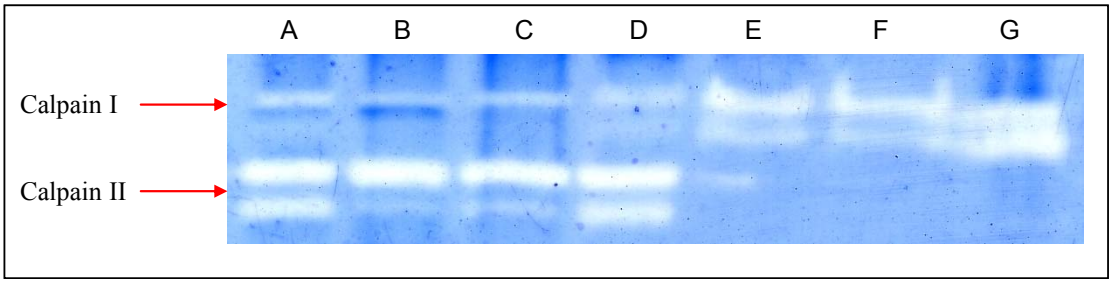

Figure 3-21 Casein Zymography of samples from calpain 1 purification steps. Lane, A) Lung Homogenate, B) Total Soluble Lung Proteins, C) After NH_4SO_4 precipitation, D) After Phenyl Sepharose, E) After DEAE Ion Exchange, F) After Reactive Red, G) HiTrap Phenyl Sepharose. Calpains 1 and 2 are as indicated.

Casein zymography was used to confirm that the collected samples contained active calpain 1. Both calpain 1 and 2 were present in the fractions until the DEAE separation following which there was only residual calpain 2 in the sample. This residual calpain 2 activity was removed from the calpain 1 fraction in the subsequent Reactive Red column and as a consequence the final fraction only contained calpain 1.

3.3.3.3 Lp82

The purity of Lp82 was first assessed by SDS-PAGE (Figure 3-22). Although each step in the purification process removed some proteins, the final Lp82 fraction contained some contaminants. The main contaminant was the α-crystallin subunits αA- and αB-crystallin (Figure 3-22 Lane D). This identification was based on the size of the two bands of 20kDa and 19kDa, as well as the spots on 2DE (Figure 5-5). The

Lp82 protein was visualised in the final sample (Lane D) and has a molecular weight of 82kDa.

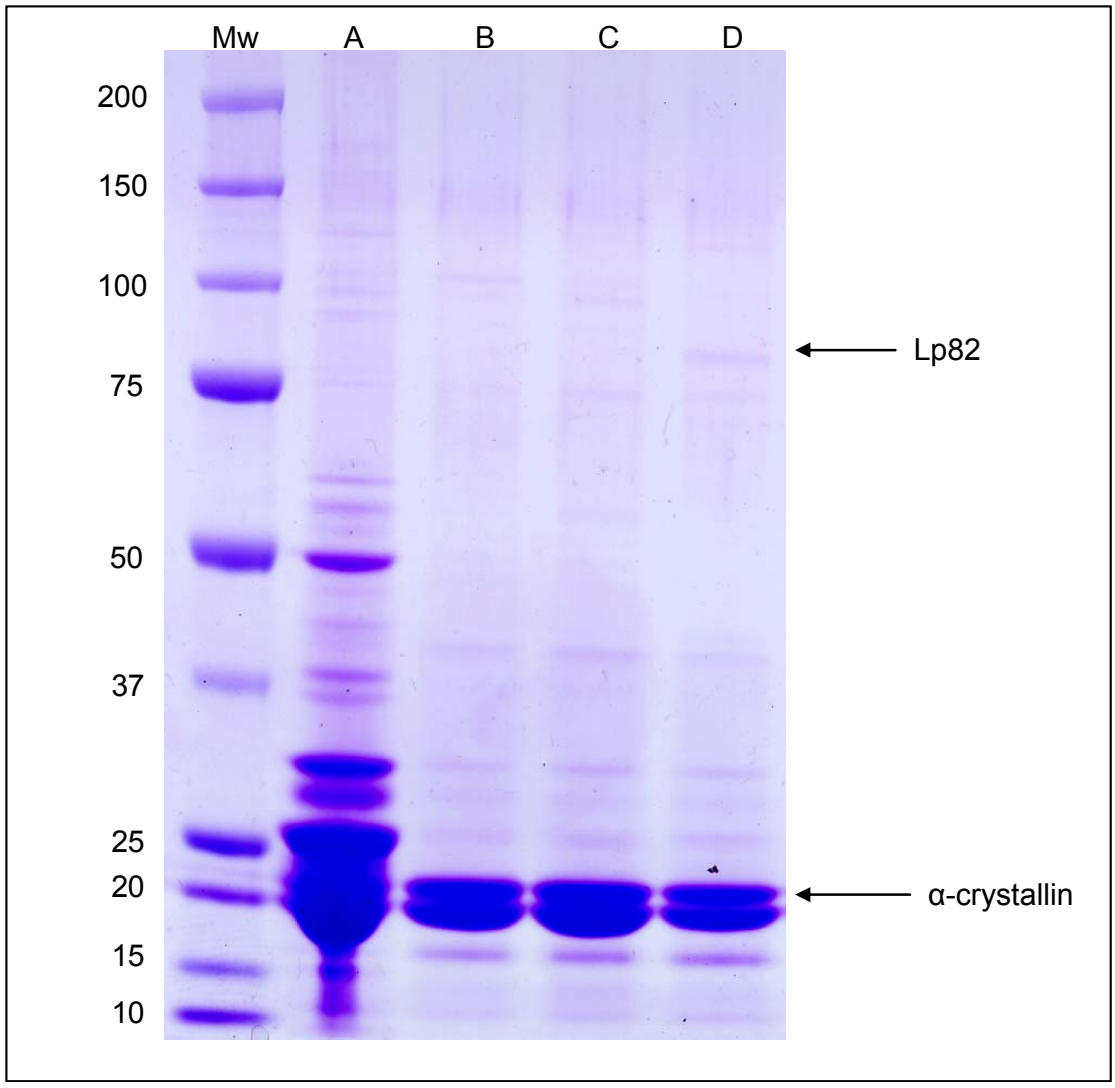

Figure 3-22 4-12% SDS-PAGE of Lp82 Purification Procedure. Mw) Molecular Weight Marker, weights in kDa on left of gel. A) Lens Homogenate, B) Lp82 Fraction after DEAE chromatography, C) After Gel Filtration chromatography, D) After Reactive Red chromatography.

Western blot analysis on each fraction at each stage of purification was carried out (Figure 3-23). In the last two purification steps (Lanes C and D) the Lp82 could be visualised by western blot. The Lp82 had a molecular weight of 82kDa.

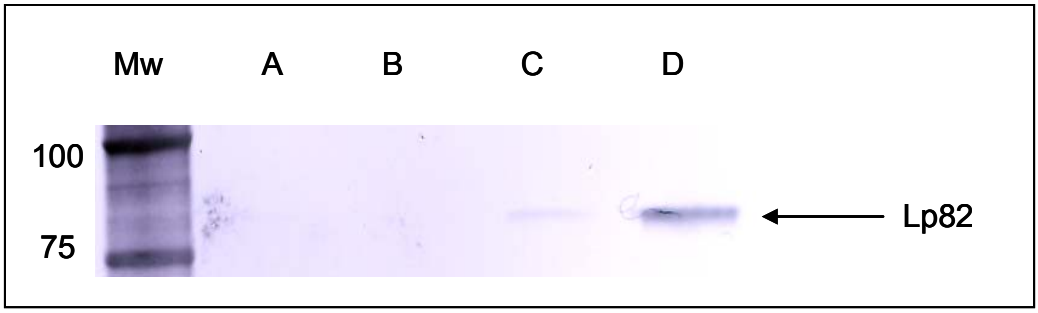

Figure 3-23 Western Blot analysis of Lp82 Purification Procedure. Mw) Molecular Weight Marker weights in kDa on left of gel. A) Lens Homogenate, B) Lp82 Fraction after DEAE chromatography, C) After Gel Filtration chromatography, D) After Reactive red chromatography.

3.3.4 Calcium and pH requirement

To determine the calcium dependence of calpain 2, the BODIPY-FL casein assay was utilised. Calpain 2 was purified in buffers without the metal ion chelators EDTA and EGTA. Calpain 2 was not active at concentrations of 50µM Ca^{2+} (Figure 3-24) and reached a maximum activity at 500µM with 50% of maximum activity occurring at 190µM Ca^{2+}.

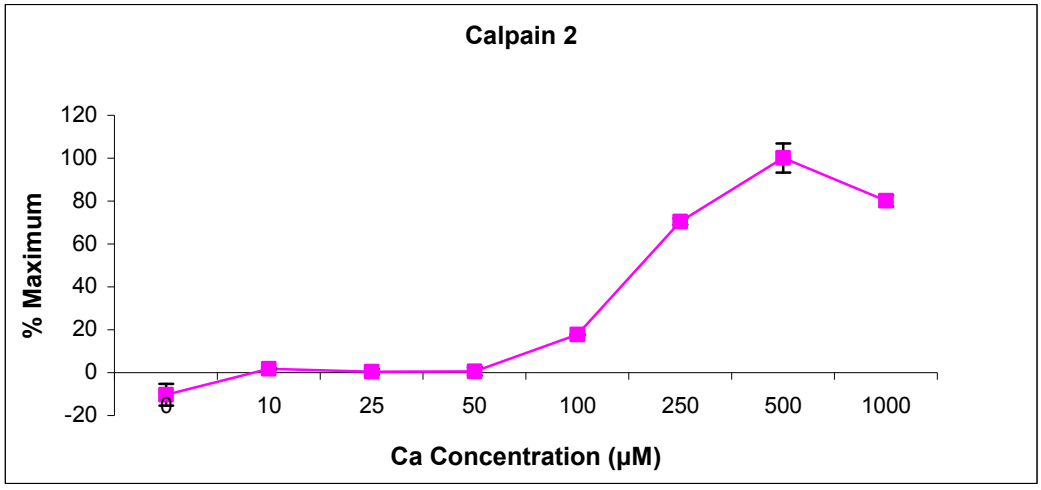

Figure 3-24 Calcium concentration vs Calpain 2 activity, expressed as a % of maximum activity. Maximum activity was seen at 500µM. Error bars represent ± 1 S.D.

Calpain 2 was active from across a wide range of pH's, it exhibited low activity below 5.4 and no activity above 8.4. The optimal pH for calpain 2 activity was 7.6.

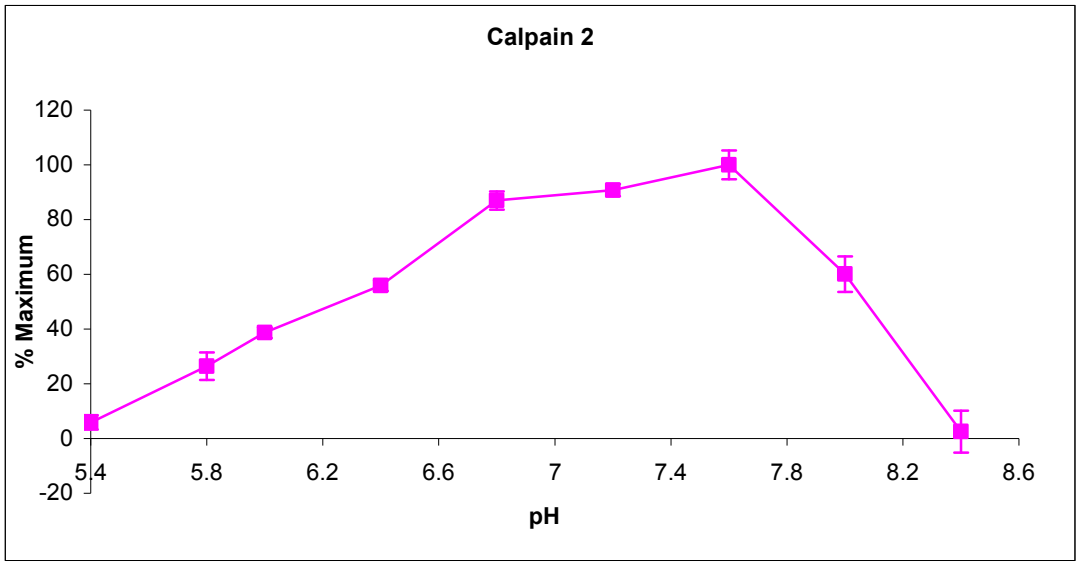

Figure 3-25 Graph of pH vs calpain 2 activity expressed as % of maximum . Bars represent ± 1 S.D. determined by t-test.

3.4 Discussion

These experiments aimed to purify and characterise the three main calpains in the ovine lens, the ubiquitous calpains 1 and 2 as well as the lens specific Lp82. All three calpains were purified using a series of FPLC steps, with several different types of chromatography media used to achieve samples specific for each of the three calpains.

Calpain 1 and 2 were both purified from lung tissue. The first purification step involved precipitation with ammonium sulphate to 50%. The calpains precipitate out between 30-45% ammonium sulphate and the calpastatin at 40-50% (Goll *et al.*, 2003). Therefore the resuspended precipitate still contained both the calpains and calpastatin.

Most calpain purifications use a combination of Phenyl Sepharose and DEAE ion exchange as the first two steps (Thompson and Goll, 2000). In this thesis Phenyl Sepharose was used as the first chromatography step for two reasons. Firstly, calpastatin does not bind to Phenyl Sepharose in the conditions used and consequently the calpains can be eluted from the column without their inhibitor (Figure 3-2). In comparison, if DEAE is used as the first chromatography step the calpastatin containing fractions overlap with the fractions containing calpain 1 (Thompson and

Goll, 2000, Goll *et al.,* 2003). The fractions then have to be subsequently applied to another column to enable a separation of calpastatin from calpain 1. Secondly, the extract after ammonium sulphate precipitation already contains a large amount of salt and would require dialysis to remove the salt before applying the extract to a DEAE column to allow calpain binding. The only negative factor about using Phenyl Sepharose first is that the two calpains elute together.

The separation of calpain 1 from calpain 2 was achieved using ion-exchange chromatography on DEAE. Calpain 1 eluted between 120mM and 200mM NaCl and calpain 2 between 230mM and 340mM NaCl (Figure 3-3), which is comparable to the 90-180mM KCl and 180-320mM KCl for calpains 1 and 2 respectively reported by Thompson and Goll, 2000. There was a small amount of overlap between the calpains with the calpain 1 fractions containing a small amount of calpain 2 (Figure 3-21, Lane E).

For the purification of calpain 1 and 2 Reactive Red Agarose was used as the third step. This step proved highly effective at separating the calpains from other unwanted proteins as well separating calpain 1 and 2 from each other. This was due to the fact that the interaction of calpains 1 and 2 with Reactive Red are completely different. Calpain 2 behaves with the Reactive Red Agarose similar to a hydrophobic interaction (DeMartino and Croall, 1983, Thompson and Goll, 2000). In this research calpain 2 was found to bind tightly to the Reactive Red matrix and elution with a low ionic strength buffer of 20mM MOPS + 5mM βME was not sufficient to elute calpain 2 from the column. H_2O was found to be an effective eluent as that had a sufficiently low ionic strength to elute the calpain 2 (Figure 3-4).

Calpain 1's interaction with the Reactive Red Agarose is the opposite of calpain 2 and is often variable in its interaction. Calpain 1 does not bind to the matrix but is retarded and can be "chased off" with 1M NaCl (Thompson and Goll, 2000) (V. Thompson, Personal Communication, 2007). This was seen with the ovine calpain 1 purification, where the calpain "eluted" with 1M NaCl (Figure 3-6). Therefore the Reactive Red provided another method to separate the two calpains and is more powerful than the DEAE, because the interactions of calpains 1 and 2 are at the opposite ends of the spectrum in terms of their elution from Reactive Red.

Calpain 2 was concentrated and further purified using Mono Q. This resulted in a sample containing no other contaminating proteins (Figure 3-16, Lane G). Overall 20mg of calpain 2 was isolated from the initial 300g of lung tissue. The recovered activity of this fraction was 8% of the total calpain activity recovered from the first Phenyl Sepharose column.

After "elution" from the Reactive Red Agarose, there was a large volume of calpain 1 which was concentrated using Hitrap® Phenyl Sepharose. This final sample contained two major proteins, calpain 1 and a unknown protein of higher molecular weight (Figure 3-19 Lane G)

Utilisation of all these purification procedures produced calpain 1 and 2 samples that were suitable for further studies.

Purification of Lp82 was more difficult than the other two calpains, due to the fact that Lp82 is lens specific and is only found in young lamb lenses (Robertson *et al.,* 2005). Lamb lenses could only be collected once a year as sheep only produce one set of lambs per year. An additional difficulty was that the lens contains large amounts of protein of which 90% is crystallins. Thus, the amount of sample that could be purified was low and consequently the yield of Lp82 was low.

Within the lamb lens, calpain 2 is the most abundant and active of the calpains (Figure 3-8, Figure 3-9 Lane A) (Robertson *et al.,* 2005). The first purification step used in the isolation of Lp82 was a DEAE ion exchange column to separate Lp82 from calpain 2. Lp82 eluted from the DEAE between 115mM to 163mM NaCl which is within the same range that calpain 1 is eluted from DEAE (120-200mM NaCl). Indeed the two calpains co-eluted (Figure 3-8). Calpain 2 eluted later with 200mM NaCl and was separated from Lp82 (Figure 3-10). Ueda *et al.,* in 2001 claimed that in the bovine lens Lp82 eluted separately from calpain 1 when using DEAE ion exchange and stated that calpain 1 eluted with calpastatin earlier than Lp82. The statements were based on a paper which purified calpain 1 from rat lens (Yoshida *et al.,* 1985). Ueda *et al.,* (2001) never tested the Lp82 containing fraction for the presence of calpain 1.

Gel filtration resulted in two peaks with BODIPY-FL casein activity (Figure 3-11) and Western blot analysis showed that the second peak was reactive to Lp82 (Figure 3-12). The first peak of activity showed no reaction to Lp82 antibody but was reactive to calpain 1 (Figure 3-13). Gel filtration of the bovine Lp82 from DEAE also produced two peaks of activity (Ueda *et al.,* 2001), but only the second peak was reactive to Lp82 antibodies. The authors attributed the activity in the first peak to leucine amino peptidase activity. In this study calpain 1 was identified as providing the BODIPY-FL casein activity. Ueda *et al.,* (2001) did not test for calpain 1. They tested for calpain 2 and Lp82 and assumed that calpain 1 had been removed in the previous ion exchange step. In this study, calpain 1 reactivity was also observed in the second peak that contained Lp82 (Figure 3-13 Peak II).

As mentioned previously calpain purification on Reactive Red Agarose can be difficult. This study is the first to purify Lp82 using Reactive Red and proved to be a very useful tool for calpain purification. During Reactive Red purification of Lp82 it was found that Lp82 has a similar affinity to Reactive Red as calpain 2 and, consequently, bound to the matrix tightly. As with calpain 2, H_2O was used to elute Lp82 from the Reactive Red (Figure 3-14). This final step was very useful as it enabled the removal of any calpain 1 that remained after gel filtration. As mentioned above calpain 1 does not bind to Reactive Red and is, therefore, washed out with any unbound proteins during purification. BODIPY-FL casein activity can be seen in the unbound protein fraction (Figure 3-14). Western blot analysis of this fraction was positive for calpain 1 but not for Lp82 (Figure 3-15 Lane PI). The second peak of BODIPY-Fl casein activity observed after elution with H_2O, was positive for Lp82 but not for calpain 1 (Figure 3-15 Lane PII).

The percentage recovery of Lp82 after each purification step was significant. Initially there was a 661% increase in activity on progression from the lens homogenate to the Lp82 containing fraction from the DEAE column (Table 3-3). This was due to the presence of the protease inhibitor cocktail in the lens homogenate which was removed during the first chromatography step. Gel filtration retained 17% of the activity applied from the DEAE column. This reduction in activity is probably due to the loss of some calpain 1. Over all in the Lp82 fraction there was a 41% recovery of BODIPY-FL casein activity from the original lens homogenate. However due to the

presence of protease inhibitors in the lens homogenate sample this figure is not indicative of the actual recovery. There was a 6% recovery of activity from the DEAE sample but the DEAE fraction also contained calpain 1 which would contribute to some of the activity.

Although the Lp82 purification procedure removed the other two calpains present in the sheep lens, it did not produce a homogenous solution containing Lp82 only. However Lp82 was concentrated to such a level that it could be visualised on SDS-PAGE (Figure 3-22 Lane D) and reacted to western blot analysis (Figure 3-23 Lane D) when 20μg of protein was loaded. The major contaminating protein was α-crystallin which was still present in the final sample (Figure 3-22 Lane D). This α-crystallin could not be removed despite several attempts at using different columns under different conditions.

3.5 Conclusion

In conclusion all three calpains; calpains 1, 2 and Lp82, that are present in the ovine lens were able to be purified. They were purified using different FPLC techniques. These purified calpains were used in further experiments (Chapter 5) to examine their role in ovine cataractogenesis

Chapter 4
Ovine Crystallins

4.1 Introduction

For the eye to function effectively it needs to focus light on to the retina. This focusing is achieved by the lens which has the ability to change shape to accommodate near or far objects. The lens needs to be completely transparent to allow the passage of all the light entering the eye onto the retina. Any disruption to the light passing through the lens can lead to blindness.

One way this transparency is generated and maintained is by the order and structure of the unique lens proteins, the crystallins. The crystallins can make up to 90% of the water soluble lens proteins (Andley, 2007). In mammalian lenses there are 3 groups of crystallins, α, β and γ crystallins. All three of these are found in the sheep lens (Robertson *et al.,* 2008).

α-crystallin is a multimeric protein made of two polypeptide subunits, αA which is acidic (pI 5.52) and the more basic αB (pI 6.57). They have molecular weights of 19875Da and 20148Da respectively (Robertson *et al.,* 2008). The α-crystallins found in sheep are similar to those found in other species. For example αA and αB share 99% and 98% sequence homology with bovine α-crystallins respectively, and 93% and 97% sequence homology with human α-crystallins (Robertson *et al*., 2008). Early sequence analysis revealed that the α-crystallins were related to the small heat shock proteins (Ingolia and Craig, 1982). Later α-crystallins were shown to act as molecular chaperones and prevent the thermal aggregation of alcohol dehydrogenase, β and γ-crystallins (Horwitz, 1992). *In vitro* α-crystallin can prevent the heat-induced denaturation of both β and γ-crystallins (Kelley *et al.,* 1993, Augusteyn *et al.,* 2002). It is proposed that α-crystallin acts as a chaperone to prevent the insolubilisation of other lens proteins during ageing and that overwhelming or disrupting this system may lead to cataract formation.

The β and γ crystallins are part of the βγ-superfamily and make up the rest of the proteins in the crystallin family. The β and γ-crystallins are structurally and evolutionary related, with both containing a Greek key motif. The β-crystallins form multimers up to 200kDa *in vivo* whereas the γ-crystallins are monomeric proteins. The major difference in their primary structure are that the β-crystallins contain N- and C-termini extensions (Graw, 1997). There are seven mammalian β-crystallins. Four are acidic (βA1, βA2, βA3 and βA4) and three basic (βB1, βB2 and βB3) (Graw, 1997). The β-crystallins form large multimeric proteins and contribute to the structural and refractive index properties of the lens. The γ-crystallin on the other hand are monomeric proteins and the smallest of the crystallins. There are seven mammalian γ-crystallin (γA-γF and γS). Like the β-crystallins the γ-crystallins role in the lens is to contribute to the structure and refractive properties of the lens (Graw, 1997).

During lens development the crystallins undergo extensive post-translational modifications including deamidation, phosphorylation, truncation and disulfide bond formation (Lampi *et al.,* 1998, Hanson *et al.,* 2000, Hains and Truscott, 2007b). All of these modifications can be seen in normal lens maturation as well as cataract formation. Of particular interest to this study is the truncation of the crystallins which occurs throughout lens maturation (Lund *et al.,* 1996, Ma *et al.,* 1998, Lampi *et al.,* 1998, Ueda *et al.,* 2002a, Zhang *et al.,* 2003). Both α and β-crystallins are truncated with lens ageing. Truncated α-crystallins are found in rat (Ueda *et al.,* 2002b), mouse (Ueda *et al.,* 2002a), bovine (Takemoto, 1995b) and human lenses (Takemoto, 1995b, Lund *et al.,* 1996, Ma *et al.,* 1998). Some of the β-crystallins are truncated in rat (David *et al.,* 1994), mouse (Ueda *et al.,* 2002a), bovine (Takemoto, 1995a, Shih *et al.,* 1998) and human lenses (Takemoto, 1995b, Lund *et al.,* 1996, Ma *et al.,* 1998).

Cataracts are described as an opacification of the lens that leads to scattering of the light that enters the eye resulting in partial and ultimately full blindness. There are significant post-translational modifications to the crystallins during cataract formation, including deamidation, phosphorylation, truncation, disulfide bond formation, oxidation and methylation (Ueda *et al.,* 2002b, Hains and Truscott, 2007b). A central interest to this study is the truncation of the crystallins during cataractogenesis. It has been shown that increased truncation of α and β crystallin are

found in cataractous lenses from sheep (Robertson *et al.,* 2008) and rats (David and Shearer, 1984, Ueda *et al.,* 2002b, Takeuchi *et al.,* 2004). It is proposed that truncation of the β-crystallins results in aggregation and insolubilisation of the crystallin which leads to light scattering. Truncation of α-crystallin has a different effect where it reduces the chaperone activity of α-crystallin. These two events contribute to the formation of cataracts.

The purpose of these experiments is to examine the ovine crystallins. In particular the purification of α and β crystallins from the lens, mass determination, the changes in crystallins profiles with ageing and maturation in the ovine lens and the changes in crystallin profiles during the different stages of cataract formation. Only α and β-crystallins were analysed in these experiments due to difficulties in resolving the γ-crystallins on gel electrophoresis. This was achieved with a combination of FPLC, gel electrophoresis and mass spectrometry. Chromatography was used to separate the crystallins into families, gel electrophoresis to visualise the changes in the crystallin profiles and mass spectrometry to identify the crystallins and their post-translational modifications.

4.2 Methods

4.2.1 Crystallin separation

Gel filtration FPLC was used to separate the crystallins into α-, β- and γ- crystallins. Gel filtration separates proteins on the basis of their size with the largest proteins leaving the column first and the smallest proteins last. A Superose 6 300/10 (Pharmacia, Uppsala, Sweden) column was equilibrated in Buffer A (20mM MOPS pH 7.5, 2mM EGTA, 2mM EDTA and 5mM β-ME). One hundred microlitres of total soluble lens protein containing approximately 650μg of protein, from a 4 month old lens, was injected on to the column and the lens proteins were separated with a 0.4mL/min flow of Buffer A, 30 x 1mL fractions were collected.

SDS-PAGE, using the BioRad Mini Protean System, was performed to determine which peaks of absorbance contained the relevant proteins. A 15% SDS resolving gel (0.4M Tris-HCl pH 8.8, 0.1%SDS, 15% Bis-acrylamide (BioRad, Ca, USA)) with 4% stacking gel (0.125M Tris-HCl pH 6.8, 0.1%SDS, 4% Bis Acrylamide (BioRad, Ca, USA)) was prepared. Each fraction from the gel filtration run was separated on the gel, by taking a 15µL aliquot and adding 3µL of SDS Loading Buffer (350mM Tris-HCl, pH 6.8: 10% SDS, 30% Glycerol, 5% βME, 0,012% Bromophenol Blue). Each sample was heated at 90ºC for 5 min to denature the protein. Samples were loaded and the gel was run at 90 V for 15 min and then 180 V for 60 min in SDS Running buffer (30mM Tris, 20mM Glycine, 1% SDS). Following electrophoresis the gels were stained in Simply Blue stain (Pierce, Il., USA). Relevant fractions with α, β and γ crystallins were pooled and protein concentrations determined by BCA assay (Pierce, USA).

4.2.2 Soluble and insoluble protein isolation

Eyes were surgically removed post-mortem from fetal, 6 month and 8 year old sheep and cataractous sheep with stage 0, 1, 3 and 6 cataracts (Figure 1-8). The lens was extracted via a posterior approach, and weighed after removal. The lens epithelium was removed and the lens homogenised using a glass homogenisor in 3ml/g of Buffer A containing Minicomplete protease inhibitor (Roche, Germany). Lens homogenates were centrifuged at 10000 x *g* for 45 min (Eppendorf, Centrifuge 5415R, Hamburg, Germany) to separate the soluble and insoluble lens proteins. The supernatant (Water Soluble Fraction, WSF) was removed and stored at -20ºC. The pellet, containing the insoluble lens proteins, was washed 3 times with buffer A to remove any residual soluble proteins. Urea soluble proteins were extracted by resuspending the pellet of insoluble proteins in 6M urea. The suspension was centrifuged at 10000 x *g* for 45 min to separate the urea soluble and insoluble proteins. The urea soluble fraction (USF) was pooled and stored at -20ºC. BCA assay was used to determine the protein concentration of the WSF and USF.

4.2.3 2DE

To identify and measure the molecular mass of the three different crystallins, two dimensional electrophoresis (2DE) was used. First dimensional focusing was performed using pH 3-10 non-linear 18cm Immobilised pH Gradient (IPG) strips (BioRad, Ca., USA). Either 100µg (for individual crystallins) or 400µg (whole lens samples) of protein was rehydrated in 300µL of rehydration buffer (8M Urea, 2% CHAPS, 50mM DTT, 2% 3-10 Biolyte buffer (BioRad, Ca., USA) 2% Glycerol and 0.001% Bromophenol Blue) and added to each strip. Focusing was performed on a Protean IEF Cell (BioRad, Ca., USA), for 70000Vh. Focused IPG strips were stored at -20ºC until the second dimension was performed.

The focused strips were equilibrated twice for the second dimension in equilibration buffer (6M Urea, 50mM tris-HCl, 30% Glycerol, 2% SDS and 0.001% Bromophenol blue). The first equilibration for 15 min contained 2% dithiothreitol and the second 15 min equilibration contained 2.5% iodoacetamide. The strips were fixed to the top of, 16cm x 20cm 15% SDS-PAGE gels (380mM Tris-HCL, pH 8.8; 15% Bis-Acrylamide, 0.1% SDS) with a 4% SDS-Stacking Gel (125mM Tris-HCL pH 6.8; 4% Bis Acrylamide, 0.1% SDS), with 1% agarose in SDS running buffer. The gels were run for 2 hr at 25V and then 20 hr at 100V, in SDS running buffer at 4ºC.

All samples were run in duplicate so that the gels could be stained in two different ways. One gel was stained with Coomassie Blue for visualisation and MALDI-TOF analysis and the other gel was zinc negative stained for protein elution and whole mass measurement.

Coomassie blue staining involved the gel being washed 3 times 5 min in 5mM HCl, then staining overnight in 0.005% Coomassie blue (R-250) in 5mM HCl. Destaining was achieved using 5mM HCl.

For zinc negative staining, the gels were washed for 5 min in 1% $NaHCO_3$, then incubated for 30 min in 200mM imidazole and 0.1% SDS. The gels was subsequently washed 3 times for 30s with dH_2O, and finally stained with 0.1M zinc acetate.

4.2.4 Mass determination

Proteins were eluted from 2-DE gels and their molecular mass determined. Following negative staining using zinc-imidazole, spots from single gels were excised, shaken twice for 15 min in 192 mM glycine, 25 mM Tris base, 50 mM DTT, 0.1% SDS, and crushed by passing through a 20μm stainless steel frit using a 0.5 mL gas tight syringe. One hundred and fifty microlitres of 96 mM glycine, 12.5 mM Tris base, 50 mM DTT was then added to the syringe to transfer the remaining gel particles into a centrifuge tube, and the resulting slurry was shaken for 30 min. The slurry was then transferred to an Ultrafree-MC microcentrifuge filter (UFC30HV00, Millipore, Bedford, MA) and centrifuged for 15 min at 13000 x *g*, an additional 50 μL of the above solution added, and the device centrifuged again. The collected liquid was then dried by vacuum centrifugation and redissolved in 50 μL of 5% formic acid. The eluted proteins were injected onto a 1.0 x 250 mm C4 column and their masses were determined on-line, by electrospray ionization mass spectrometry on a model LCG iontrap (ThermoFinnigan, San Jose, California, USA). A 20 μL/min flow rate and 2-60% acetonitrile gradient over 50 min was used, and 0.05% TFA was added to the mobile phase to prevent formation of SDS-protein adducts during mass analysis. Samples were autoinjected and concentrated using a micro protein trap cartridge.

In-gel trypsinization was performed as previously described (Wilmarth *et al.,* 2004) and digests were analyzed by LC-MS (Wilmarth *et al.,* 2004) to collect MS/MS spectra using an LCQ Classic ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Proteins were identified using SEQUEST software (Thermo Finnigan) to correlate experimental MS/MS spectra with theoretical MS/MS spectra calculated from peptide sequences in a bovine subset of the Swiss-Prot protein database (Swiss-Prot, Swiss Institute of Bioinformatics, Geneva, Switzerland) supplemented with the sequences of sheep αA, αB, and βB3 crystallins (Robertson *et al.,* 2008). SEQUEST results were filtered using DTASelect (Tabb *et al.,* 2002). DeltaCN values greater than 0.08 were required and identified peptides had to exceed XCorr values of 2.0, 2.5, and 3.8 for +1, +2, and +3 ions, respectively. All identified proteins also required two or more unique peptide matches per entry for a positive identification.

4.3 Results

4.3.1 Crystallin separation

Gel filtration chromatography was used to separate the crystallins into their subunits. One hundred microlitres of total lens soluble proteins from a 4 month old lens was separated on a Superose 6 gel filtration column (Figure 4-1).

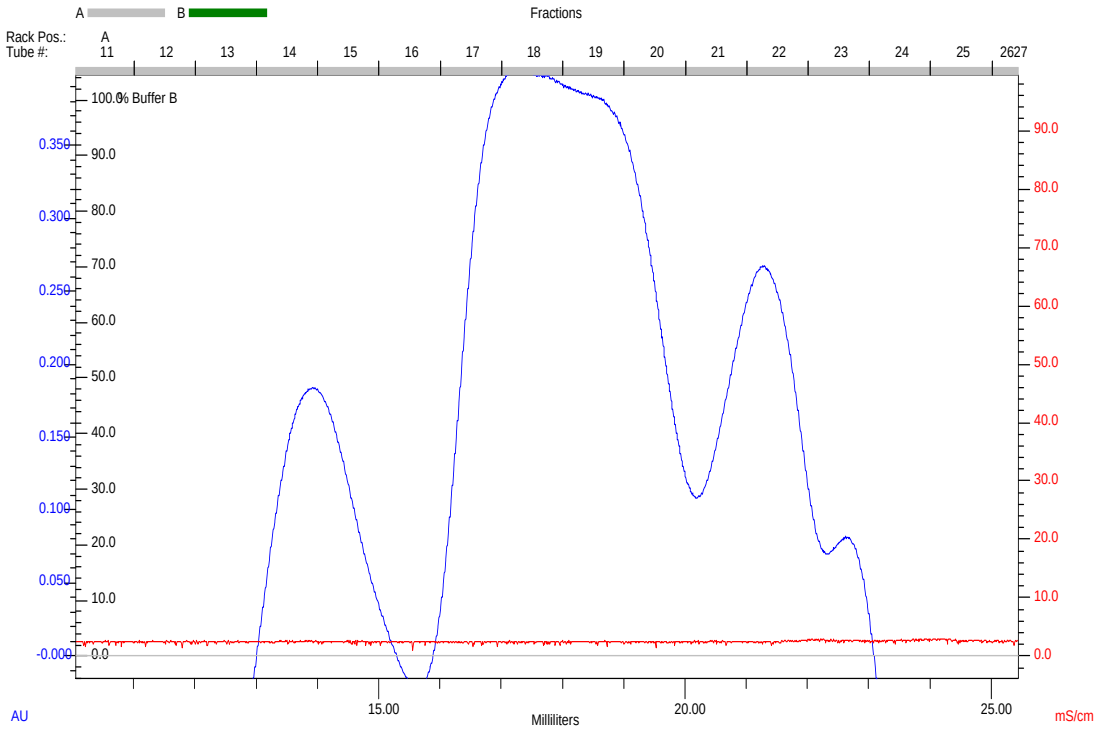

Figure 4-1 Gel filtration chromatogram of the total soluble proteins from a 4 month old sheep lens. UV absorbance @ 280nm is indicated by the Blue line. Fraction numbers (11-25) are on the upper x axis.

To determine which crystallins were in each fraction and peak of the chromatogram (Figure 4-1), the fractions were run on a 15 % SDS-PAGE gel (Figure 4-2).

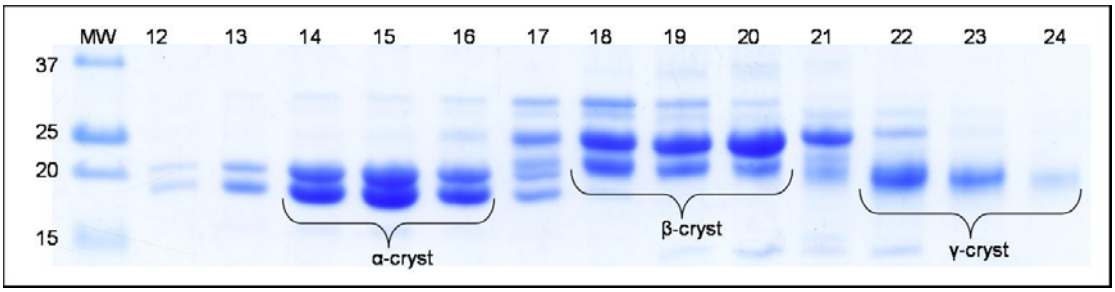

Figure 4-2 15 % SDS-PAGE of each fraction from the gel filtration separation of total soluble lens proteins. Numbers (12-24) indicate the fractions from (Figure 4-1), MW- Molecular Weight Marker (kDa). α, β, and γ-cryst labels show the crystallin fractions pooled for further analysis.

Gel filtration of the total lens soluble proteins resulted in separation of the lens crystallins into their three groups (Figure 4-1 and Figure 4-2). The large α-crystallins were the first to elute from the column followed by the β-crystallins and then the monomeric γ-crystallins. The fractions were pooled according to the labels in Figure 4-2. The α-crystallins had masses between 18 and 21kDa, β-crystallins 21-30kDa and γ-crystallin 19kDa. The BCA assay (3.2.3.4) was used to determine protein concentrations. The pooled crystallin fractions were used for further analysis.

4.3.2 Individual crystallins

The separated crystallins were run on 2DE for further identification and their whole mass measured by mass spectrometry.

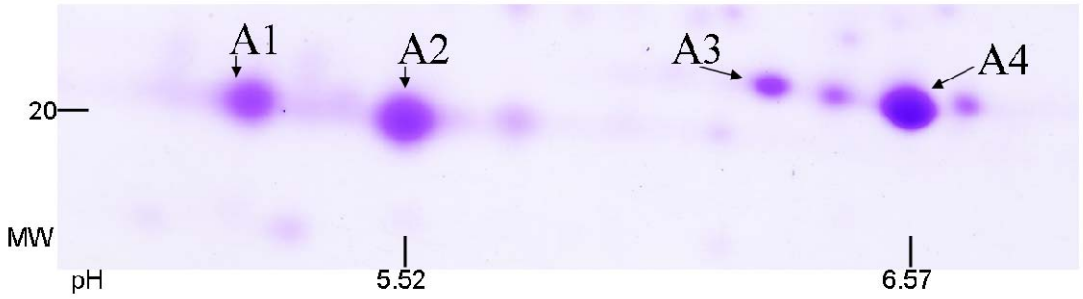

Figure 4-3 2DE of the α-crystallin fraction from separation using gel filtration. Numbers indicate the different α-crystallins according to (Table 4-1). A1-αA+phos, A2- αA, A3, αB+phos, A4-αB. Molecular weight (MW) in kDa and pH range are as indicated.

2DE of the α-crystallin showed four major protein species (Figure 4-3 A1-A4). The four spots were extracted, the protein identified and the whole mass measured (Table

4-1). Two of the four proteins found were intact αA (A2) and αB (A4). The same proteins were found with a mass increase of 80Da and a positive shift on the gel (A1 and A3 respectively) which is indicative of a single phosphorylation (αA+phos (A1) and αB+phos (A3)). The other spots on the gel were too small to be sequenced

Spot	ID	Mass (Da)	Modification
A1	αA	19954.5	+Phos
A2	αA	19874.5	Intact
A3	αB	20187.5	+Phos
A4	αB	20108.6	Intact

Table 4-1 Table of α-crystallin spots from Figure 4-3, Masses and modifications were determined by whole mass measurement. Identification of each spot was performed by MALDI-TOF analysis and comparison to known ovine sequences.

2DE of the β-crystallin fraction revealed 20 different β-crystallin species which were identified and their masses measured. At present the only ovine β-crystallin sequence that has been determined is βB3 (Robertson *et al.,* 2008), therefore to identify the isolated β-crystallin spots the sequences derived from MADLI-TOF analysis were compared with the complete bovine lens proteins. Post translational modifications of the ovine β-crystallins were determined by comparison to the equivalent bovine 2DE (Shih *et al.,* 1998) and the comparison of the whole mass measurement to that of the bovine β-crystallins and the ovine βB3 sequence (Robertson *et al.,* 2008)

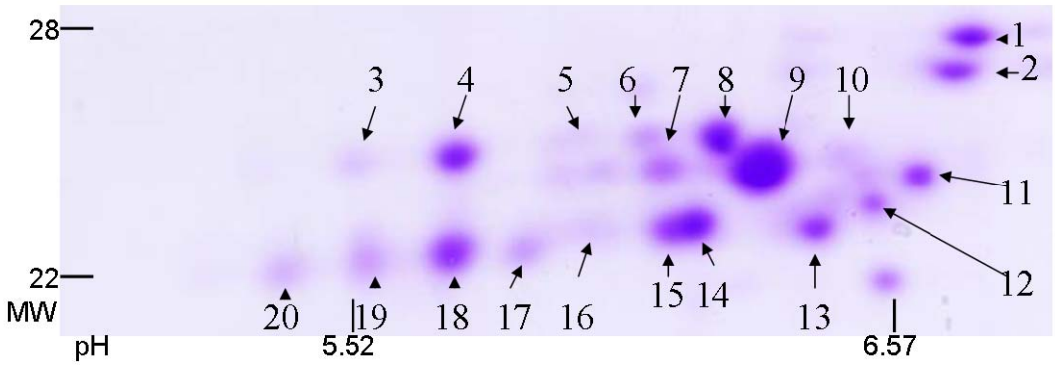

Figure 4-4 2DE of the β-crystallin. Numbers indicate the different β-crystallins according to Table 4-2. Molecular weight (MW) in kDa and pH range are as indicated.

Spot	Protein (ID)	Mass	Bovine Equivalent
1	βB1	28055.9	$\beta B1_{1-252}$
2	βB1	26743.0	$\beta B1_{15-252}$
3	βA3	25828.3	βA3 Mod
4	βA3	25572.3	βA3
5	βA3/βA1	25756.3	N/A
6	βB3	24323.8	βB3 Deamidation
7	βB2	23322.8	βB2 Deamidation
8	βB3	24321.9	βB3
9	βB2	23321.3	βB2
10	βB3	24323.9	N/A
11	βB3	23302.4	$\beta B3_{10-210}$
12	βA3/βA1	24212.1	$\beta A3_{12-215}$
13	βA3/βA1	23038.2	$\beta A3_{23-215}$
14	βA3/βA1	23497.3	βA1
15	βA2	22387.2	βA2
16	βA3/βA1	23496.6	βA1
17	βA4	22879.1	βA4 Mod
18	βA4	22696.4	βA4
19	βA4	22912.6	βA4 Mod
20	βA4	22752.9	βA4 Mod

Table 4-2 Table of protein spots from Figure 4-4. Masses were determined by whole mass measurement. Identification of each spot was performed by MALDI-TOF analysis (see 4.2.4) Bovine equivalents were determined by comparison with Shih *et al.,* (1998) and personal communication with L. David, 2005. N/A = not available.

4.3.3 Crystallin modification with age

To determine the exact crystallin modifications that are associated with cataractogenesis, the crystallin changes that occur in normal ovine lenses during ageing were established first. Then comparison could be made to the changes during cataractogenesis. Lens maturation was determined by comparing the crystallin profiles of lenses from fetal, 6 month and 8 year old sheep. The WSF and USF proteins from lenses of the different ages were compared.

The percentage total soluble protein content decreased as the lens aged from fetal to 8 years of age (Figure 4-5). There was little insoluble protein in the fetal lens and the amount of insoluble protein increased as the lens aged until it made up 30% of the total lens wet weight.

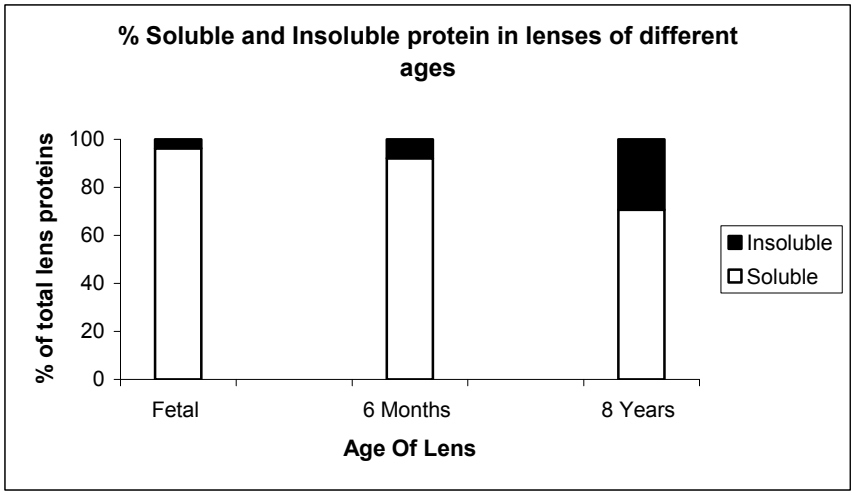

Figure 4-5 Total soluble (□) and insoluble (■) protein from lenses of different ages, expressed as a percentage of total lens wet weight.

4.3.3.1 Modifications of α-crystallins with age

During maturation of the lens there is modification of the α-crystallins (Figure 4-6 and Figure 4-7). Both αA and αB were phosphorylated in the fetal lens (Figure 4-6 Gel A Spots A1 and A3 respectively). This phosphorylation increased as the lens aged as seen by the increase in the size of spots A1 and A3 in gels A-C. The phosphorylation increased to the extent that the phosphorylated form of αA (Spot A1) was the major form of αA in the WSF of the 8 year old lens. The phosphorylation of αB did not increase to the same extent. Consequently, unmodified αB (A4) was still the most prominent form of αB in the 8 year old lens.

Truncation of the α-crystallins occurs at all ages in the lens. There are three truncations of αA crystallin $\alpha A_{1\text{-}154}$ (A6), $\alpha A_{1\text{-}151}$ (A7) and $\alpha A_{1\text{-}157}$ (A18) which are all found in the WSF of the fetal and 6 month old lenses. However in the WSF of the 8 year old lens $\alpha A_{1\text{-}151}$ (A7) was absent but was identified in the USF. The opposite is

found for αA_{1-154} (A6) where it was identified in the USF but not the WSF. In the aged lens a new protein species was identified (A12) but the whole mass and truncation could not be determined. There are two truncations of αB_{1-175}, αB_{1-170} (A5) and αB_{1-174} (A15) with 5 and 1 residues missing from the C-terminus respectively. The truncation αB_{1-174} was also found in a phosphorylated form (A15) based on its position after 2DE but the mass could not be determined. Within the WSF, as the lens ages, the αB_{1-174} truncation and its phosphorylated forms are found at approximately the same abundance. αB_{1-170} is present in fetal lenses and those of a 6 month old sheep but there is none in the aged lens (Figure 4-6 Gels A,B and C).

In the fetal lens αA was deamidated (A13) and the amount of deamidated αA did not increase as the lens aged. There is also deamidation of the phosphorylated form of αA-phos (A14). In contrast to the deamidation of the unmodified α-crystallins, the deamidation of the phosphorylated form of αA increased as the lens aged. There is no deamidation in the fetal lens or 6 month old lens of αA-phos but there are large amounts of deamidated αA-phos in the 8 year old lens.

Within the USF (Figure 4-7) of the same lenses there are modifications similar to those seen in the WSF. There are however some differences in the proteins found in the WSF and the USF. The truncation product αA_{1-151} (A7) is not present in the WSF of the aged lens but is present in the USF. Within the USF there is an increase in αB_{1-174} (A15) to the point that the truncated form of αB_{1-174} (A15) is the most dominant species of αB crystallin in the lens. The truncation product αB_{1-170} (A5) is only present in the USF fraction of the 8 year old lens.

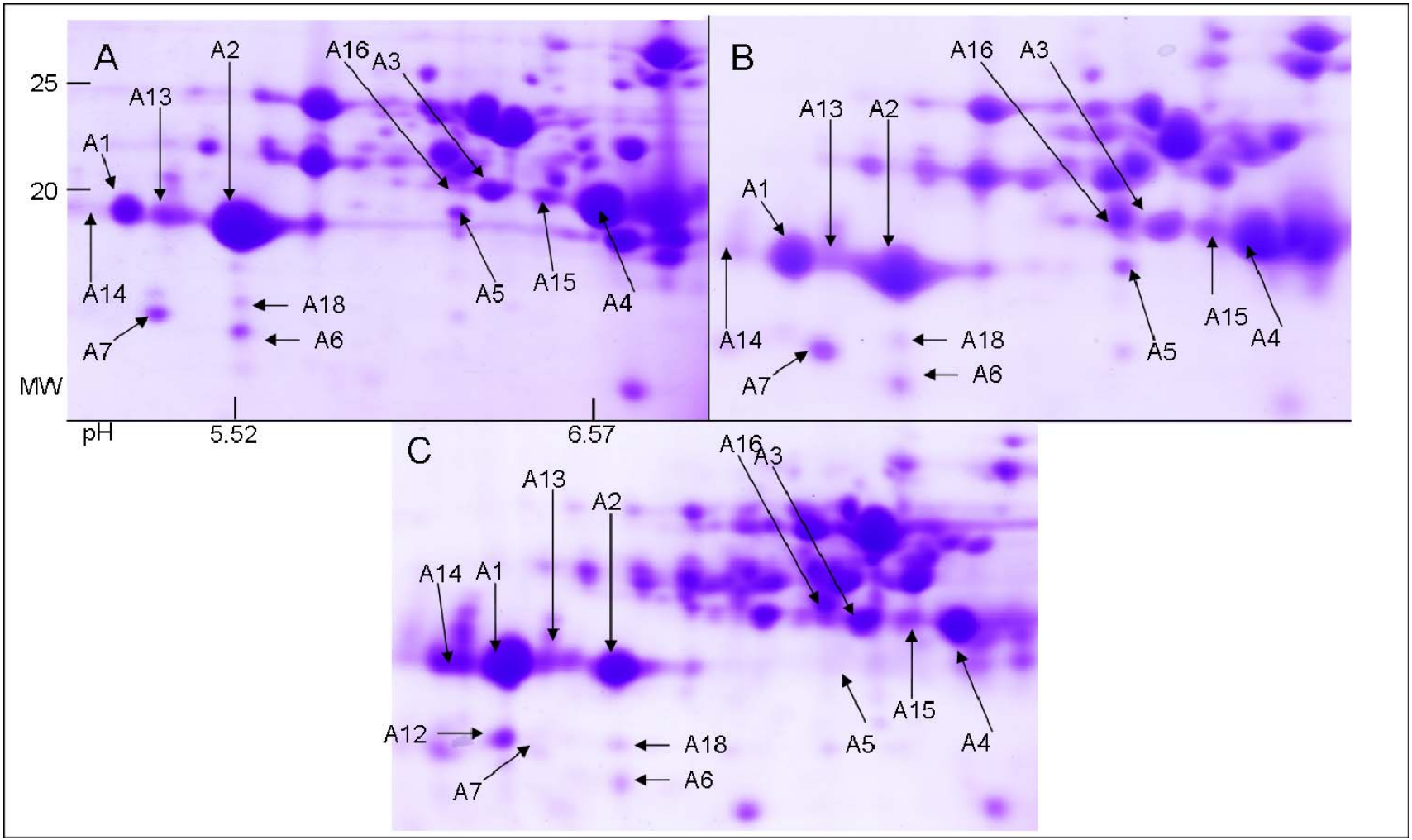

Figure 4-6 Total soluble lens proteins from sheep of different ages. A) fetal lens, B) 6 month old lens, C) 8 year old lens. Spots are labelled for the α-crystallins only and correspond to Table 4-3. Molecular weight (MW) in kDa and pH range are as indicated for gel A only.

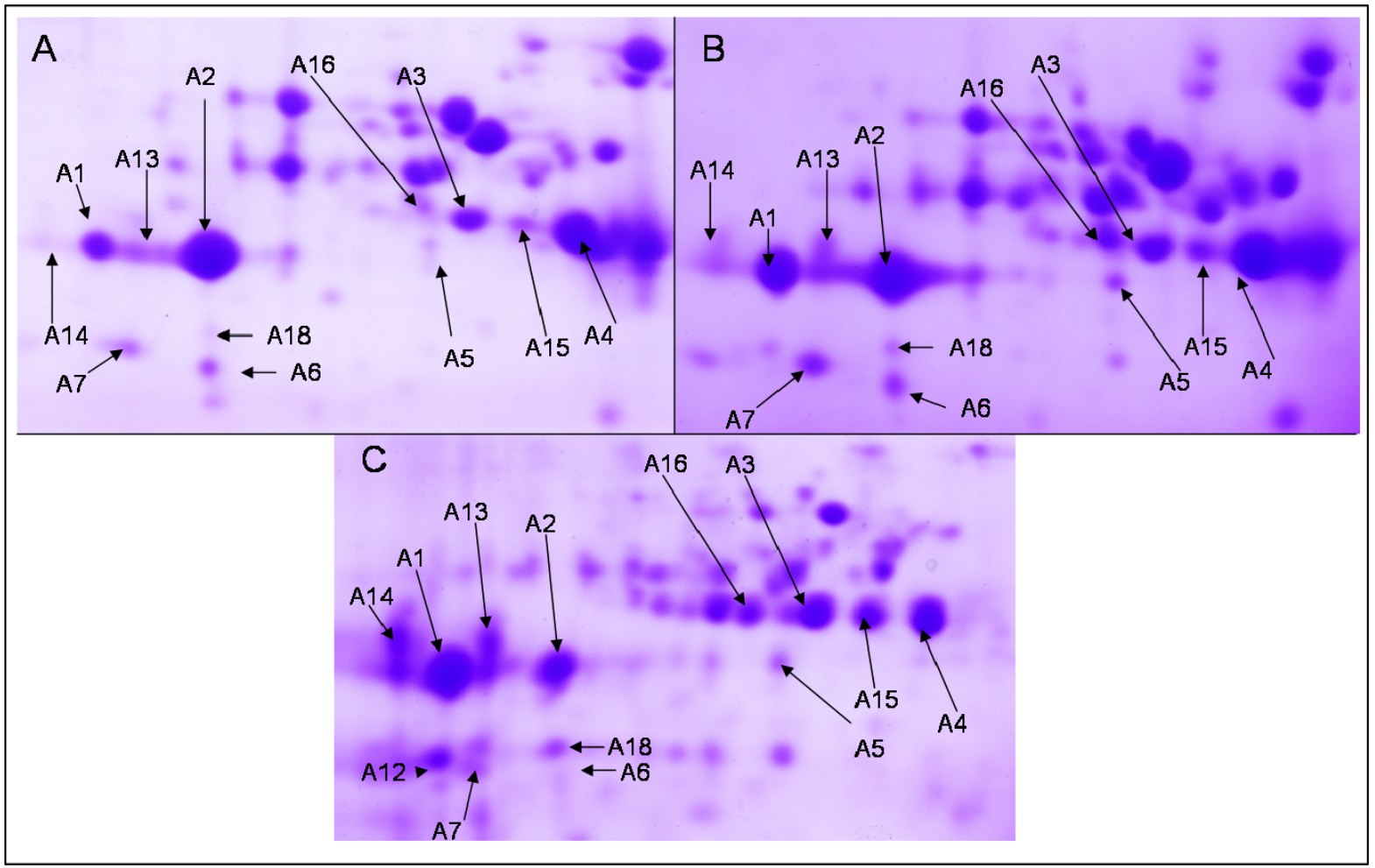

Figure 4-7 Urea soluble lens proteins from sheep of different ages. A) fetal lens, B) 6 month old lens, C) 8 year old lens. Spots are labelled for the α-crystallins only and correspond to Table 4-3.

Spot	ID	Mass (Da)	Predicted Mass (Da)	Modification
A1	αA	19954.5	19955.1	αA-Phos
A2	αA	19874.5	19875.1	αA
A3	αB	20187.5	20188.8	αB-Phos
A4	αB	20108.6	20108.8	αB
A5	αB	19614.3	19613.1	αB_{1-170}
A6	αA	17879.3	17879.0	αA_{1-154}
A7	αA	17614.3	17613.7	αA_{1-151}
A18	αA	18251.3	18251.0	αA_{1-157}
A12	αA	N/A	N/A	N/A
A13	αA	N/A	19876.1	αA Deamidation
A14	αA	N/A	19956.1	αA-Phos Deamidation
A15	αB	19980.3	19980.6	αB_{1-174}
A16	αB	N/A	20060.6	αB_{1-174}-Phos

Table 4-3 Masses and modifications of the α-crystallins seen during ageing of the ovine lens from Figure 4-6 and Figure 4-7. N/A = not available

4.3.3.2 Modifications of β-crystallins with age

Within the fetal lens there is a full complement of intact ovine β-crystallins (Figure 4-8 A) βB1 (1), βB2 (9), βB3 (8), βA1 (14), βA3 (4), βA4 (18) and βA2 (15). The intact unmodified β-crystallins are the most abundant of the total β-crystallin species in the fetal lens. All of these crystallins are also found in the fetal lens in a modified form due to deamidation or truncation. βB2, βB3 and βA1 are all deamidated in the fetal lens (Spots 6, 7, and 16 respectively). βB1 is truncated at the N-terminus with species missing 15 and 24 amino acids (2, 21). βA3 also undergoes truncation at the N-terminus in the fetal lens, removing 12 and 23 amino acids (Spots 12 and 13). There is a single modification to βB3 missing 10 amino acids from the N-terminus (spot 11). βA4 is also modified (spot 17) with a mass increase of 180Da from the native βA4.

There are changes in the β-crystallins as the lens ages and matures. At 6 months (Figure 4-8 B) and 8 years (Figure 4-8 C) of age the major intact β-crystallins are all

present in the WSF. There is also an increase in the post translational modifications of the β-crystallins.

Intact βB1 is the most abundant species of this crystallin in the fetal lens. However, as the lens matures, the amount of the intact βB1 decreases. The major truncation of βB1 is missing 15 amino acids from the N-terminus (2). At 6 months of age there are approximately equal amounts of the intact form (1) and the major truncated form (2). By 8 years old only a small amount of intact βB1 was found and the major form of βB1 is the truncation $\beta B1_{16\text{-}252}$ (2). There are other minor truncations of βB1 that change during lens maturation. Spot 21, which is a βB1 species missing 24 amino acids from the N-terminus (21), is found in the fetal and 6 month old lens but is missing from the mature 8 year old lens. Spot 22, which is βB1 missing 24 amino acids from the N-terminus and 5 amino acids from the C-terminus, is absent in the fetal lens but present in the 6 month and 8 year old lens. Spot 23, which is βB1 missing 10 amino acids from the N-terminus, is present in the fetal lens but its abundance decreases as the lens matures through to 8 years of age.

The intact form of βB2 is the dominant β-crystallin in the lenses from sheep of all ages (Figure 4-8 A,B and C 9). The only modification seen to βB2 during lens maturation is deamidation (7) which increases in quantity as the lens ages.

There is a large amount of intact βB3 found in the fetal lens, however by 6 months of age the amount of βB3 has decreased and in a 8 year old lens intact βB3 only makes up a small amount of the β-crystallins. βB3 modifications are found in both deamidated and truncated forms. The deamidated form (6) is found in the same amount in lenses of all ages. The truncated form, missing 10 amino acids from the N-terminus (11), decreases in quantity from fetal to 6 month to 8 years of age.

βA3 undergoes truncation, deamidation and possibly glycation with the amounts of the intact and modified forms of βA3 changing throughout lens maturation. Intact βA3 decreases as the lens matures (Figure 4-8 A, B, and C 4). βA3 is truncated in the fetal lens to produce 2 spots, one spot is missing 11 amino acids and the other 22 amino acids from the N-terminus (12 and 13, respectively). Both of these truncations are found at increased levels by 6 months of age. However at 8 years of age the

species missing 11 amino acids (12) is decreased and there is an increased amount of the -22 amino acid species (13).

βA4 (18) in the intact form is present in lenses at all ages but is only found in small amounts in the 8 year old lens. The only modification that changes during lens ageing is spot 17 which is an unknown modification adding a mass of 180Da to the normal βA4. This could be glycation since the addition of a monosaccharide moiety to a protein increases the mass by 180Da.

βA1(14) is present in lenses of all ages and becomes increasingly deamidated as the lens matures (16). There is no change to the βA2 (15) profile as the lens ages.

For the fetal lens the profile of the β-crystallins in the WSF (Figure 4-8 Gel A) and USF (Figure 4-9 Gel A) are similar. By 6 months of age there start to be small differences between the WSF (Figure 4-8 Gel B) and USF (Figure 4-9 Gel B) of the lens. For example in the USF there are increased amounts of the modified β-crystallins compared with the WSF. In the USF the truncated form of βB1 (missing 15 amino acids) is increased (2) and is more prevalent than the unmodified βB1. There is increased deamidation of βB2 (7), and βB3 (6) in the USF at 6 months of age. There are decreased amounts of all the β-crystallins in the USF of an 8 year old lens. With intact βA3 and βB1 missing from the USF of an 8 year old lens.

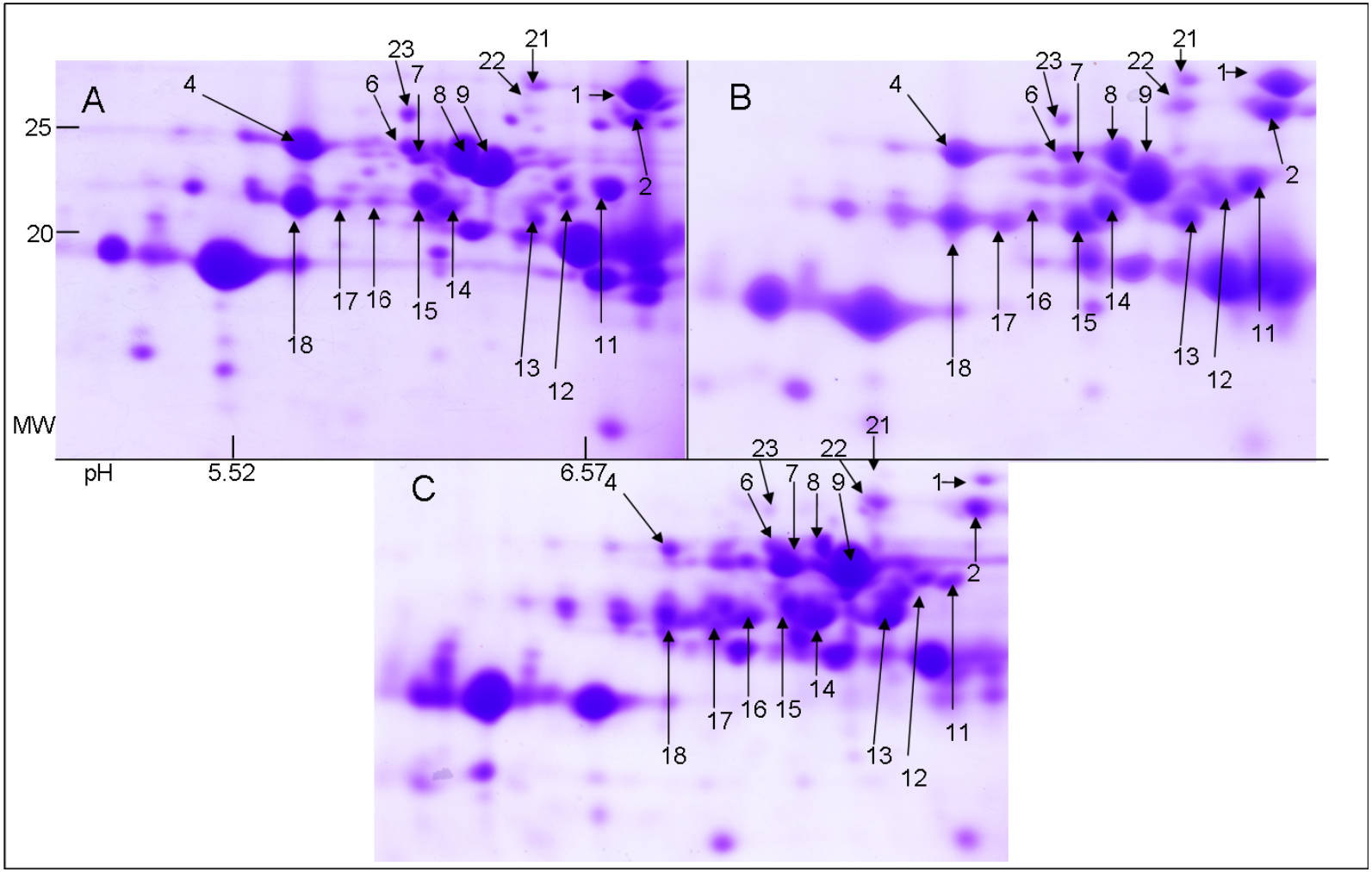

Figure 4-8 Total soluble lens proteins from sheep of different ages. A) fetal lens, B) 6 month old lens, C) 8 year old lens. Spots are labelled for the β-crystallins only and correspond to Table 4-4 and Table 4-6. Molecular weight (MW) in kDa and pH range are as indicated for gel A only.

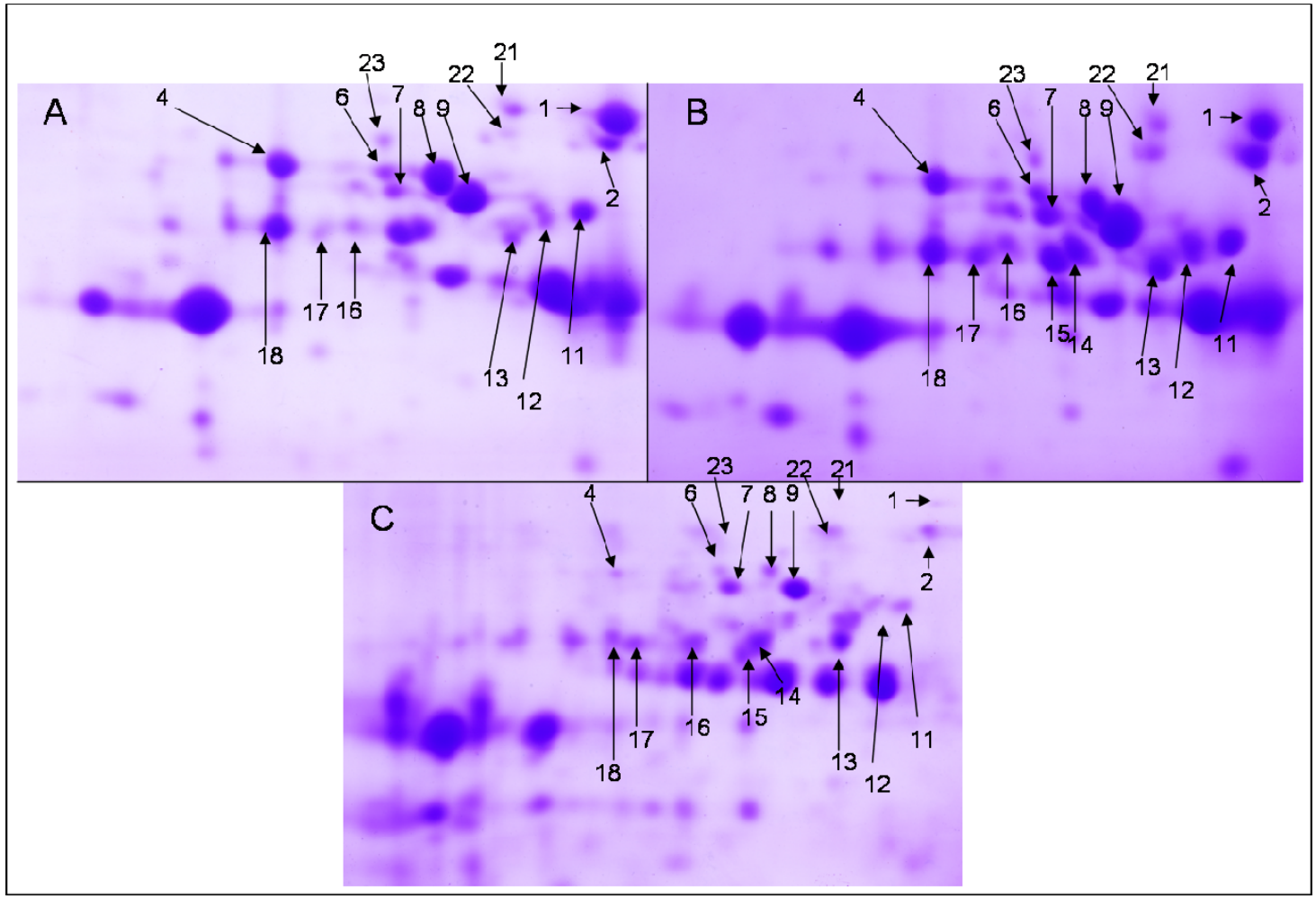

Figure 4-9 Total Urea Soluble lens proteins from sheep of different ages. A) fetal lens, B) 6 month old lens, C) 8 year old lens. Spots are labelled for the β-crystallins only and correspond to Table 4-4 and Table 4-6.

Spot	Protein (ID)	Mass	Bovine Equivalent
1	βB1	28055.9	βB1
2	βB1	26743.0	$\beta B1_{16-252}$
3	βA3	25828.3	βA3 Modification
4	βA3	25572.3	βA3
5	βA3/βA1	25756.3	N/A
6	βB3	24323.8	βB3 Deamidation
7	βB2	23322.8	βB2 Deamidation
8	βB3	24321.9	βB3
9	βB2	23321.3	βB2
10	βB3	24323.9	N/A
11	βB3	23302.4	$\beta B3_{10-210}$
12	βA3/βA1	24212.1	$\beta A3_{12-215}$
13	βA3/βA1	23038.2	$\beta A3_{23-215}$
14	βA3/βA1	23497.3	βA1
15	βA2	22387.2	βA2
16	βA3/βA1	23496.6	βA1
17	βA4	22879.1	βA4 Mod
18	βA4	22696.4	βA4
19	βA4	22912.6	βA4 Mod
20	βA4	22752.9	βA4 Mod
21	βB1	25935.3	$\beta B1_{25-252}$
22	βB1	25414.9	$\beta B1_{25-247}$
23	βB1	27199.8	$\beta B1_{10-252}$

Table 4-4 Table of modifications to the β-crystallins seen during ageing (Figure 4-8 and Figure 4-9). The crystallins were identified by MADLI-TOF analysis and mass determined by LC/MS. Bovine equivalents were determined by comparison with Shih *et al.,* (1998) and personal communication with L. David, 2005. N/A = not available.

4.3.4 Crystallin modification with cataract progression

2DE was used to analyse the changes in lens proteins during cataract formation from lenses with stage 0 (normal), 1, 3 and 6 cataracts (Figure 1-8). The lenses were separated into the WSF and USF and run on 2DE.

As cataracts develop in the lens there are changes in the lens protein profile (Figure 4-10 and Figure 4-11). The most obvious changes are in the crystallin profiles and in particular truncation of the α- and β-crystallins.

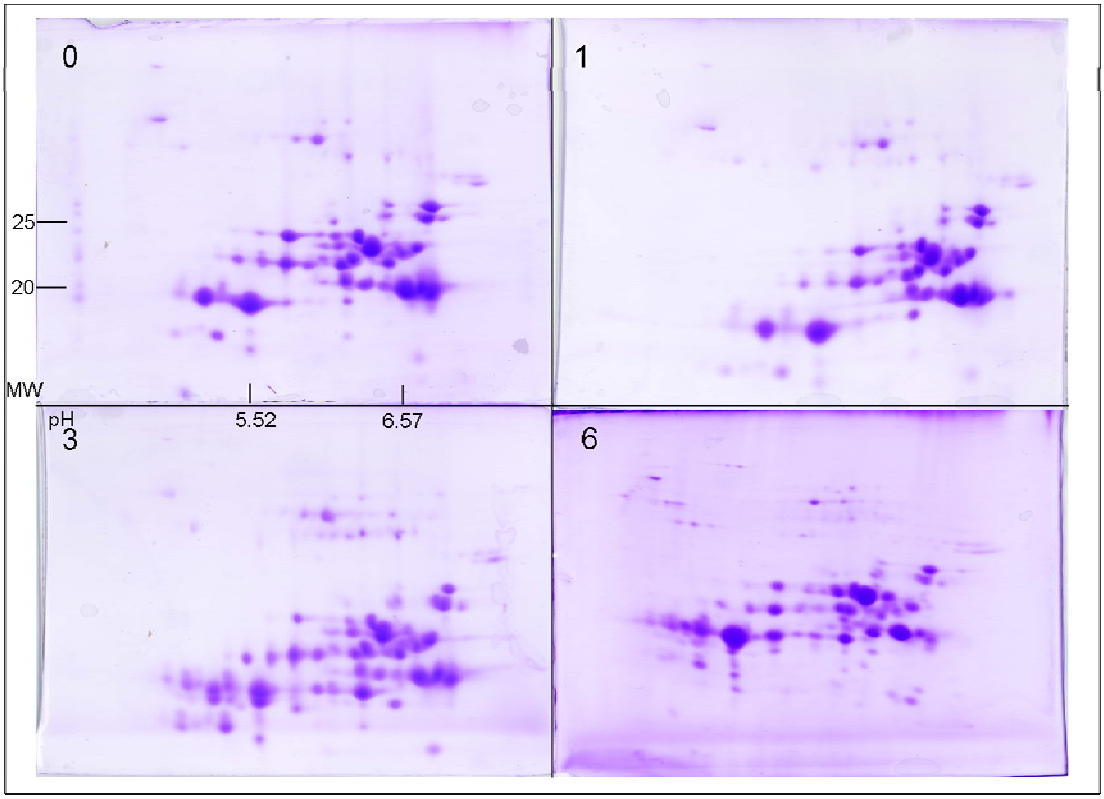

Figure 4-10 2DE of WSF from cataractous lenses 0) Normal lens, 1) Stage 1 cataractous lens, 3) Stage 3 cataractous lens and 6) Stage 6 cataractous lens. Molecular weight (MW) in kDa and pH range are as indicated for gel 0 only.

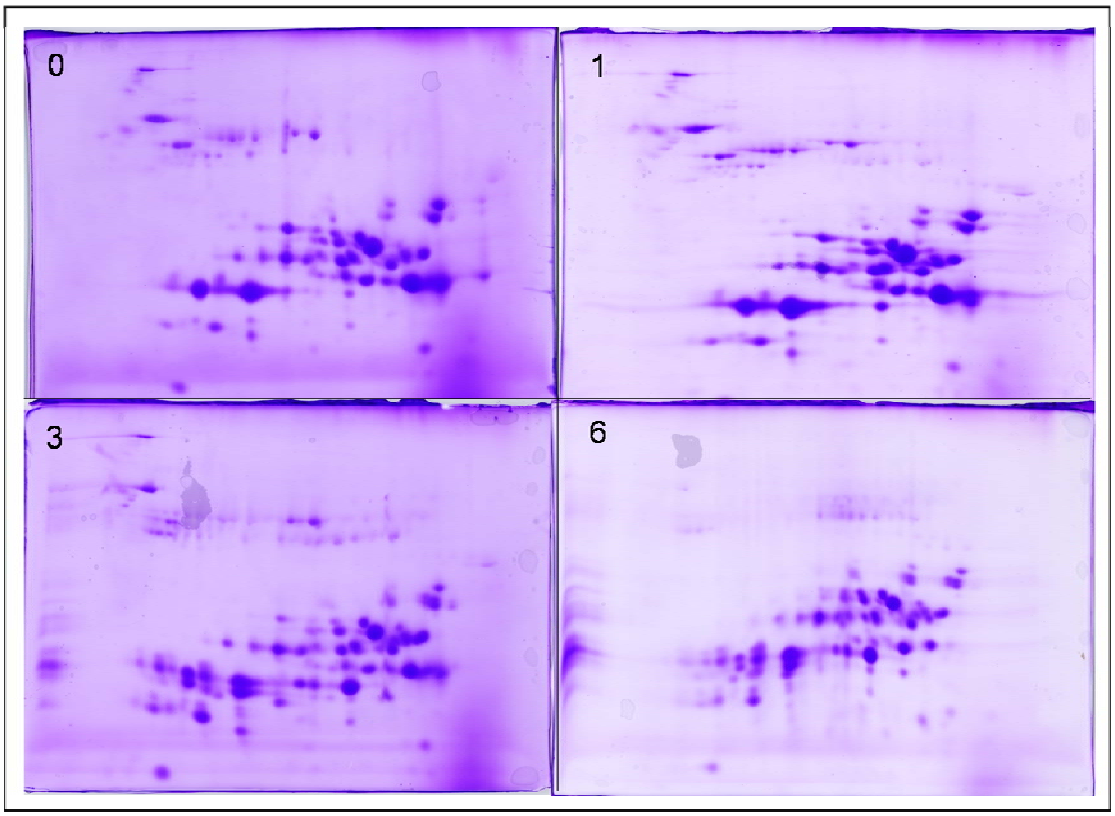

Figure 4-11 2DE of the USF from cataractous lenses 0) Normal lens, 1) Stage 1 cataractous lens, 3) Stage 3 cataractous lens, 6) Stage 6 cataractous lens.

4.3.4.1 α-crystallin changes with cataract formation

There are modifications to the α-crystallins as the cataract progresses from normal (0) through to full cataract (6) (Figure 4-12, Figure 4-13 and Table 4-5).

At stage 1 of the cataract there are few changes in the profile of the α-crystallins. The only visible change is an increase in $\alpha B_{1\text{-}170}$ (A5). The equivalent truncation removing 5 amino acids from the C-terminus of αA is not seen however that may be due to the large intact αA spot overshadowing the smaller truncated product.

By stage 3 of cataract formation there is extensive truncation and modification of the α-crystallins. The most abundant modification of αA is a truncation removing 5 amino acids from the C-terminus forming $\alpha A_{1\text{-}168}$ (A8). This truncation product is in equal amounts to its parent species (A2). A second new truncation seen at the stage 3 cataract is $\alpha A_{1\text{-}163}$ (A9). The $\alpha A_{1\text{-}151}$ (A7) truncation that is seen in the control lens is

increased in abundance at the stage 3 cataract. The amount of αA-phos (A1) is decreased in the stage 3 cataract and there is a truncated form of αA-phos missing 5 amino acids, αA-phos$_{1-168}$ (A17) present. There is increased deamidation of αA (A13) and αA-phos (A14) in the stage 3 lens compared with stage 1 and the control lens. The αB$_{1-170}$ truncation product (A5) is increased in the stage 3 cataract and is more abundant than its parent compound. There is a decrease in the amount of αB-phos (A3) in the stage 3 cataract and it is also truncated to form αB-phos $_{1-170}$ (A10). There is a second truncation of αB in the stage 3 cataract αB$_{1-163}$ (A11) missing 12 amino acids from the C-terminus. There is no change in the deamidated forms of αB (A15) and αB-phos (16) in the stage 3 cataract.

During progression from stage 3 to stage 6 cataract there are further changes in the α-crystallins in the WSF. The amount of αA-phos (A1) and its truncation product αA-phos$_{1-168}$ (A17) are reduced in the stage 6 cataract. There is an increase in deamidated αA (A13) and no changes in any other αA species. By stage 6 cataract there is no αB-phos (A3) present and reduced amounts of the truncation product αB-phos$_{1-170}$ (A10). There is an increase in the deamidated form of αB (A15) and an increase in the deamidated αB-phos (A16).

The α-crystallins are also modified in the USF as the cataract progresses (Figure 4-13). These changes are similar to those seen in the soluble fraction for α-crystallin. The only differences between the WSF and USF are seen in the stage 6 cataract. Both αA$_{1-163}$ (A9) and αA$_{1-154}$ (A6) are absent in the USF of the stage 6 cataract. There is a reduced amount of αB (A4) and increased amount of αB$_{1-170}$ (A5) in the USF of the stage 6 cataract compared with the USF in the other stages of cataract and with the WSF of the same lens.

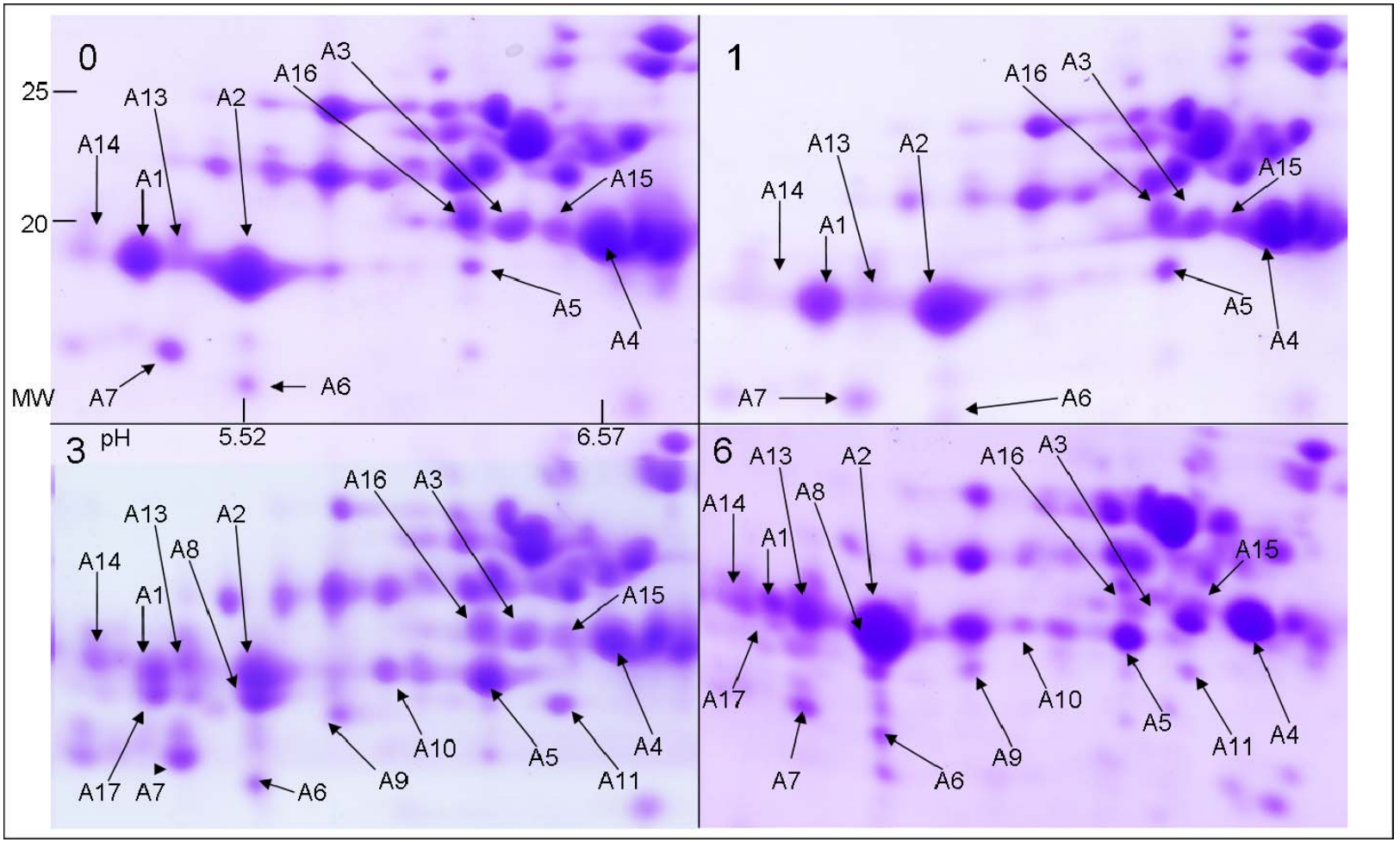

Figure 4-12 2DE of the WSF from cataractous lenses 0) Normal lens, 1) Stage 1 cataractous lens, 3) Stage 3 cataractous lens, 6) Stage 6 cataractous lens. Molecular weight (MW) in kDa and pH range are as indicated for gel 0 only.

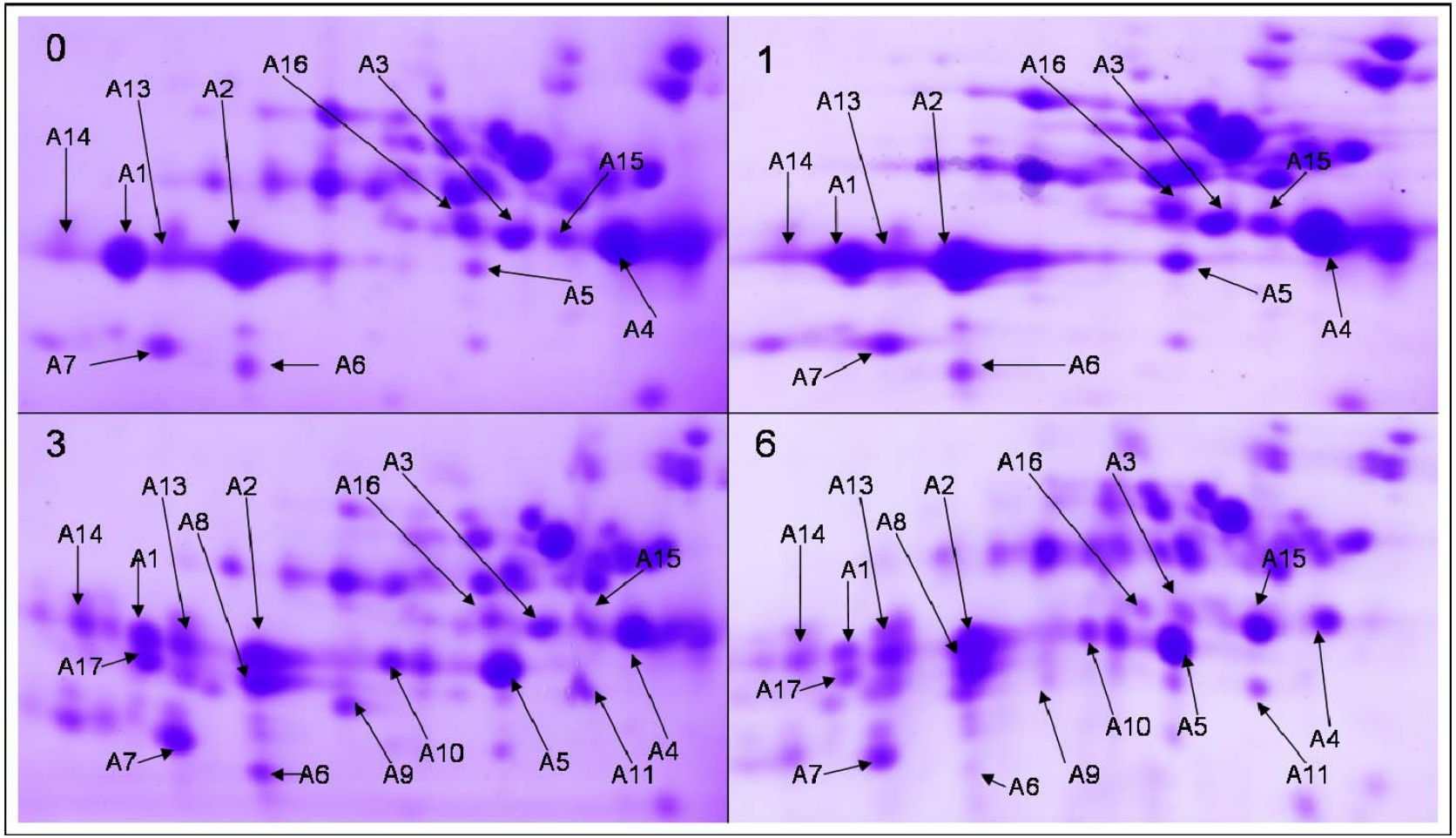

Figure 4-13 2DE of the USF from cataractous lenses 0- Normal lens, 1- Stage 1 cataractous lens, 3- Stage 3 cataractous lens, 6- Stage 6 cataractous lens.

Spot No	Crystallin	Mass (Da)	Predicted Mass(Da)	Modification
A1	αA	19954.4	19955.1	αA-phos
A2	αA	19873.1	19875.1	Intact
A3	αB	20187.5	20188.8	αB-phos
A4	αB	20108.6	20108.8	Intact
A5	αB	19614.3	19613.1	αB_{1-170} (-5aa)
A6	αA	17879.3	17879.0	αA_{1-154} (-19aa)
A7	αA?	17614.3	17613.7	αA_{1-151} (-22aa)
A8	αA	19446.0	19445.7	αA_{1-168} (-5aa)
A9	αA	18873.3	18875.1	αA_{1-163} (-10aa)
A10	αB	19692.7	19693.1	αB-phos $_{1-170}$ (+phos -5aa)
A11	αB	18860.0	18858.3	αB_{1-163} (-12aa)
A13	αA	N/A	19876.1	αA Deamidation
A14	αA	N/A	19956.1	αA-phos Deamidation
A15	αB	19980.3	19980.6	αB_{1-174}
A16	αB	N/A	20060.6	αB_{1-174}-phos
A17	αA	N/A	19526.7	αA-$phos_{1-168}$

Table 4-5 Table of modifications to soluble α-crystallins during cataract formation, Spot numbers correspond to Figure 4-12 and Figure 4-13. Predicted masses and modifications were made by comparison with the known ovine sequence (Robertson *et al.*, 2008).

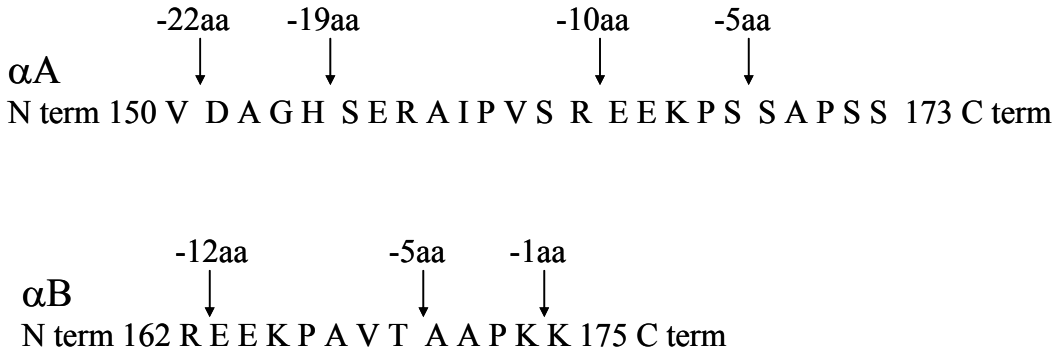

Figure 4-14 C-terminal regions of αA and αB-crystallin showing the points at which they are cleaved during ageing and cataract formation.

4.3.4.2 Changes in β-crystallins with cataract formation

The β-crystallins are modified during cataract formation (Figure 4-15 0-6) with truncation and deamidation being the main modifications. There is a decrease in the intact form of βB1 (1) during cataract formation. In the control lens there are three truncations of βB1, $\beta B_{16\text{-}252}$ (2), $\beta B1_{25\text{-}252}$ (21) and $\beta B1_{25\text{-}247}$ (22). All of these truncations decrease in the water soluble fraction as the cataract progresses. During cataract formation three other modifications of βB1 appear and increase as the cataract progresses but only two of these truncations could be determined, $\beta B1_{9\text{-}252}$ (24) and $\beta B1_{13\text{-}252}$ (26). These truncations first appear in the stage 3 cataract and are decreased in the stage 6 cataract. In the stage 6 cataract there is a reduced amount of βB1 in all forms.

The intact form of βB2 is the dominant β-crystallin in all cataract stages and the deamidated βB2 (7) increases as the cataract progresses. A truncated product of βB2 with 7 amino acids missing from the N-terminus (25), appears in the stage 6 cataract.

The intact form of βB3 (8) decreases as cataract forms, and the deamidated form of βB3 (7) stays consistent throughout formation. The only truncation of βB3, with 9 amino acids from the N-terminus missing (11), stays consistent throughout cataract formation.

With cataract progression there is a decline in the intact form of βA3 (4). The two βA3 truncations seen in the control lens, $\beta A3_{12\text{-}215}$ and $\beta A3_{23\text{-}215}$ (12 and 13, respectively), stay at consistent levels in stage 1 and 3 cataracts and decrease in stage 6 cataract. Both βA1 (15) and βA2 (16) decrease during cataract formation and βA4 (18) stays the same throughout cataract formation.

In the USF there are changes to the β-crystallins which are similar to those seen in the WSF of the cataract lenses (Figure 4-15 and Figure 4-16). The only differences are in the USF of the stage 6 cataract, where βA3 (4) and $\beta B2_{8\text{-}204}$ are absent in the USF.

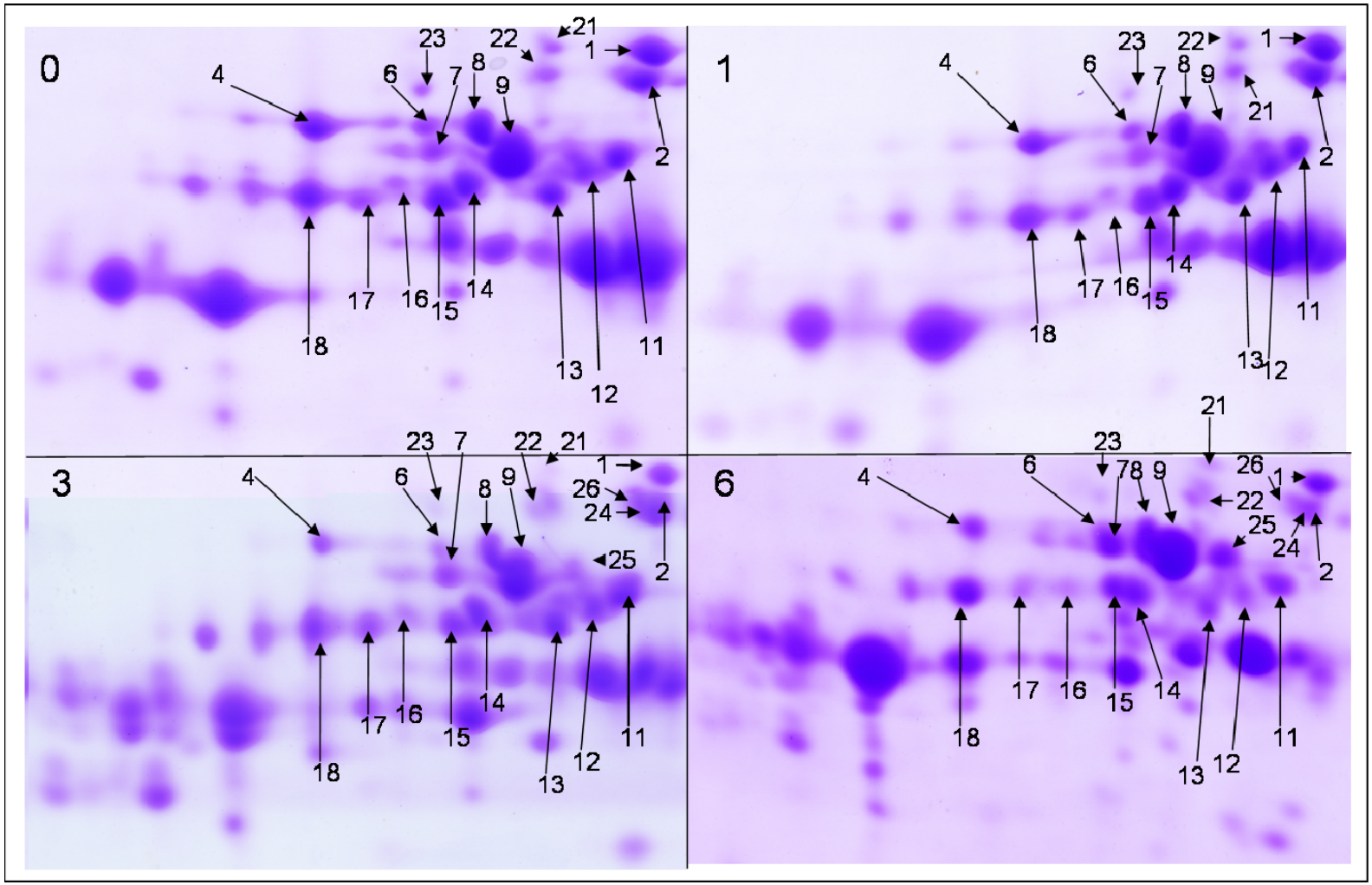

Figure 4-15 2DE of the WSF from cataractous lenses 0) Normal lens, 1) Stage 1 cataractous lens, 3) Stage 3 cataractous lens, 6) Stage 6 cataractous lens.

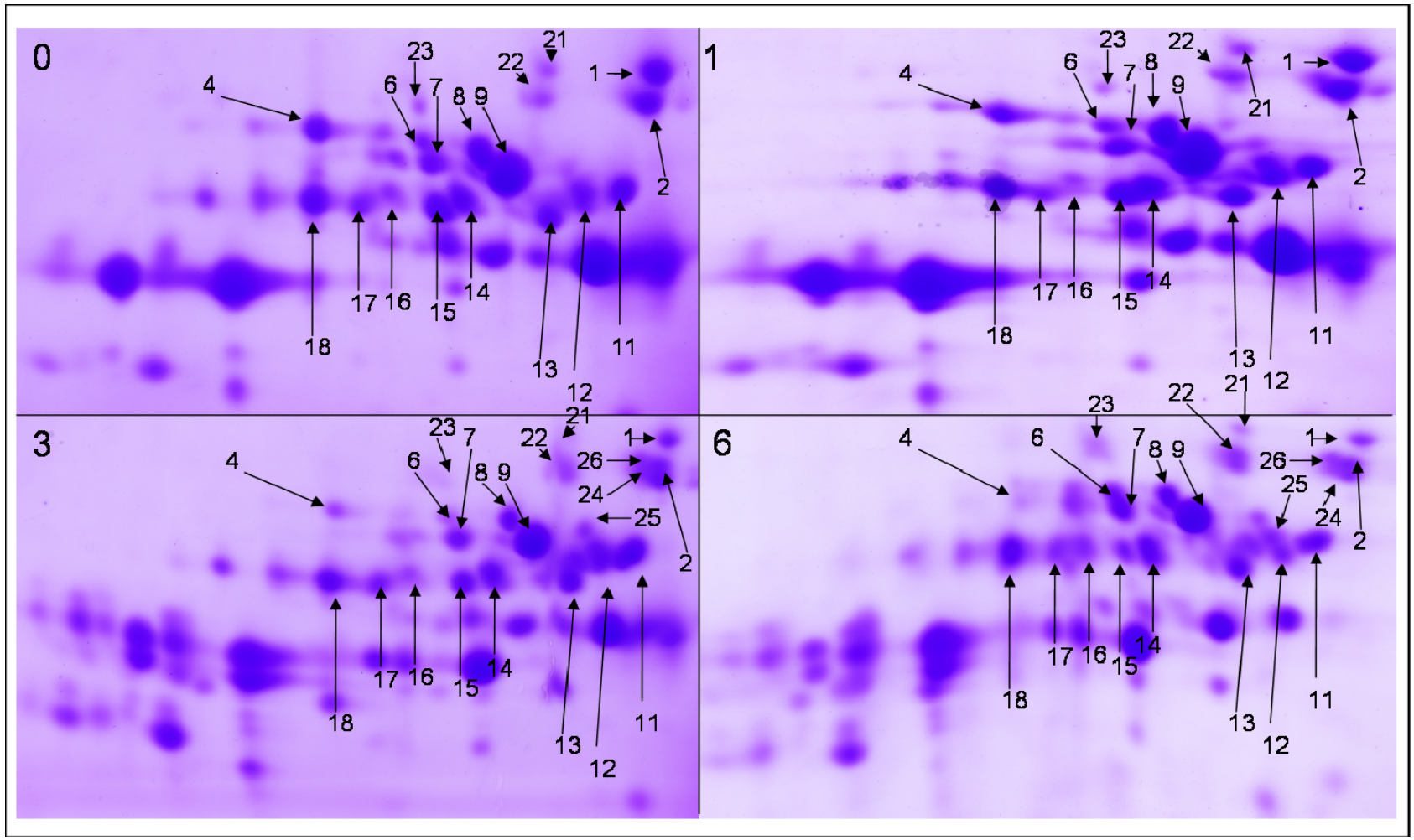

Figure 4-16 2DE of the USF from cataractous lenses 0) Normal lens, 1) Stage 1 cataractous lens, 3) Stage 3 cataractous lens, 6) Stage 6 cataractous lens

Spot	Protein (ID)	Mass	Bovine Equivalent
1	βB1	28055.9	βB1
2	βB1	26743.0	$\beta B1_{16-252}$
4	βA3	25572.3	βA3
5	βA3/βA1	25756.3	N/A
6	βB3	24323.8	βB3 Deamidation
7	βB2	23322.8	βB2 Deamidation
8	βB3	24321.9	βB3
9	βB2	23321.3	βB2
11	βB3	23302.4	$\beta B3_{10-210}$
12	βA3/βA1	24212.1	$\beta A3_{12-215}$
13	βA3/βA1	23038.2	$\beta A3_{23-215}$
14	βA3/βA1	23497.3	βA1
15	βA2	22387.2	βA2
16	βA3/βA1	23496.6	βA1
17	βA4	22879.1	βA4 Mod
18	βA4	22696.4	βA4
21	βB1	25935.3	$\beta B1_{25-252}$
22	βB1	25414.9	$\beta B1_{25-247}$
23	βB1	27199.8	$\beta B1_{10-252}$
24	βB1	27358.7	$\beta B1_{11-252}$
25	βB2	N/A	$\beta B2_{8-204}$
26	βB1	27028.6	$\beta B1_{13-252}$

Table 4-6 Table of modifications to soluble β-crystallins during cataract formation, Spot numbers correspond to Figure 4-15 and Figure 4-16. Bovine equivalents were determined by comparison with Shih *et al.,* (1998) and personal communication with L. David, 2005. N/A = not available.

4.4 Discussion

This study focused on the ovine lens crystallins, their masses and post translational modifications using 2DE and mass spectrometry techniques. This allowed the visualisation and determination of the changes in the crystallins during ageing and cataractogenesis in both the WSF and USF of the lens. The described research

expands on early work by Robertson (2005), where normal and cataract lenses were compared by 2DE. This work investigated the changes during normal ageing of the lens and the changes at each stage of cataractogenesis.

In the fetal lens there was the full complement of the α and β-crystallins; αA, αB, βB1, βB2, βB3, βA1, βA3, βA2 and βA4 (Figure 4-6 and Figure 4-7) which are the same crystallins found in newborn mouse (Ueda *et al.,* 2002a), 16 day old rat (Lampi *et al.,* 2002b), bovine (Takemoto, 1995b, Shih *et al.,* 1998) and human (Lampi *et al.,* 1998, Lapko *et al.,* 2003) lenses.

4.4.1 α-Crystallin modification during lens ageing and maturation

With increasing age both αA and αB undergo post-translational modification including phosphorylation, truncation and deamidation.

Phosphorylated forms of αA and αB were present at all ages in ovine lenses. Phosphorylated α-crystallins have also been detected in human (Miesbauer *et al.,* 1994, Takemoto, 1996, Ma *et al.,* 1998), mouse (Ueda *et al.,* 2002a), rat (Wang *et al.,* 2000) and bovine (Carver *et al.,* 1996) lenses. Ovine αA is phosphorylated at serine 122 (Robertson *et al.,* 2008). The site of phosphorylated αB has not been determined in ovine lens. However, both human (Miesbauer *et al.,* 1994) and bovine (Voorter *et al.,* 1989) αB have three phosphorylation sites at serine residues 19, 45 and 59, these serines are found in ovine αB-crystallin.

αA becomes increasingly phosphorylated as the lens ages in both the WSF and USF of the lens, so much so that by 8 years of age αA+phos is the prominent form of αA-crystallin in the lens. A similar pattern has been observed in the ageing of mouse lens with phosphorylated αA comprising one third of total αA by 51 weeks of age (Ueda *et al.,* 2002a). αB-crystallin phosphorylation also increased with age but not to the same extent as αA. Consequently the level of phosphorylated αB was less than that of normal αB. In the mouse lens phosphorylated αB does not increase after 10 weeks of age (Ueda *et al.,* 2002a). In human lenses no phosphorylation was detected in infant lens (up to 4 months of age) but phosphorylation was detected in 12 year old lenses

and increased as the lens aged (Takemoto, 1996). There are two mechanisms for phosphorylation detected in the lens, a cAMP-dependent kinase pathway and both α-crystallins also show autokinase activity (Kantorow and Piatigorsky, 1998). There is evidence that the phosphorylation may be reversible as indicated by the discovery of a lens phosphatase (Chiesa and Spector, 1989).

Phosphorylation is important as it can alter the oligomerisation (Ito *et al.,* 2001) and chaperone activity (Kamei *et al.,* 2001, Ecroyd *et al.,* 2007) of the α-crystallins. There is evidence that phosphorylation of αB decreases its chaperone ability by up to 30% *in vitro* (Kamei *et al.,* 2001, Aquilina *et al.,* 2004, Ecroyd *et al.,* 2007). However research into the effect of phosphorylation of αA on its chaperone ability has been contradictory. Some groups report that phosphorylation has no effect on the chaperone function of αA (Wang *et al.,* 1995, Carver *et al.,* 1996), whilst others have found an increase in chaperone activity (van Boekal *et al.,* 1996). More research is needed to determine (i) the exact role of phosphorylation of α-crystallin in the lens, (ii) why αA continues to become phosphorylated in the aged ovine lens and (iii) why αB phosphorylation does not increase as the lens ages.

Truncation of α-crystallins during lens maturation is well documented in rat (Ueda *et al.,* 2002b), mouse (Ueda *et al.,* 2002a), cow (Takemoto, 1995b) and human lenses (Takemoto, 1995b, Lund *et al.,* 1996, Ma *et al.,* 1998). In this study there was only minor truncation of the α-crystallins, with only three truncations of αA being identified. They are: $\alpha A_{1\text{-}154}$, $\alpha A_{1\text{-}151}$ and $\alpha A_{1\text{-}157}$. All three of these truncations were present in the fetal and 6 month old lens but only $\alpha A_{1\text{-}157}$ and $\alpha A_{1\text{-}154}$ were present in the 8 year old lens. The $\alpha A_{1\text{-}151}$ modification is found in several other animal lenses during maturation. For example $\alpha A_{1\text{-}151}$ is in 16 day, 6 week old (Ueda *et al.,* 2002b) and 85 day old lenses from rats (Takeuchi *et al.,* 2004). $\alpha A_{1\text{-}151}$ has also been found in two year old bovine lenses (Smith *et al.,* 1991). $\alpha A_{1\text{-}154}$ missing 19 residues from the C-terminus of αA crystallin, has previously been detected in 85 day old rat (Takeuchi *et al.,* 2004) and 2 year old bovine lenses (Smith *et al.,* 1991). The $\alpha A_{1\text{-}157}$ truncation, missing 16 residues from the C-terminus, is only a minor component in ovine lenses of all ages. The same modification has been found in 16 day and 6 week old rat lenses (Ueda *et al.,* 2002b) as well as 85 day old rats (Takeuchi *et al.,* 2004).

These were the only αA-crystallin truncations that could be detected in the ovine lens during ageing. Other αA-crystallin truncations found during maturation in other species include $\alpha A_{1\text{-}172}$ and $\alpha A_{1\text{-}168}$, which were found in human (Takemoto, 1995b, Lund *et al.,* 1996), rat (Ueda *et al.,* 2002b) and bovine (Takemoto, 1995b) lenses. Within the rat lens, $\alpha A_{1\text{-}163}$ and $\alpha A_{1\text{-}162}$ have also been found (Ueda *et al.,* 2002b, Takeuchi *et al.,* 2004) during ageing. None of these truncations were found in the ovine lens. This maybe due to the detection sensitivity limitations of the electrophoretic and mass spectrometric techniques used in this study. $\alpha A_{1\text{-}172,}$ and $\alpha A_{1\text{-}168}$ may have comigrated with $\alpha A_{1\text{-}173}$ in the 2DE, and been overshadowed by the intact αA. In some of the above studies these two truncations were detected by direct mass spectrometry of the lens sample without prior separation on 2DE as was the case here.

Within normal ovine lens the only truncation of αB observed was $\alpha B_{1\text{-}170,}$ removing 5 residues from the C-terminal region. This modification was seen only in the fetal and 6 month old lenses. The same truncation product is found in human (Colvis *et al.,* 2000) and rat (Ueda *et al.,* 2002b, Takeuchi *et al.,* 2004) lenses. $\alpha B_{1\text{-}174}$ has been found in human (Lund *et al.,* 1996, Colvis *et al.,* 2000) and rat (Takeuchi *et al.,* 2004) lenses. A second αB truncation $\alpha B_{1\text{-}163}$ has also been detected in human (Colvis *et al.,* 2000) and rat (Ueda *et al.,* 2002b, Takeuchi *et al.,* 2004) lenses. Neither of these truncations were detected in the ovine lens at any age.

The C-terminal region of the α-crystallins is important for chaperone function (Takemoto *et al.,* 1993, Smulder *et al.,* 1996). For further discussion on chaperone function and the C-terminal extension of α-crystallin see 1.4.2. Briefly modifications that affect the C-terminal region of α-crystallins can alter the chaperone function of α-crystallin within the lens. In particular truncation of the C-terminal region reduces the chaperone ability of α-crystallin (Takemoto *et al.,* 1993).

Although truncation of the C-terminal region of the α-crystallins reduces their chaperone function, there is only a limited amount of truncation of both α-crystallins during ovine lens ageing. Therefore the majority of α-crystallin within the aged lens is in the intact form and would function as a "normal" chaperone and so prevent the insolubilisation of other proteins in the lens. However during cataract formation there

is an increase in truncation of the α-crystallins which may reduce the chaperone function within the lens.

4.4.2 α-Crystallin modification during cataract formation

During cataract formation there is extensive truncation of the α-crystallins. At stage 1 cataract there is little change to αA-crystallin profile in either the WSF or the USF. However by stage 3 cataract there is extensive truncation of αA-crystallin. The major truncation seen in the stage 3 cataract is $\alpha A_{1\text{-}168}$ where αA has lost the 5 C-terminal residues. This same modification is one of the major αA-crystallin truncations seen in the rat selenite cataract (Ueda *et al.,* 2002b) and is also found in the ICR/f rat cataract (Takeuchi *et al.,* 2004). Interestingly $\alpha A_{1\text{-}168}$ has been found in the aged lens of human, bovine and rat lenses, but not the ovine lens (See 4.4.1 for further discussion). Another new truncation seen in the stage 3 cataract is $\alpha A_{1\text{-}162}$ missing 10 amino acids from the C-terminus. This protein species was only a minor truncation seen in the stage 3 cataract. This modification has been observed in two types of rat cataract, the selenite (Ueda *et al.,* 2002b) and ICR/f hereditary cataract (Takeuchi *et al.,* 2004), and in aged human, bovine and rat lenses (4.4.1). $\alpha A_{1\text{-}151}$ is found in aged ovine lens and the amount of this protein species increased in the stage 3 cataract. The phosphorylated form of αA also underwent truncation in the stage 3 cataract, at the same truncation sites as the unmodified αA to produce $\alpha A_{1\text{-}168}$+phos, $\alpha A_{1\text{-}162}$+phos and $\alpha A_{1\text{-}151}$+phos.

With progression from stage 3 to stage 6 cataract there are a number of changes in the αA-crystallin profile. There was a decrease in the truncated forms of αA within the stage 6 cataract with the most notable changes being a decrease in the amount of αA+phos and $\alpha A_{1\text{-}151}$ observed in the WSF and USF. $\alpha A_{1\text{-}162}$ and $\alpha A_{1\text{-}154}$ are not present at all in the USF. These observed differences could be attributed to the physical loss of cortical sample when dissecting stage 6 lenses. That is, the cortical region of the stage 6 lens is almost entirely liquid and the nucleus is normal (solid). Consequently when dissecting the lens there is some loss of the cortical region of the lens, which would reduce the amount of cortical proteins compared to the nuclear proteins within the sample.

There is also increased truncation of αB during cataract formation. Three truncations are seen in αB during cataract formation, they are $\alpha B_{1\text{-}174}$, $\alpha B_{1\text{-}170}$ and $\alpha B_{1\text{-}163}$. The only truncation seen in early cataract (stage 1) is $\alpha B_{1\text{-}170}$, and this truncation increases in abundance as the cataract progresses, and $\alpha B_{1\text{-}170}$ is the main form of αB found within the USF of a stage 6 cataract. The equivalent truncation is found in the phosphorylated form. This truncation is found in cataractous lenses from selenite (Ueda *et al.,* 2002b) and ICF/r rats (Takeuchi *et al.,* 2004), as well as from human lenses with Soemmering's ring (Colvis *et al.,* 2000), which is a phenomenon that occurs after cataract surgery. At stage 3 of cataract formation, $\alpha B_{1\text{-}163}$ is present. This modification has also been detected in selenite (Ueda *et al.,* 2002b) and ICR/f (Takeuchi *et al.,* 2004) rat cataracts and human Soemmering's ring (Colvis *et al.,* 2000). The final modification of αB is the removal of the C-terminal lysine ($\alpha B_{1\text{-}174}$) which is seen in the final stage of cataract development. This modification has been found in aged and cataractous human lenses (Lund *et al.,* 1996, Colvis *et al.,* 2000).

4.4.3 Chaperone function of α-crystallin

One of the important features of α-crystallin is its ability to function as a molecular chaperone, where it binds to denatured proteins and prevents them aggregating and being insolublised. During bovine lens ageing there is a decrease in the chaperone function of α-crystallin (Augusteyn *et al.,* 2002).

In this study there is an increase in phosphorylation of α-crystallin within the lens during ageing. However the role of phosphorylation in chaperone function is not entirely understood with some research reporting no change (Wang *et al.,* 1995, Augusteyn *et al.,* 2002) and others reporting an increase (Ecroyd *et al.,* 2007) or decrease (Aquilina *et al.,* 2004) in chaperone activity. The effect of increased phosphorylation of α-crystallins within the ovine lens is therefore unclear.

Many studies have shown the importance of the C-terminal region of α-crystallin to chaperone function. During ovine cataract formation αA-crystallin was found to be truncated at the C-terminus. Previous studies have shown that removal of C-terminal

residues dramatically alters the chaperone function of α-crystallin (Kelley *et al.,* 1993, Takemoto *et al.,* 1993, Takemoto, 1994). Kelley *et al.,* (1993) showed that in the selenite cataract there is truncation of the α-crystallin and that this truncation resulted in a reduction of the chaperone function of α-crystallin. In the ovine cataract there were truncations that produced $\alpha A_{1\text{-}168}$, $\alpha A_{1\text{-}163}$, $\alpha A_{1\text{-}154}$ and $\alpha A_{1\text{-}151}$. The effect of some of these truncations on chaperone function has been determined. The major αA modification in the cataractous lens is the $\alpha A_{1\text{-}168}$ truncation, which has been shown to reduce the chaperone function by 12-23% (Aziz *et al.,* 2007). As more residues (from 5-11) are removed from the C-terminus there is greater reduction of the chaperone function. For example, $\alpha A_{1\text{-}162}$ showed a 65% reduction in chaperone protection (Rajan *et al.,* 2006). The observation that there is increased truncation in cataract lens and that C-terminal truncation of α-crystallin reduces chaperone function suggests that within the ovine lens there is a decrease in chaperone function during cataract formation. This reduction in chaperone function could result in an increase in insolubilisation and aggregation of lens proteins and as a consequence promote cataract development.

4.4.4 β-Crystallin modification during lens ageing and maturation

The β-crystallins role in the lens is to provide structure. They pack tightly and generate a refractive index for the lens to focus light. During ageing of the lens the β-crystallins undergo many post-translational modifications including truncation and deamidation, which assist in the functioning and maturation of the lens. Within the ovine lens all 7 β-crystallins were found; βB1, βB2, βB3, βA1, βA2, βA3 and βA4. The same 7 crystallins are found in mice (Ueda *et al.,* 2002a), rat (Lampi *et al.,* 2002b) and bovine (Shih *et al.,* 1998) lenses. Early studies of human lenses showed that only 6 β-crystallins were present as βA2 was not detected (Lampi *et al.,* 1998, Ma *et al.,* 1998). However later work identified βA2 in the human lens (Lapko et al., 2003). At present only one of the ovine lens crystallins (βB3) has been sequenced (Robertson *et al.,* 2008). Protein identification in this study was therefore performed by comparison to the bovine crystallin sequences. This was able to be performed because of the closeness of the bovine and ovine genomes. For example, ovine and bovine βB3 are both 210 amino acids long and have masses of 24352 Da and 24322

Da respectively. Also the mass of bovine βB1 is 28054.5 Da and the measured mass of ovine βB1 is 28055.9 Da suggesting that they have identical protein sequences with 1-252 residues.

Within the fetal lens there is already truncation of βB1 with the loss of residues from the N-terminus to produce $\beta B1_{16\text{-}252}$, $\beta B1_{25\text{-}252}$ and $\beta B1_{10\text{-}252}$. By 6 months of age another truncation appears when $\beta B1_{25\text{-}252}$ loses 5 residues from its C-terminal end to form $\beta B1_{25\text{-}197}$. The major βB1 modification in the ovine lens is βB1 missing 15 residues from the N-terminus. This is also found in human (David *et al.,* 1996, Lampi *et al.,* 1997, Zhang *et al.,* 2003) and bovine (Shih *et al.,* 1998) lenses. During maturation of the ovine lens the intact βB1 decreases in abundance and the truncation product $\beta B1_{16\text{-}252}$ increases in abundance. This is also observed during bovine (Shih *et al.,* 1998) and human (Ma *et al.,* 1998) lens maturation. This would suggest that this truncation is "normal" and is required for lens maturation. In human lenses there are more truncations of βB1 between residues 33 and 41 (David *et al.,* 1996, Lampi *et al.,* 1997), and in the rat there have been truncations of βB1 from 27-49 residues (David *et al.,* 1994). None of these truncations were found in the ovine lens during maturation. Loss of the N-terminus of βB1 results in a reduction in the βH aggregate which is an important part of the lens proteome (Lampi *et al.,* 1998, Ma *et al.,* 1998). Removal of the entire N-terminus of βB1 resulted in an increased rate of heat-induced denaturation which meant more αA-crystallin was required to prevent denaturation than wildtype βB1 (Lampi *et al.,* 2002a).

βB3 is the dominant β-crystallin in the fetal lens. However as the lens ages there is a decrease in the amount of βB3 within the WSF and USF. During ageing of the bovine lens the same results were observed with a decrease in βB3 occurring (Shih *et al.,* 1998). Within the rat lens there is a decrease in βB3 gene expression after birth (Aarts *et al.,* 1989) and during mouse lens maturation there is decrease in βB3 during ageing (Ueda *et al.,* 2002a). Within the human lens βB3 is only detected in lenses aged less than 3 years (Ma *et al.,* 1998). There are two post-translational modifications in the ovine fetal lens, deamidation and truncation. The truncation between residues $^{9}Q/A^{10}$ of βB3 which produces $\beta B3_{10\text{-}210}$ in the ovine lens has also been found in rat lenses (David *et al.,* 1994). A βB3 spot in a similar position was found in the bovine lens but the truncation could not be confirmed by Edman sequencing as it contained a

blocked N-terminus (Shih *et al.,* 1998). Deamidation of βB3 was evident in the fetal lens by a mass shift of +1 and a positional shift on the 2DE to be more positive.

The sequence of ovine βA3 has not been determined. However the N-terminal sequence has been discovered by a BLAST search of the sheep genome (J Wood, Personal communication, 2008), which means that any truncation to the N-terminus should be detectable. There were two truncations of βA3 found in the fetal lens, $\beta A3_{12\text{-}215}$ and $\beta A3_{23\text{-}215}$. In the ovine lens these two βA3 species have been identified in similar positions on 2DE to those found in the bovine lens (Shih *et al.,* 1998, Werten *et al.,* 1999) which confirms the ovine truncations. These same truncations to βA3 have been found in bovine (Shih *et al.,* 1998), rat (David *et al.,* 1994) and mouse (Ueda *et al.,* 2002a) lenses. Only $\beta A3_{23\text{-}215}$ has been found in human lenses (Lampi *et al.,* 1997, Ma *et al.,* 1998). During maturation of the ovine lens there is a decrease in βA3 in the lens which also happens in the bovine lens (Werten *et al.,* 1999). This decrease in intact βA3 sees an increase in $\beta A3_{12\text{-}215}$ and $\beta A3_{23\text{-}215}$ at 6 months of age. Interestingly, in the 8 year old lens there is little βA3 and $\beta A3_{12\text{-}215}$ and the amount of $\beta A3_{23\text{-}215}$ is increased. This situation is mirrored in the bovine lens (Werten *et al.,* 1999). Any discussion of truncation of βA3 larger than 17 amino acids needs to consider βA1. Both βA3 and βA1 are translated from the same βA3/A1 mRNA. The two proteins have start codons in the same reading frame which means that they are identical except for βA3 having an extra 17 amino acids at its N-terminus. Therefore the truncation $\beta A3_{23\text{-}215}$ could also be $\beta A1_{5\text{-}197}$. In the bovine lens there is only a small decrease in the βA1 protein during lens ageing and there is a total disappearance in βA3. Therefore, it has been suggested that $\beta A3_{23\text{-}215}$ arises from truncation of βA3 and not βA1 (Werten *et al.,* 1999). Within the ovine lens there is no change in βA1 during ageing which means the same conclusion could be reached about the origins of the $\beta A3_{23\text{-}215}$ truncation. Truncation of βA3 results in a protein species that is more prone to association and has a higher affinity for association (Sergeev *et al.,* 2000, Zhang *et al.,* 2003).

βB2 is the most prominent β-crystallin within the ovine lens, there is little deamidation of this protein during lens ageing which is similar to what is seen in human lenses (Ma *et al.,* 1998). There was no truncation of βB2 recorded in this

study. In contrast, the bovine lens shows a truncation of βB2 missing 8 residues in the aged lens (Shih *et al.,* 1998).

The changes in the profile of the ovine β-crystallins with age appear to be consistent with those found in rat, mouse, bovine and human lenses. This suggests that the maturation of the crystallins, like the crystallins themselves, is a well conserved process amongst species. The purpose of the numerous post-translational modifications to the β-crystallins during lens ageing is thought to help in the packing of the lens proteins. That is, removal of the N-terminal extensions of the β-crystallins allows the crystallins to pack tighter together as the lens ages, which contributes to setting up the refractive index within the lens.

4.4.5 β-Crystallin modification cataract formation

During ovine cataract formation there are changes in the β-crystallins within the lens. Interestingly many of these changes are the same as those observed during the ageing of the ovine lens. During cataractogenesis there is a loss of βA3 which is also seen in aged ovine lenses. What is interesting to note is that there is no increase in the two truncation products of βA3 ($\beta A3_{12\text{-}215}$ and $\beta A3_{23\text{-}215}$), suggesting that these are truncated in the ovine cataract to smaller products or that the reduction in intact βA3 is via a different mechanism in cataractous lenses. In human cataractous lenses there is an increase in βA3 fragments (Harrington *et al.,* 2004). During selenite cataract formation there is an increase in the truncation of βA3, between residues 11/12, and 21/22 (David and Shearer, 1993, David *et al.,* 1993). Overall the changes seen to βA3 during cataractogenesis are the same as those seen during ageing and maturation of the lens. For example, the βA3 profile of a 6 month old cataractous lens is similar to an 8 year old normal lens.

One difference between normal, cataractous and aged lenses is the truncation of βB1. In aged and normal lenses the major truncation of βB1 is $\beta\beta1_{15\text{-}252}$ and this truncation decreases with cataract development. Within the cataract lens there are three truncations $\beta B1_{9\text{-}252}$, $\beta B1_{13\text{-}252}$, and an unknown truncation. None of these truncations are seen in aged matched controls. In human cataractous lenses there is an increase in

βB1 fragments compared with controls. In the human study the exact location of the truncation was not determined except that it did occur at the N-terminus (Harrington *et al.,* 2004). No other research has demonstrated these unique truncations seen in the ovine cataract in either normal or cataractous lenses.

βB2 is the main β-crystallin in the ovine lens. During ageing the only modification to βB2 is deamidation, but during cataract formation there is truncation of βB2 in the ovine lens. The truncation removes 7 residues from the N-terminus and a limited amount of $\beta B2_{8\text{-}204}$ was detected in the stage 3 cataract and increased in the stage 6 cataract. This truncation has been reported in aged mouse (Ueda *et al.,* 2002a) and bovine (Shih *et al.,* 1998) lenses only. Interestingly, in this study $\beta B2_{8\text{-}204}$ was only found in the cataractous lens.

N-terminal cleavage of rat β-crystallins results in an increase in turbidity and insolubilisation of the β-crystallins (David *et al.,* 1992).

Overall the changes in the ovine β-crystallins during cataract formation can be split into two types: ageing and cataract-specific changes. Ageing changes are those that are seen in an aged ovine lens and in the younger cataractous lens, suggesting that cataract formation is an advanced disease of ageing. The cataract-specific changes are modifications only seen in the cataractous lenses indicating that the ovine cataract is a specific disease and is independent of age. Therefore it appears that the ovine cataract is a combination of advanced ageing and specific cataract changes.

4.5 Conclusion

During ageing of the lens there is a specific pattern of post-translational modifications that occur to the lens proteins. This enables the lens fibre cells to age and mature without the ability to synthesis new proteins. The highly conserved nature of these modifications amongst lenses of the same species and between lenses of different species suggests that this process is essential for normal lens growth. When this process is disrupted in some way, it can lead to cataract formation. During cataract formation in the ovine lens there are patterns of modification to the β-crystallins that

are similar to those observed in the aged lens (8 year old). However the aged lens is completely transparent. In the aged lens there is a large amount of intact α-crystallin which can act as a chaperone and prevent the aggregation and insolubilisation of the modified β-crystallins. On the other hand in the cataractous lens, α-crystallins are truncated at the C-termini, which has been shown to reduce the chaperone function of the α-crystallins. Therefore a possible mechanism for cataract formation can be proposed. There is an increase in truncation of the crystallins. These truncated crystallins cannot be prevented from aggregating because of the reduced chaperone capability of α-crystallin and therefore the denatured crystallins become insoluble and as a consequence initiate the onset of cataractogenesis.

Chapter 5
Proteolysis of the Lens Proteins by the Calpains

5.1 Introduction

During cataract formation the lens proteins undergo uncontrolled proteolysis which disrupts their structure and function. In many cortical cataracts there is an increase in Ca^{2+} concentration within the lens (Duncan and Bushell, 1975, Hightower *et al.,* 1987, Robertson *et al.,* 2005). This has lead to the hypothesis that the increased calcium activates the calcium dependent cysteine proteases, (calpains) which then truncate the crystallins and this leads to cataract formation. In rodents the crystallins are particularly susceptible to uncontrolled enzymatic proteolysis by the calpain family (David and Shearer, 1984, David and Shearer, 1993, Ueda *et al.,* 2002b).

Calpains have been implicated in several models of cataract including the selenite (David and Shearer, 1984), galactose (Azuma *et al.,* 1995), diamide (Azuma and Shearer, 1992) and Shumiya (Inomata et al., 1997a) cataracts in rats and the heritable sheep cataract (Robertson *et al.,* 2005, Robertson *et al.,* 2008). In many of these models the calpains have been shown to cleave either the α or β-crystallins in a similar way to the truncated forms of the crystallins found in cataract.

Within the lens there are three ubiquitous calpains; calpain 2 (Yoshida *et al.,* 1984), calpain 1 (Roy *et al.,* 1983), and calpain 10 (Ma *et al.,* 2001). There are also two splice variants of calpain 3, Lp82 (Ma *et al.,* 1998b) and Lp85 (Ma *et al.,* 2000b) both of which are lens specific. So far Lp85 has only been found in rodent lenses. Calpain 1, 2 and Lp82 but not calpain 10 have been discovered in the ovine lens (Robertson *et al.,* 2005). The expression of the ovine calpains varies across the lens and with the age of the lens (Robertson, 2003). Calpain 2 is found in all fractions of the sheep lens and in lenses of various ages, however calpain 1 is only detected in the cortex of ovine lenses. Lp82 is found in both the cortex and nucleus of young lamb lenses (Robertson *et al.,* 2005). Calpain 10 has been found in rat, mouse and human lenses and is

concentrated in the epithelial cells (Ma *et al.,* 2001). Within the same study it was shown that calpain 10 mRNA levels increased during selenite cataract formation. No calpain 10 has been discovered in the ovine lens to date (personal communication, J. Wood, 2008).

Bovine and rat calpain 2 and Lp82 both truncate αA and αB-crystallin at the C-terminus *in vitro*. Calpain 2 removes 10 or 11 amino acids from the C-terminus of αA (Yoshida *et al.,* 1986, Ueda *et al.,* 2002b), Lp82 also removes 10 amino acids but not 11 from αA-crystallin. Lp82 has a second, unique, truncation site for αA removing 5 amino acids (Ueda *et al.,* 2002b). Both calpain 2 and Lp82 remove 5 amino acids from the C-terminus of αB-crystallin and calpain 2 also removes 12 residues (Yoshida *et al.,* 1986, Ueda *et al.,* 2002b). The removal of these amino acids from the C-terminal end by the calpains has been shown to reduce the chaperone function of α-crystallin (Kelley *et al.,* 1993).

The role of β-crystallins in the lens is to provide structure and the refractive index. In rats, calpain 2 has been shown to truncate the β-crystallins in a similar way to that seen in selenite cataract (David *et al.,* 1992, David and Shearer, 1993). Rat calpain 2 truncates βB1, βB3, βA3 and βA4-crystallins from rat lenses by removing residues from the N-terminus of these proteins (David and Shearer, 1993). When the sequences for the rat β-crystallins were determined the exact cleavage points could be identified and were found to correspond to the crystallins isolated from cataractous lenses (David *et al.,* 1993). Incubation of bovine β-crystallins with calpain 2 also produced cleavage at the N-termini of βB1, βB2, βB3 and βA3 (Shih *et al.,* 1998). Some of these truncation products were found in normal and aged bovine lenses.

Incubation of β-crystallins with calpain 2 resulted in the formation of insoluble proteins which could be isolated by centrifugation (David *et al.,* 1992). Increases in the proportion of insoluble protein are a hallmark of cataractous lenses.

The purpose of these experiments is to observe the effect ovine calpain 1, 2 and Lp82 have on ovine α and β-crystallin and cytoskeletal proteins and to determine the specific truncation points for each of these calpain isoforms.

5.2 Methods

5.2.1 Crystallin incubations

The purified calpains 1 and 2 (Chapter 2), were incubated with the purified crystallins (Chapter 3) according to Table 5-1. Two different controls were used with all the incubations; (i) a calcium control where the crystallins where incubated with calcium alone to determine if there was any endogenous protease activity in the crystallin samples, and (ii) a calpain control where the calcium chelator EDTA was added to the incubation mixture to determine if there was any non-calcium dependent protease activity. The calpain activity in each incubation was 82 Fluorescence Units (FU) (units released per minute using the BODIPY-FL casein assay) for calpain 1 and 164 FU for calpain 2.

	Calpain	Crystallin	Ca^{2+}	EDTA pH 7.5	Total Volume
Calpain	50µL	200µg	2mM	X	200µL
Calcium Control	X	200µg	2mM	X	200µL
Calpain Control	50µL	200µg	2mM	4mM	200µL

Table 5-1 Protocol for incubation of calpain 1 and 2 with the lens crystallins. Concentrations given are the final concentrations in the incubation.

The mixtures were incubated at 37ºC for 4 hours, the reaction was stopped with the addition of excess 100mM EDTA pH 7.5 and Minicomplete protease inhibitor (1 tablet per 10ml) (Roche, Manneheim, Germany).

The purified Lp82 still contained some residual α-crystallin after the final chromatography step (see Chapter 2). Therefore for the α-crystallin incubations, calcium was added directly, to a concentration of 2mM, to the Lp82 fraction which contained endogenous α-crystallin. The Lp82 fraction contained 22 FU of activity. This mixture was incubated at 37ºC for 4 hours and the reaction stopped as before. For the β-crystallin incubations 200µg of purified β-crystallin was added to the Lp82 and incubated with 2mM calcium. Calcium and calpain controls were as for calpain 1 and 2 (Table 5-1).

To visualise the changes in α- and β-crystallin profiles after the calpain incubations, all the samples were separated and visualised by 2DE (4.2.3)

5.2.2 Calpain 2 and Lp82 with cytoskeletal proteins

Lens cytoskeletal proteins were isolated from a 4 month old lamb lens. The lens was dissected into cortical and nuclear regions. The cortex was homogenised in 3ml/g of Buffer A containing Minicomplete protease inhibitor (Roche, Manneheim, Germany). The insoluble proteins were isolated by centrifugation at 15000 x *g* for 1 hr at 4ºC. The supernatant containing the soluble proteins was discarded and the pellet containing the insoluble protein was washed three times with Buffer A to remove any residual soluble proteins. To isolate the cytoskeletal proteins the pellet was resuspended by sonication in 6M Urea. The urea soluble fraction (USF) was isolated by centrifugation at 15000 x *g* for 1 hr at 4ºC. The USF, containing the cytoskeletal proteins, was removed and the protein concentration determined using the BCA assay (3.2.3.4).

To determine if Lp82 and calpain 2 can modify the lens cytoskeletal proteins, each was incubated with lens cytoskeletal proteins in the presence of calcium (Table 5-2). Calpain and calcium controls were performed as before (5.2.1). 164 FU of calpain 2 was used and 22 FU of Lp82. The reaction was started with the addition of calcium, and incubated for 4 hours at 37ºC. The reaction was stopped with the addition 12.5µL of six times SDS loading buffer and the samples boiled for 5 minutes to denature the proteins.

	Calpain	Cytoskeletal Proteins	Ca^{2+}	EDTA pH 7.5	Total Volume
Calpain (C2 or Lp82)	50µL	50µg	12mM	X	62.5µL
Calcium Control	X	50µg	12mM	X	62.5µL
Calpain Control	50µL	50µg	X	24mM	62.5µL

Table 5-2 Protocol for incubation of calpain 2 an Lp82 with the lens cytoskeletal proteins.

To visualise the changes in the cytoskeletal proteins 1-DE SDS-PAGE was used. Fifteen micrograms of each sample was loaded on to a 4-12% NuPAGE® Novex® Bis-Tris Mini Gel (Invitrogen, Ca., USA) along with a molecular weight marker (All Blue Prestained Marker®, BioRad, Ca., USA). The gel was run at 200V for 50 min in NuPAGE® MOPS Running buffer in a XCell *SureLock*™ Mini-Cell. After running the gel was stained with Simply Blue™ Stain (Pierce, Il., USA). Cytoskeletal proteins were identified by comparison to western blots by McDermott (2007).

5.2.3 Insolubilisation of lens crystallins

To determine if calpain 2 truncation of the crystallins could induce their insolubilisation, purified calpain 2 was incubated with whole soluble lens proteins in the presence of calcium and centrifugation used to pellet any insoluble protein produced.

Calpain 2 was purified as per chapter 2 but without using the Phenyl Sepharose column and using Buffer C (20mM MOPS, 0.1mM EGTA and 2mM DTT) during the final step. Proteins were isolated from both normal and cataractous lenses. The lenses were homogenised in 3ml/g of Buffer C and centrifuged at 8000 x *g* for 15 min to separate the soluble and insoluble lens proteins. A BCA assay was used to determine the protein concentrations. The soluble proteins from the normal lens were incubated with calpain 2 as per Table 5-3, the samples were incubated for 2 hours and the reaction terminated using excess 100mM EDTA pH 7.5. Controls were performed as before (see 5.2.1). After termination of the incubation the insoluble proteins were separated by centrifugation at 8000 x *g* for 15 min. The pellet containing the insoluble protein was washed three times with Buffer C, and then resolubilsed by sonication in Buffer C and the amount of insoluble protein was determined using the BCA assay.

	Calpain	Lens Proteins	Ca^{2+}	EDTA	Total Volume
Calpain 2	83µL	100µg	5mM	X	332µL
Calcium Control	X	100µg	5mM	X	332µL
Calpain Control	83µL	100µg	5mM	6mM	332µL

Table 5-3 Protocol for incubation of calpain 2 with the total soluble lens proteins.

To visualise the insoluble proteins produced after incubation 2DE was performed using the BioRad Criterion-XT system. First dimensional focusing used pH 3-10 non-linear 11cm IPG strips (BioRad, Ca., USA), 100µg of protein was rehydrated in 175µL of rehydration buffer (8M Urea, 2% CHAPS, 50mM DTT, 2% 3-10 Biolyte buffer (BioRad, Ca., USA) 2% Glycerol and 0.001% Bromophenol Blue) and added to each strip. Focussing was performed on a BioRad Protean IEF Cell for 33000Vh. Focussed IPG strips were stored at -20ºC until the second dimension was completed. Second dimension was performed on 12% XT-MES gels using the Criterion XT system (BioRad, Ca., USA) at 200 V for 45 min. The separated proteins were visualised with Simply Blue™ Stain (Pierce, Il.,, USA).

5.2.4 Protein identification and mass spectrometry analysis

Proteins were eluted from 2-DE gels and molecular mass determined as per (4.2.4)

5.3 Results

Incubation of calpain 1, 2 and Lp82 with the lens proteins resulted in truncation of some of the lens proteins.

5.3.1 Calpain 2

Purified calpain 2 (Chapter 2) was incubated with purified α- and β-crystallins (Chapter 3). This resulted in truncation of both the α- and β-crystallins.

The incubation of calpain 2 with α-crystallin produced 15 truncations which were visible on 2DE (Figure 5-1 and Table 5-4). Proteolysis of αA crystallin by calpain 2 produced 6 unique protein spots (CA1-6). The truncations removed 10-28 residues from the C-terminus of αA_{1-73}, the truncations seen were αA_{1-162} (CA1), αA_{1-157} (CA2), αA_{1-154} (CA3), αA_{1-151} (CA5) and αA_{1-145} (CA6). Calpain 2 also cleaved the

phosphorylated form of αA-crystallin 6 times (CA7-12). αA_{1-163}+phos (CA12) was the only mass that could be measured, the masses for all of the other protein spots could not be determined directly. However, the mass and modification (Table 5-4) could be predicted from the equivalent protein species in the unphosphorylated form of αA, eg CA9 can be identified by comparison to CA3. Therefore all the modifications seen with the intact αA were also seen with αA+phos. The most prevalent truncation, based on size and intensity of the protein spot on 2DE, of αA and αA+phos were αA_{1-163} (CA4) and αA_{1-154} (CA3), which were missing 10 and 19 amino acids respectively

Calpain 2 truncated αB_{1-175} in two places, αB_{1-170} and αB_{1-163} (CA14 and CA15), missing 5 and 12 residue respectively from the C-terminus. The phosphorylated form of αB was also truncated by 5 residues (CA13). Calpain 2 truncated almost all of the αB crystallin so that only a small amount of intact αB and no αB-phos was present after incubation.

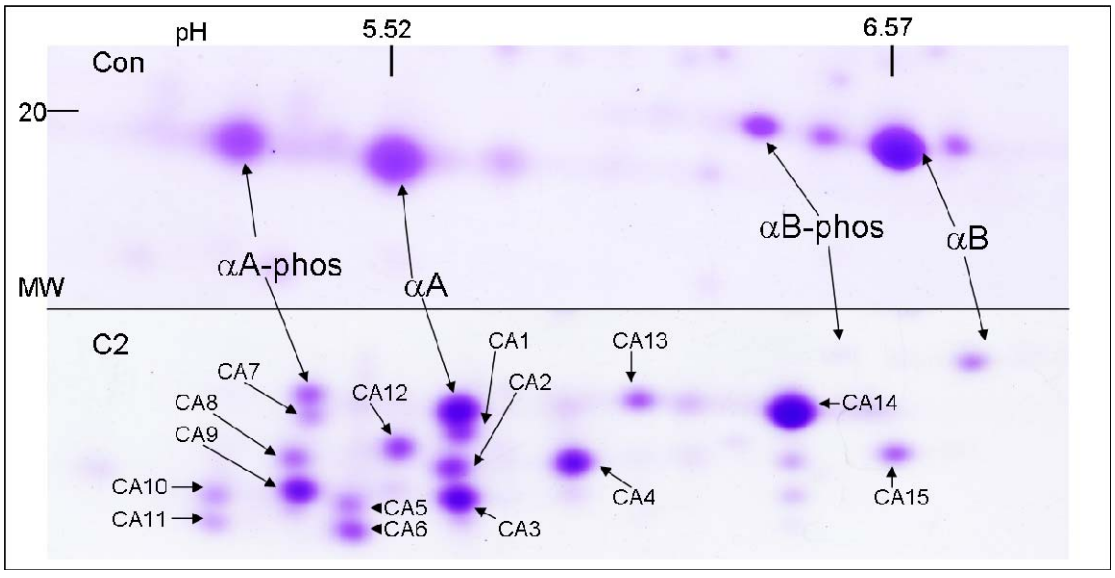

Figure 5-1 Incubation of α-crystallin with calpain 2. Con) α-crystallin before incubation, C2) Control incubation with no calpain 2. αA, αA-phos, αB and αB-phos are labelled and all other spots are identified in Table 5-4. Molecular weight (MW) in kDa and pH range are as indicated.

Spot	ID	Mass (Da)	Predicted Mass (Da)	Modification
αA	αA	19874.0	19875.1	αA_{1-173}
αA-phos	αA	19954.5	19955.1	αA_{1-173}+phos
αB	αB	20108.6	20108.8	αB_{1-175}
αB-phos	αB	20187.5	20188.8	αB_{1-175}+phos
CA1	αA	18720.0	18718.9	αA_{1-162}
CA2	αA	18251.0	18251.3	αA_{1-157}
CA3	αA	17879.3	17879.0	αA_{1-154}
CA4	αA	18873.3	18875.1	αA_{1-163}
CA5	αA	17614.3	17613.7	αA_{1-151}
CA6	αA	17059.5	17059.1	αA_{1-145}
CA7	αA	N/A	18798.9	αA_{1-162}+phos
CA8	αA	N/A	18331.3	αA_{1-157}+phos
CA9	αA	N/A	17959.0	αA_{1-154}+phos
CA10	αA	N/A	17693.7	αA_{1-151}+phos
CA11	αA	N/A	17139.1	αA_{1-145}+phos
CA12	αA	18957.2	18955.1	αA_{1-163}+phos
CA13	αB	N/A	19693.1	αB_{1-170}+phos
CA14	αB	19613	19613.1	αB_{1-170}
CA15	αB	18860.0	18858.3	αB_{1-163}

Table 5-4 Table of truncations of α-crystallin after incubation with calpain 2 (Figure 5-1) and calpain 1 (Figure 5-3). Proteins were identified by MALDI-TOF and masses were determined by whole mass measurement. N/A indicate spots where whole mass measurement was not able to be performed.

Incubation of purified β-crystallin with purified calpain 2 resulted in truncation of βB1, βB3 and βA3 (Figure 5-2). After incubation with calpain 2 the intact form of βB1 (spot 1) was not present, and there was an increase in the truncated forms of βB1 (Box), the spots within the box could not be resolved or massed. Another crystallin that was truncated by calpain 2 is βB3 (8), both the intact and deamidated form (6) of βB3 were not present after calpain 2 incubation (Figure 5-2 C2). In the control sample before incubation there was a truncation product $\beta B3_{10-210}$ (11) already present. This gel spot increased in size and intensity following calpain incubation (Figure 5-2). In the control sample there were two truncation products of βA3 already present $\beta A3_{12-215}$ and $\beta A3_{23-215}$ (12 and 13 respectively). After incubation with calpain 2 there is an

increase in the amount of $\beta A3_{12\text{-}215}$ (12) protein, but no increase in $\beta A3_{23\text{-}215}$. As a consequence of this increased truncation, the intact $\beta A3_{1\text{-}215}$ (4) was not present after incubation with calpain 2. Incubation with calpain 2 had no effect on βB2, βA4, βA2 and βA1.

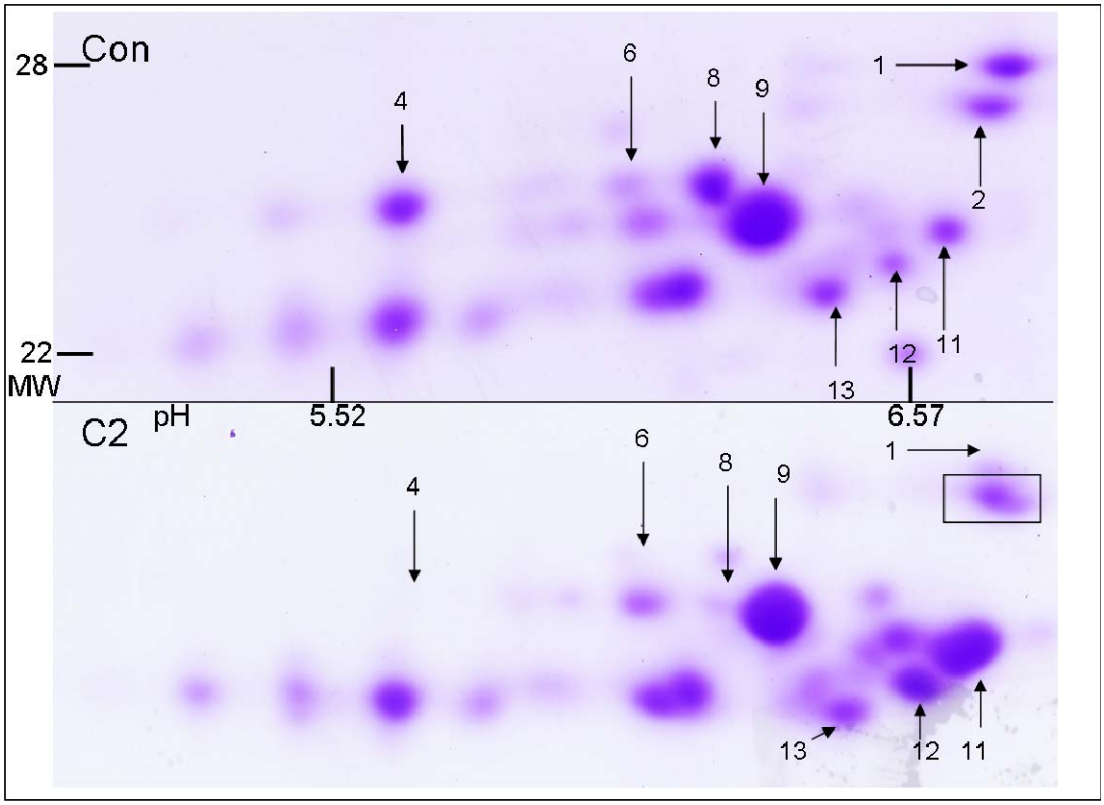

Figure 5-2 Incubation of β-crystallin with purified calpain 2. Con) Control incubation with no calpain 2, C2) Incubation with calpain 2 and calcium. Spots are labelled as in Figure 4-4 and identified in Table 5-5. Molecular weight (MW) in kDa and pH range are as indicated.

Spot	Protein (ID)	Mass	Bovine Equivalent
1	βB1	28055.9	$\beta B1_{1-252}$
Box	βB1	N/A	N/A
4	βA3	25572.3	$\beta A3_{1-215}$
6	βB3	24323.8	$\beta B3_{1-210}$ Deamidation
8	βB3	24321.9	$\beta B3_{1-210}$
9	βB2	23321.3	βB2
11	βB3	23302.4	$\beta B3_{11-210}$
12	βA3/βA1	24212.1	$\beta A3_{12-215}$
13	βA3/βA1	23038.2	$\beta A3_{23-215}$

Table 5-5 Table of modifications to β-crystallins after incubation with calpain 2 (Figure 5-2). Masses were determined by whole mass measurement. Each spot was identified by MALDI-TOF analysis and comparison to total bovine cDNA from the lens except for βB3 which was compared to the known ovine sequence. Bovine equivalent modifications were determined by comparison with Shih *et al.*, (1998).

5.3.2 Calpain 1

Purified calpain 1 (Chapter 2) was incubated with purified α and β-crystallin (Chapter 3). This resulted in truncation of the α and β-crystallins (Figure 5-3 and Figure 5-4).

There were 13 truncations of the α-crystallins when incubated with calpain 1 and the protein profile was similar to that seen with calpain 2 (Figure 5-1 and Table 5-4).

αA-crystallin was truncated five times to produce αA_{1-162} (CA1), αA_{1-157} (CA2), αA_{1-154} (CA3), αA_{1-145} (CA6) and $\alpha A_{?}$ (CAX). Once again the equivalent truncations were seen with the phosphorylated form of αA spots αA_{1-162}+phos (CA7), αA_{1-157}+phos (CA8), αA_{1-154}+phos (CA9) and αA_{1-145}+phos (CA11) (Figure 5-3 and Table 5-4). Comparing the calpain 1 and 2 incubations showed a number of differences. Firstly, spot αA_{1-151} (CA5) and $\alpha A1-_{151}$+phos (CA10) were not present in the Calpain 1 sample, secondly that the major spot in the calpain 2 incubation, αA_{1-154} (Figure 5-1 CA3) was only a minor spot in the calpain 1 incubation (Figure 5-3 CA3). Thirdly, the presence of a new protein species (CAX), whose mass could not be measured, which was absent in the calpain 2 sample.

αB, once again, was only truncated twice. Firstly to produce $\alpha B_{1\text{-}170}$ (CA14) and the equivalent truncation on the phosphorylated form of $\alpha B\text{-phos}_{1\text{-}170}$ (CA13) and secondly, $\alpha B_{1\text{-}163}$ (CA15). These truncations were identical to those found with calpain 2.

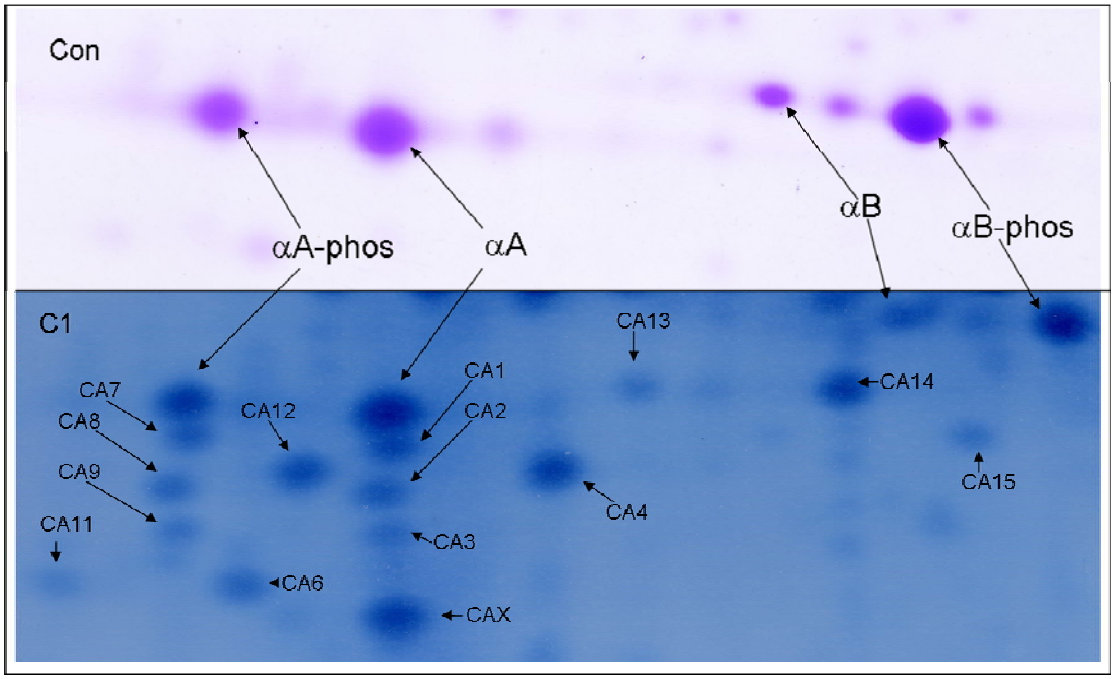

Figure 5-3 Incubation of calpain 1 with α-crystallin. Con) Control incubation with no calpain 1, CI) calpain 1 with α-crystallin and calcium. Spots labelled correspond to Table 5-4. The control gel was stained with Commassie blue and the lower gel with Zinc negative staining for greater sensitivity.

Incubation of calpain 1 with the β-crystallins resulted in the truncation of βB1 and βA3 only (Figure 5-4). The intact form of βB1 (1) was decreased after incubation and four truncation products were observed (box) with each truncation missing amino acids from their N-terminus. The amount of the intact form of $\beta A3_{1\text{-}215}$ (4) decreased after incubation with calpain 1 and the truncation product $\beta A3_{12\text{-}215}$ (12) increased.

Unlike calpain 2, calpain 1 had no effect on $\beta B3_{1\text{-}210}$. The intact form (8) and truncated $\beta B3_{11\text{-}210}$ (11) form were present in the same amounts before and after incubation with calpain 1. In contrast incubation with calpain 2 truncated βB3 (Figure 5-2) (8) and the truncation product $\beta B3_{11\text{-}210}$ (11) increased after incubation.

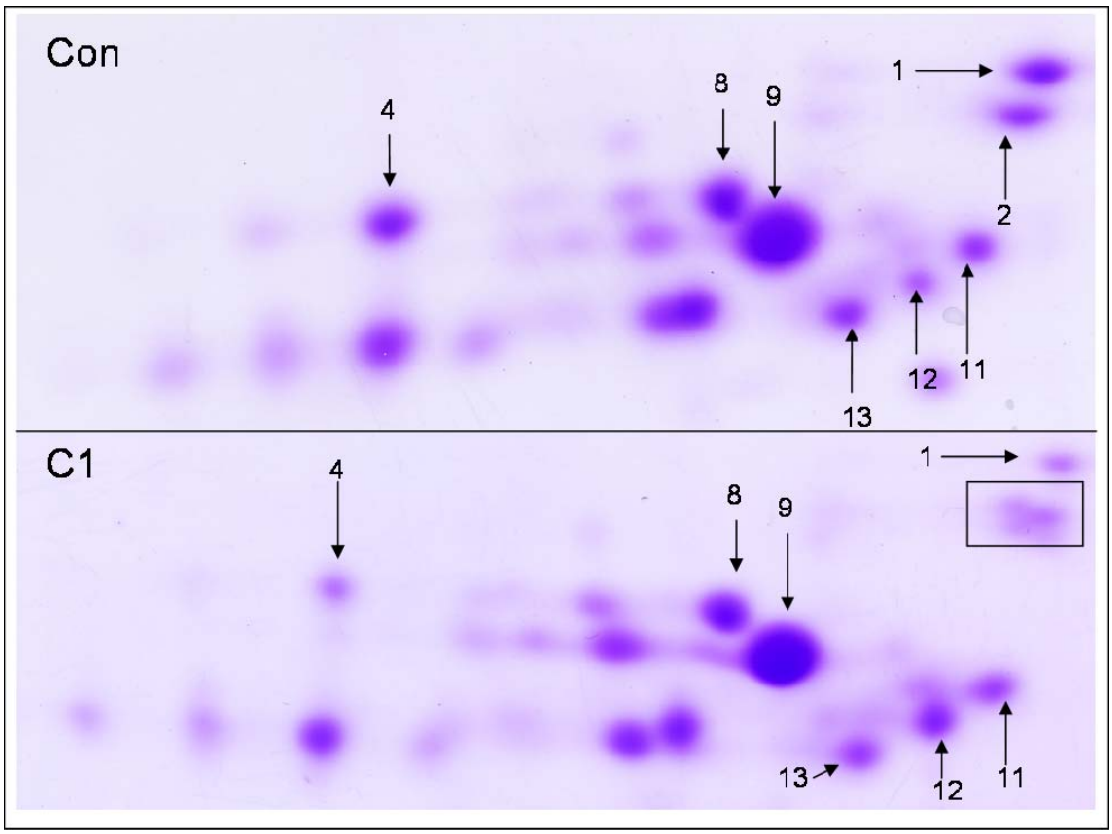

Figure 5-4 Incubation of β-crystallin with purified calpain 1. Con) Control incubation with no calpain 1, CI) Incubation of β-crystallin with calpain 1 and calcium. Spots are labelled as in Figure 4-4 and identified in Table 5-5

5.3.3 Lp82

Purified Lp82 was incubated with α and β-crystallin. The purified Lp82 was incubated with calcium and the endogenous α-crystallin from the purification procedure (Chapter 2) (Figure 5-5). Lp82 truncated αA in two places (L2) and the same truncation was seen in the phosphorylated form (L1). There was also another truncation, L3. The masses of the spots could not be determined. However the truncation $\alpha A_{1\text{-}168}$ can be tentatively applied by comparison to cataract gels and the literature (see 5.4.1).

αB was truncated in only one place (L5) and the phosphorylated form of αB was truncated in the same place (L4). Again the masses of the spots could not be determined from these spots but by comparison to equivalent spots in other gels and comparison with the literature they were assigned $\alpha B_{1\text{-}170}$.

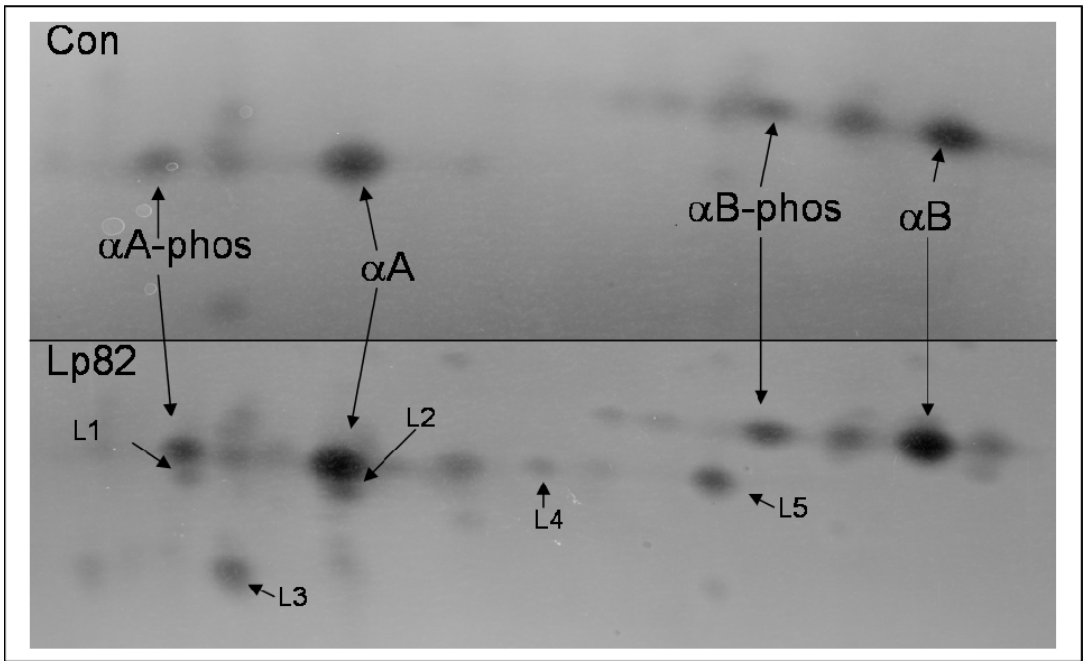

Figure 5-5 Incubation of Lp82 with α-crystallin, Con) Lp82 fraction without Ca^{2+}, Lp82) Lp82 and α-crystallin with Ca^{2+}. Spots are identified and masses predicted in Table 5-6.

Spot	ID	Mass	Predicted Mass	ID
L1	αA	N/A	19445.7	αA_{1-168}
L2	αA	N/A	19525.7	αA_{1-168}+phos
L3	αA	N/A	N/A	N/A
L4	αB	N/A	19592.0	αB_{1-170}+phos
L5	αB	N/A	19512.0	αB_{1-170}

Table 5-6 Truncations of α-crystallin with Lp82. N/A indicate spots where whole mass measurement was not able to be performed. Modifications were determined by comparison to Ueda *et al.,* (2002b) and previously run gels.

Purified Lp82 was incubated with β-crystallin and truncated both βB1 (1) and βA3 (4) (Figure 5-6 and Table 5-5). The intact form of βB1 and the truncation product $\beta B1_{12-252}$ (2) are less abundant in the Lp82 gel. Four new truncation products can be identified (Box) with each missing amino acids from their N-terminus. The intact form of βA3 (4) also decreased after incubation with Lp82 whilst the truncation product $\beta A3_{11-215}$ (12) increased. As found with calpain 1, Lp82 had no effect on βB3 and the intact form (8) was present in the same amount before and after incubation.

The truncated form $\beta B3_{10\text{-}210}$ (11) had not increased. This was in contrast with calpain 2 incubation where there was a decrease in βB3 and increase in $\beta B3_{10\text{-}210}$ (Figure 5-1).

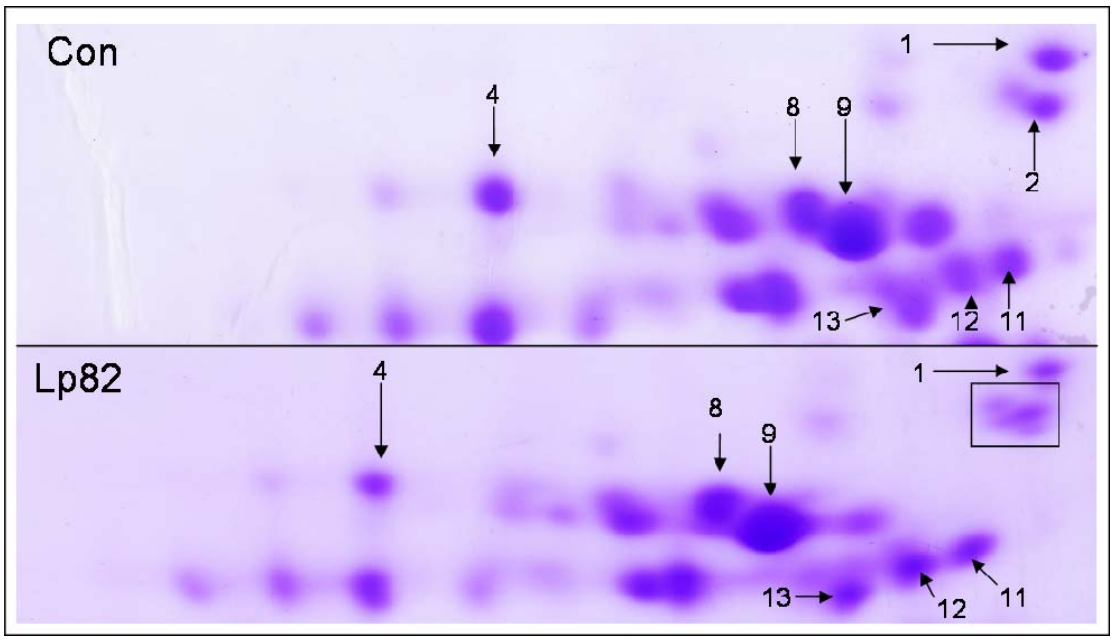

Figure 5-6 Incubation of Lp82 with β-crystallin. Con) Control incubation with no calcium, Lp82) Incubation with lp82 and calcium. Spots are labelled as in Figure 4-4 and identified in Table 5-5.

5.3.4 Insolubilisation of lens proteins

Incubation of calpain 2 with the soluble lens proteins resulted in the production of an insoluble protein pellet. The controls produced no pellet and consequently no insoluble protein was detected. Analysis of the insoluble proteins from the calpain 2 incubations revealed a number of changes to the crystallins. Spot identifications were determined by comparing the 2DE pattern with patterns from previous calpain incubations with α-crystallin (Figure 5-1 and Table 5-4) and β-crystallins (Figure 5-2 and Table 5-5) and cataractous 2DE gels (4.3.4). The soluble and insoluble proteins that were produced were compared directly to cataractous soluble and insoluble protein after being run on the same 2DE systems (see 5.2.3)

The major crystallin modification found in the insoluble proteins produced after incubation with calpain 2 was truncation. The crystallins that were modified were αA, αB, βA3, βB3 and βB1.

The calpain 2 specific cleavage products αA_{1-162} (CA4) and αA_{1-162}+phos (CA12) (Figure 5-7 SC2, IC2) were found in both the insoluble and soluble fraction after incubation with calpain 2. The same truncations were found in the cataractous lenses (SCA, ICA A9). Other truncations of αA-crystallin seen after incubation with calpain 2 were αA_{1-157} (CA2), αA_{1-154} (CA3), αA_{1-151} (CA5) and αA_{1-145} (CA6) and their phosphorylated equivalents αA_{1-157}+phos (CA8), αA_{1-154}+phos (CA9), αA_{1-151}+phos (CA10) and αA_{1-145}+phos (CA11).

Calpain 2 truncated αB to produce the αB_{1-170} (CA14) (Figure 5-7 SC2, IC2) fragment which was seen in both the insoluble and soluble fraction after incubation. The same fragment was also found in the soluble and insoluble cataract (SCA, ICA A5). The equivalent truncation to the phosphorylated form of αB was found in the calpain 2 incubation (SC2, IC2 CA13) and cataractous lenses (SCA, ICA A10).

Amongst the β-crystallins only βB1, βA3 and βB3 were truncated by calpain 2. Intact βA3 (Figure 5-7 SC2, IC2 4) was absent from both the insoluble and soluble fractions of the crystallins after incubation with calpain 2. The intact form of βB1 was also absent from the soluble and insoluble fractions of the calpain 2 incubation (1). Truncation products of βB1 were found in both the soluble and insoluble fraction of the calpain 2 incubations (Boxed area). Similar proteins were found in the soluble and insoluble cataract fractions (SCA and ICA boxed area). Another β-crystallin that is truncated by calpain 2 is βB3 (8). It is less abundant in the calpain 2 soluble and insoluble fractions and the truncation product $\beta B3_{10-210}$ (11) increased after incubation with calpain 2.

There were no visible differences between the soluble and insoluble proteins produced after incubation with calpain 2.

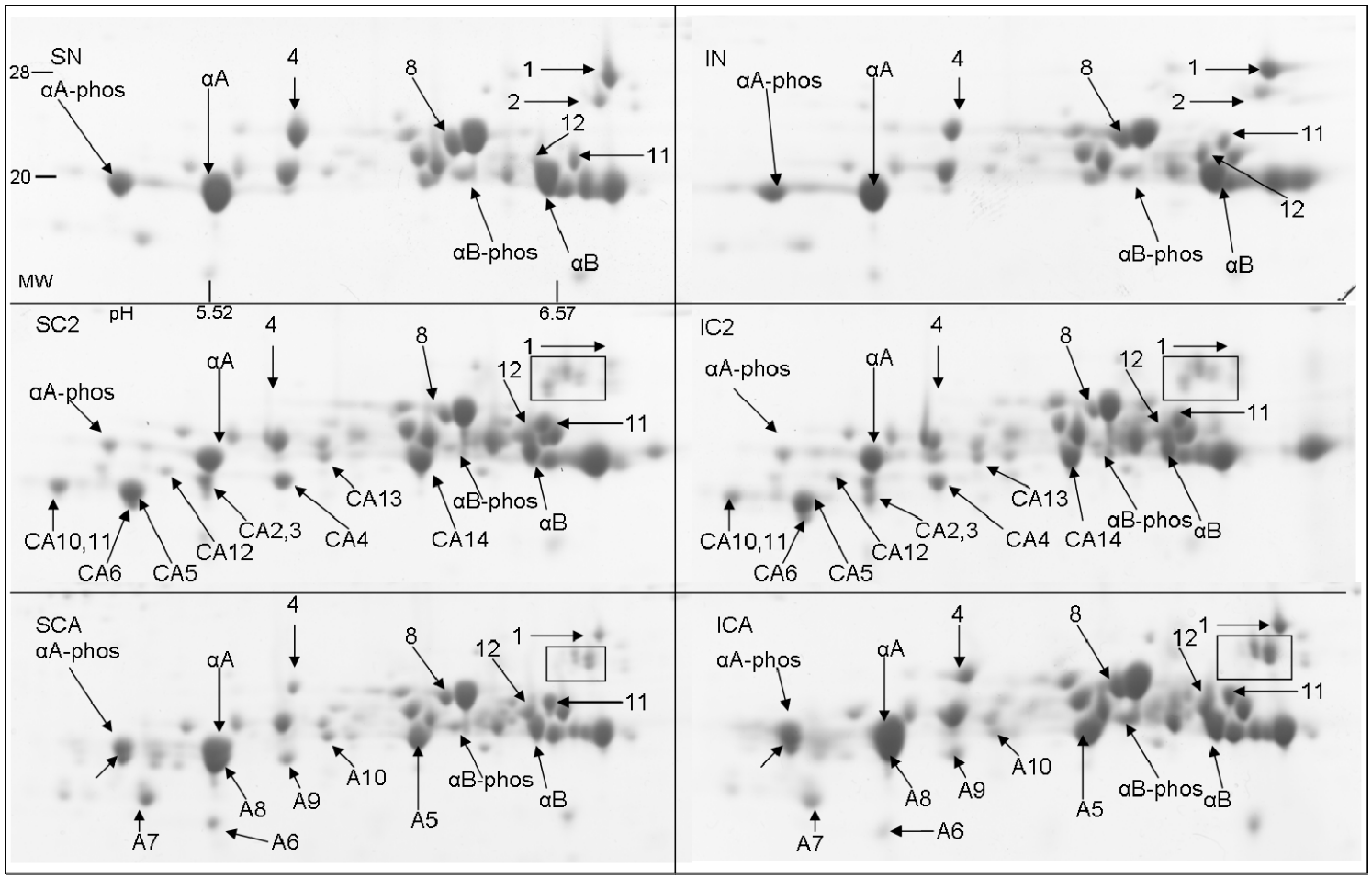

Figure 5-7 2DE of total lens proteins. SN) Soluble protein from a normal lens, IN) Insoluble protein from a normal lens, SC2) soluble protein after incubation with calpain 2, IC2) Insoluble protein produced after incubation with calpain 2, SCA) Soluble protein from a cataractous lens, ICA) Insoluble protein from a cataractous lens. α-crystallins are labelled. CAX=α-crystallin species produced after incubation with calpain 2 Table 5-4. AX=α-crystallin species in cataractous lenses Table 4-5 . Numbers=β-crystallin species Table 5-5. Molecular weight (MW) in kDa and pH range are as indicated for SN gel only.

5.3.5 Cytoskeletal incubations

The purified calpain 2 and Lp82 were incubated with the isolated cytoskeletal proteins and their profiles compared using SDS-PAGE (Figure 5-8). Both calpain 2 (Lane 1) and Lp82 (Lane 4) proteolysed spectrin, Lp82 produced breakdown fragments at 150kDa, whereas calpain 2 produced smaller fragments at 130kDa. Filensin and vimentin were also proteolysed by calpain 2 and Lp82.

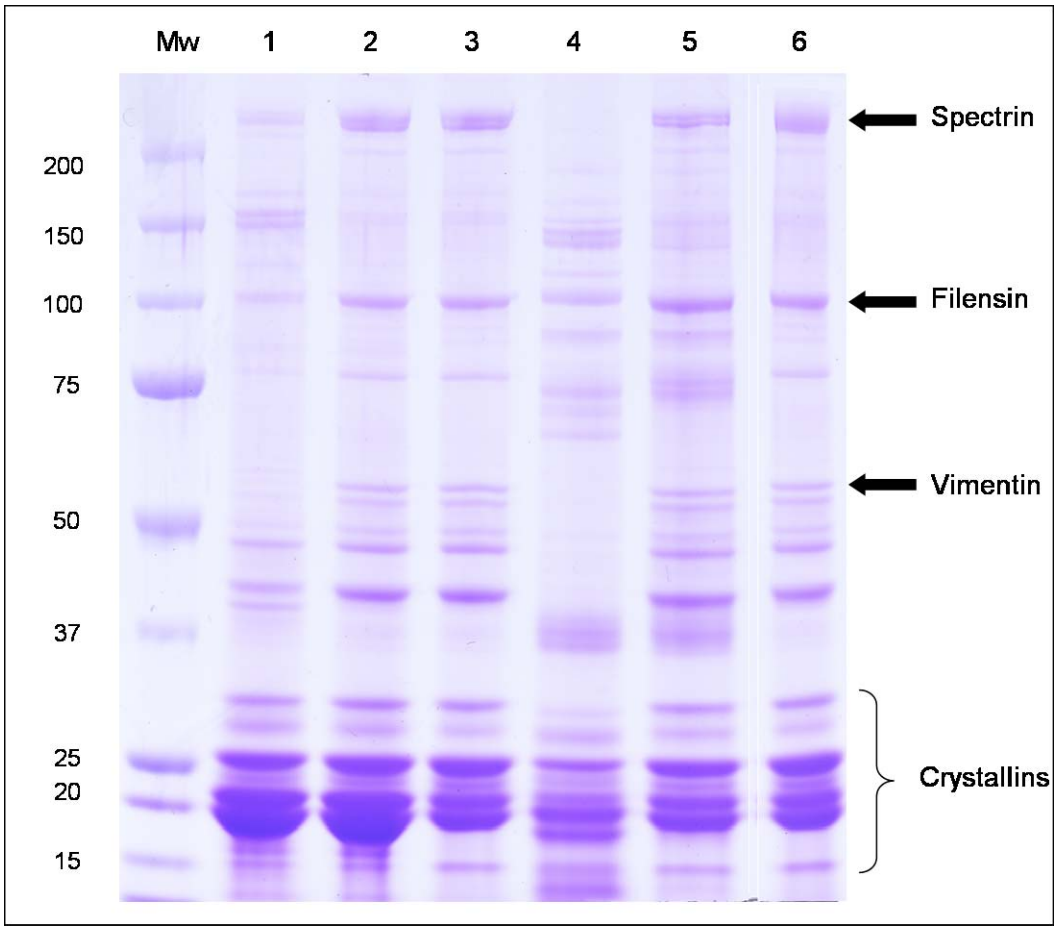

Figure 5-8 4-12% SDS PAGE of Lens Cytoskeletal Proteins after incubation with Lp82 and Calpain 2.. Lane Mw: Molecular Weight marker, 1) Urea Soluble Fraction (USF) + Lp82 + Ca^{2+} 2) USF + Lp82 – Ca^{2+} 3) USF – Lp82 + Ca^{2+} 4) USF +C2+ Ca^{2+} 5) USF +C2 – Ca^{2+}, 6) USF only. Spectrin, Filensin, Vimentin and the crystallins were identified by comparison to Robertson *et al.,* (2005) and McDermott, (2007).

5.4 Discussion

During cataract formation some of the lens proteins are truncated, specifically α and β crystallins and the cytoskeletal proteins: spectrin, vimentin and filensin. Purified calpains were incubated with these proteins to determine whether members of the calpain family of proteases are responsible for the uncontrolled proteolytic cleavage seen in cataract formation.

5.4.1 α-Crystallins incubated with the calpains

Incubation of the α-crystallins with calpain 1, 2 and Lp82 resulted in truncation at the C-termini of both αA and αB-crystallin. Some of these truncations were the same as those found during lens maturation and cataractogenesis. Originally it was proposed that calpain 2 activity was responsible for the cleavage of α-crystallins seen *in vivo* (Yoshida *et al.,* 1986). However since the discovery of the lens specific Lp82, there has been evidence that this is the major calpain isoform within the rat lens (Ueda *et al.,* 2002b). Calpain 1, calpain 2 and Lp82 have all been found in sheep lenses (Robertson *et al.*, 2008)

In this present study Lp82 cleaved αA at two places but the masses of the products and therefore exact truncation sites could not be determined. The reported literature shows that Lp82 *in vitro* can remove 5 residues from the C-terminus of rat (Ueda *et al.,* 2002b), mouse (Nakamura *et al.,* 2000) and bovine (Ueda *et al.,* 2001) αA-crystallin to produce the fragment $\alpha A_{1\text{-}168}$. The seven C-terminal amino acids for all the above species are identical, ^{167}PSSAPSS^{173}, and Lp82 truncates between ^{167}PS/SA^{170}. Ovine αA-crystallin (AY819022) has the same C-terminal residues ^{167}PSSAPSS^{173}, showing that the Lp82 truncation site ^{167}PS/SA^{170} is present. Therefore the truncation of αA by Lp82 in this study was assigned $\alpha A_{1\text{-}168}$, based on the literature, sequence analysis and the location of the spot on 2DE when compared to the $\alpha A_{1\text{-}168}$ species in the 2DE of cataract proteins (Figure 4-12 and Figure 4-13). This $\alpha A_{1\text{-}168}$ truncation was not observed with calpain 1 or calpain 2. The inability for calpain 2 to produce $\alpha A_{1\text{-}168}$ *in vitro* has been well documented in other species

(Yoshida *et al.,* 1986, Nakamura *et al.,* 2000, Ueda *et al.,* 2001, Inomata *et al.,* 2002, Ueda *et al.,* 2002b). No $\alpha A_{1\text{-}168}$ was found in normal ovine lens of any age studied. $\alpha A_{1\text{-}168}$ has been found within the aged lenses of cattle (Takemoto, 1995b, Ueda *et al.,* 2001) and rats (Inomata *et al.,* 2002, Ueda *et al.,* 2002b). Interestingly, $\alpha A_{1\text{-}168}$ has been found in aged human lenses (Takemoto, 1995b) despite there being no Lp82 in human lenses due to the presence of a stop codon in the sequence (Fougerousse *et al.,* 2000). This suggests that there might be an Lp82 like protease in human lens, filling the role that Lp82 plays in other mammalian lenses. The Lp82 specific $\alpha A_{1\text{-}168}$ was found in stage 3 cataractous ovine lenses and onwards and was the major αA-crystallin truncation product in the cataractous lenses. It is possible that the $\alpha A_{1\text{-}168}$ species in the stage 1 lens was overshadowed by its parent protein αA, which resolves closely to $\alpha A_{1\text{-}168}$ (Figure 4-12 and Figure 4-13). $\alpha A_{1\text{-}168}$ has been found at increased levels in the Shimuya (Inomata *et al.,* 2002) and selenite (Ueda *et al.,* 2002b) rat cataracts. Although this was the only truncation seen with ovine Lp82, rat Lp82 has been shown to cleave the C-terminus of αA at multiple sites to produce $\alpha A_{1\text{-}165}$, $\alpha A_{1\text{-}163}$, $\alpha A_{1\text{-}157}$, $\alpha A_{1\text{-}156}$ and $\alpha A_{1\text{-}151}$ (Ueda *et al.,* 2002b).

Incubation of calpain 2 with the α-crystallins produced multiple truncations of αA at the C-terminus with the removal of between 10-28 amino acids. Of particular interest is the truncation $_{1\text{-}162}$ with 11 residues removed, which has been shown to be an *in vitro* calpain 2 specific truncation with rat (Ueda *et al.,* 2002b) and bovine (Yoshida *et al.,* 1986, Ueda *et al.,* 2001) calpain 2 and αA-crystallin . This provides a unique marker for calpain 2 activation within the lens. This truncation was not found in normal lenses at any age but was found in cataractous lenses from stages 3 and 6, showing that calpain 2 is active within the ovine cataract. However, $\alpha A_{1\text{-}162}$ is less abundant than other truncations within the cataractous ovine lens. Shimuya rat cataract (Inomata *et al.,* 2002), selenite rat cataract (Ueda *et al.,* 2002b) and buthinone sulphoximine mouse cataract (Nakamura *et al.,* 2000), have all contained $\alpha A_{1\text{-}162}$ in the lens. The other calpain 2 truncations of αA include $\alpha A_{1\text{-}163,}$ $\alpha A_{1\text{-}157,}$ $\alpha A_{1\text{-}154,}$ $\alpha A_{1\text{-}151}$ and $\alpha A_{1\text{-}145}$. Some of these truncations namely, $\alpha A_{1\text{-}157,}$ $\alpha A_{1\text{-}154,}$ and $\alpha A_{1\text{-}151,}$ were found in the fetal ovine lens and their levels increased as the age of the lens increased. Within the cataract lens there are increased levels of the calpain 2 truncation products, $\alpha A_{1\text{-}163}$ and $\alpha A_{1\text{-}151}$. This shows a role for calpain 2 within ovine cataractogenesis. However some of these protein species: $\alpha A_{1\text{-}163,}$ $\alpha A_{1\text{-}157}$ and $\alpha A_{1\text{-}151}$ have also been

shown to be produced by Lp82 in the rat lens (Ueda *et al.,* 2002b). These truncations were not observed with Lp82 in this study but this may be due to the limited amount of Lp82 that was purified and incubated with α-crystallins (22 FU for Lp82 compared with 162 FU for calpain 2).

In this study calpain 1 also produced truncations in a similar position to that of calpain 2 as observed by 2DE. Further analysis needs to be performed to determine if the truncations are the same truncations as are produced by calpain 2.

All three calpains, when incubated with αB, produced the same truncation where 5 residues were removed from the C-terminus to produce $\alpha B_{1\text{-}170}$. Calpain 2 also produced a second truncation removing 12 amino acids to produce $\alpha B_{1\text{-}163}$. $\alpha B_{1\text{-}170}$ was found in normal, fetal and 6 month old ovine lenses but not in 8 year old ovine lenses. $\alpha B_{1\text{-}170}$ is a major component of the cataractous ovine lens, to the extent that it is the most prevalent form of αB in the USF of a stage 6 cataractous lens. This truncation has been well documented and occurs during lens maturation and cataractogenesis. Calpain 2 and Lp82 from rat (Ueda *et al.,* 2002b) and bovine (Yoshida *et al.,* 1986, Ueda *et al.,* 2001) lenses both produce $\alpha B_{1\text{-}170}$. This truncation was found in the Shimuya rat cataract (Inomata et al., 1997a), selenite rat cataract (Ueda *et al.,* 2002b) and ICR/f rat (Takeuchi *et al.,* 2004) lenses. In human lenses $\alpha B_{1\text{-}170}$ has been found in normal lenses and at increased levels in lenses with Soemmerrings rings (Colvis *et al.,* 2000). From these results it can be concluded that αB truncation *in vivo* could be attributed to any of the three calpains present within the ovine lens.

When the calpains were incubated with the α-crystallins they also truncated the phosphorylated forms of αA and αB in the same place as the unmodified forms. There was no evidence of preference for either form of the α-crystallins which suggests that phosphorylation of target proteins has no effect on their proteolysis.

As previously described, the C-terminal end of α-crystallin is important for chaperone function and truncation of this tail has a detrimental effect on α-crystallins (for more information see 3.4). Therefore, calpain truncation of the α-crystallins should reduce the chaperone function of the α-crystallins. This is supported by the research of Kelly,

et al., (1993) who showed that when calpain 2 was incubated with α-crystallin it reduced the chaperone function of α-crystallin to a similar level as that seen in the selenite cataract.

In conclusion it appears that all three calpains present in the ovine lens, calpain 1, 2 and Lp82, could be responsible for the truncation of α-crystallin observed during lens ageing and cataractogenesis. The majority of the truncations within the cataractous lens could be attributed to all three calpains. However, the most common, $\alpha A_{1\text{-}168}$ found in the cataractous lens was produced only by Lp82. This suggests that Lp82 is the major calpain protease active in the ovine lens and responsible for the majority of the proteolytic damage. A similar conclusion was reached by Ueda *et al.,* (2002b) in the selenite rat cataract. However the calpain 2 specific $\alpha A_{1\text{-}162}$ was also found in the ovine cataract, which means that calpain 2 could also be contributing to the cataractogenesis. Further investigation with calpain 1 needs to be performed before its exact role in ovine cataractogenesis can be determined.

5.4.2 β-Crystallins incubated with the calpains

All three calpains found in the lens truncated some of the β-crystallins. The three β-crystallins affected by the calpains were βB1, βB3 and βA3. βB1 was truncated by all three calpains examined. Calpain 2, Lp82 and calpain 1 truncated βB1 to produce four truncation products but the masses of the spots could not be determined. The spots however, were in the same place as the four βB1 truncations found in the ovine cataractous lens (4.3.4.2). This would suggest that they are the same truncations as those that occur in cataractogenesis. Mass analysis will be needed to confirm this. The results show that activation of calpain 1, calpain 2 or Lp82 occurred during cataractogenesis, where they truncated the N-terminus of βB1, in a similar manner to that observed within the ovine cataract. In other species calpain 2 has been shown to truncate βB1 in multiple places. Bovine calpain 2 was shown *in vitro* to truncate βB1 in two places to produce $\beta B1_{15\text{-}252}$ and $\beta B1_{12\text{-}252}$ (Shih *et al.,* 1998). In humans, calpain 2 truncates βB1 and removes 47 residues from the N-terminus (Shih *et al.,* 2001). A similar truncation was found with rat calpain 2 which removed 49 residues (David and Shearer, 1993). Another rat calpain 2 truncation product is missing 27

residues (David *et al.,* 1994). None of these truncations were identified with any of the calpains studied in this research.

βB3 is one of the major lens crystallins in the fetal lens and becomes increasingly truncated as the lens ages. Calpain 2 truncated all of the intact βB3 crystallin when incubated in the presence of calcium and consequently, no βB3 was left in the sample. Corresponding with this decrease in βB3 was an increase in the truncation product $\beta B3_{12\text{-}215}$. Neither calpain 1 nor Lp82 had a discernable effect on βB3. $\beta B3_{11\text{-}210}$ is found in fetal lenses and decreases as the lens ages, compared with cataract formation where it increases in abundance. The same results are seen with rat βB3 and calpain 2 (David *et al.,* 1994). A second truncation was seen with rat calpain 2 *in vitro* which removed 5 residues from the N-terminus (David *et al.,* 1994). These same two truncations, missing 5 and 10 amino acids, were found after incubation with bovine β-crystallins and calpain 2 (Shih *et al.,* 1998). The fact that $\beta B3_{11\text{-}210}$ increases as cataract formation progresses and that calpain 2 can produce this truncation provides convincing evidence that calpain 2 is involved in cataractogenesis within the ovine lens.

The third β-crystallin that is truncated by the three lens calpains is βA3. After the β-crystallins were incubated with calpain 2 there was complete disappearance of intact βA3 and with calpain 1 and Lp82 there was a reduction of intact βA3. This disappearance is seen during lens ageing and cataractogenesis in the ovine lens. Associated with the reduction in βA3 was an increase in $\beta A3_{12\text{-}215}$ with all of the three calpains. This truncation is already present in the fetal ovine lens and decreases as the lens ages. This decrease in βA3 and $\beta A3_{12\text{-}215}$ is also seen during maturation of the bovine lens (Werten *et al.,* 1999). In the ovine cataract there is a decrease in $\beta A3_{11\text{-}210}$ as the opacity develops and there is an increase in the other truncation product of βA3, $\beta A3_{23\text{-}215}$. This second truncation was not observed with the calpain incubations. This suggests that a different protease could be responsible for this βA3 modification *in vivo*. Bovine calpain 2 also failed to produce this truncation (Shih *et al.,* 1998) and a similar conclusion was reached by the authors. $\beta A3_{12\text{-}215}$ was produced *in vitro* with human (Shih *et al.,* 2001) and rat (David *et al.,* 1993, David *et al.,* 1994) calpain 2 and it was also found in the selenite rat cataract (David and Shearer, 1993, David *et al.,* 1994). Lp82 also reduced the amount of intact βA3 which was accompanied by an

increase in βA3$_{12\text{-}215}$ after incubation with Lp82. Ueda *et. al.,* in 2001 also showed that Lp82 could truncate βA3 at the same location. Thus the modification βA3$_{12\text{-}215}$ *in vivo* could be due to cleavage by any one of the three calpains, calpain 1, calpain 2 or Lp82.

βB1, βB3 and βA3 were the only β-crystallins that were truncated by the calpains in this study. Researchers have found that the calpains also truncate βB2 in other species. In particular bovine, rat and human calpain 2 can remove seven amino acids from βB2 (Shih *et al.,* 2001). This truncation was not observed with ovine calpain 2 possibly due to it comigrating with βB3$_{11\text{-}210}$ on 2DE, which is where it was observed in the bovine lens 2DE and in previous ovine cataractous 2DE (Robertson *et al.,* 2008).

In conclusion the β-crystallins can be truncated by all three calpains found in the ovine lens and the observed truncations are similar as those found in the ovine cataract. As all three calpains produce the same modifications it is difficult to confirm which calpain is the most active and responsible for the modifications to the β-crystallins within the ovine lens.

5.4.3 Calpain 2 induced insolubilisation of the lens crystallins

It has been proposed that after truncation by members of the calpain family the crystallins precipitate and become insoluble (David *et al.,* 1993, Fukiage *et al.,* 1997, Shih *et al.,* 2001). When rat calpain 2 was incubated with the lens crystallins there was an increase in the insoluble protein within the sample. When this insoluble protein produced by calpain 2 was compared to the insoluble fraction of a selenite lens it was shown that the β-crystallins contained similar cleavage sites (David and Shearer, 1993). Rat and mouse crystallin solutions incubated with activated calpain 2 showed an increase in light scatter as measured by an increase of absorbance. The increase in light scatter corresponded with an increase in the proteolytic cleavage of the β-crystallins (Fukiage *et al.,* 1997). However no increase in light scatter was detected with guinea pig crystallins. Similar results experiments chicken, bovine or human crystallins showed that it was only rat crystallins that were susceptible to

calpain-induced insolubilisation (Shearer *et al.,* 1996). Further work, however, showed that human, bovine and rat crystallins were susceptible to heat induced light scatter after incubation with calpain 2 (Shih *et al.,* 2001). In this research calpain 2's ability to insolublise the lens crystallins was studied. When purified calpain 2 was incubated with lens crystallins, insoluble protein was formed but no precipitate was seen when calcium or calpain was left out of the sample. This increase in insoluble protein corresponded with proteolytic cleavage of α and β-crystallin. The insoluble protein from the incubation had a similar profile to the insoluble protein from an ovine cataractous lenses on 2DE. This suggests that calpain 2 truncation *in vivo* could cause the increase in insoluble protein that is seen in the ovine cataract.

5.4.4 Calpains and cytoskeletal proteins

The cytoskeletal proteins within the lens are important for its structure and integrity. During ovine cataract formation there was breakdown of spectrin and vimentin (Robertson *et al.,* 2005). In this study incubation of calpain 2 and Lp82 with the lens cytoskeletal proteins resulted in cleavage of spectrin, vimentin and filensin. Spectrin incubated with calpain 2 produced 130kDa breakdown products and Lp82 produced 150kDa. Robertson *et al.,* (2005) showed that during cataract formation there is a decrease in intact spectrin and in increase in the 145 and 150kDa breakdown products. These results would suggest that Lp82 alone is responsible for the formation of the 145kDa and 150kDa products seen in cataracts. However calpain 2 has been previously shown to produce 145kDa and 150kDa truncations (Goll *et al.,* 2003, McDermott, 2007) and in this study they may have been further broken down by calpain 2 (Yoshida *et al.,* 1995). Vimentin was also broken down when incubated with calpain 2 and Lp82. Again in the ovine cataractous lens there is breakdown of vimentin (Robertson *et al.,* 2005), in a similar pattern to that seen here *in vitro*. In this study there was breakdown of the cytoskeletal protein filensin by calpain 2 and Lp82. Breakdown of filensin was seen in cultured bovine and rat lenses after incubation with a calcium ionophore (Sanderson *et al.,* 2000). In cultured ovine lenses, there is breakdown of spectrin, vimentin and filensin after incubation with a calcium ionophore (Lee *et al.,* 2008). This is the first study to show that Lp82 can breakdown the cytoskeletal proteins spectrin, vimentin and filensin.

These results show that calpain 2 and Lp82 have the ability to proteolyse cytoskeletal proteins. Previously it was thought that calpain 2 was responsible for the cytoskeletal truncation seen with ovine cataracts (Robertson *et al.*, 2005, McDermott, 2007) but the results in this research suggest that Lp82 could also have a role in this breakdown.

5.5 Conclusion

In summary, all three calpains found in the ovine lens posses the ability to truncate the α and β-crystallins. Lp82 and calpain 2 produced unique truncations with αA-crystallin. This provides important markers for the activation of these calpains within the lens. All three calpains truncated αB-crystallin in the same place. The β-crystallins that were truncated by the calpains were βB1, βB3 and βA3. βB1 and βA3 were truncated in the same place with all three calpains. The βA3 truncations were already present within the lens and were associated with lens maturation. Only calpain 2 truncated βB3. Incubation of the WSF from a young lens with calpain 2 resulted in the truncation of the crystallins and the truncated crystallins became insoluble. Calpain 2 and Lp82 also truncated spectrin, vimentin and filensin which are cytoskeletal proteins within the lens. The profile of the truncated crystallins produced by incubating the crystallins with the calpains was similar to the truncated crystallin profile observed during cataract formation.

Chapter 6
Overall Conclusion and Future Directions

6.1 Overall Conclusion

Cataracts are the leading cause of blindness in the world. Understanding the biochemical mechanism that is responsible for this debilitating disease is essential for finding an alternative treatment to expensive surgery. This thesis explored changes in the protein of the ovine lens in ageing and cataractogenesis. It also determined possible mechanisms for the changes observed within the ovine lens proteins during cataractogenesis, specifically the involvement of the calpains in this process.

Three calpains have been discovered within the ovine lens, the ubiquitous calpains 1 and 2 and the lens specific calpain 3 splice variant, Lp82. In Chapter 3 all three of these calpains were purified. Calpain 1 and 2 were purified from ovine lung and Lp82 from lamb lenses. This resulted in samples that were specific for each of the calpains. These samples were used for further experiments in Chapter 5.

During lens maturation there are numerous biochemical changes to the lens proteins (Table 6-1, Table 6-2). Chapter 4 deals with the changes that occur to the crystallins during ageing. Lenses from fetal, 6 month and 8 year old sheep were analysed by 2DE. The results showed that there is increased phosphorylation of the α-crystallin as the lens ages. They also showed that both α and β-crystallin undergo minor truncation during ageing of the lens. The truncation of the β-crystallins is believed to help the crystallins pack tightly within the lens and to aid in the formation of the refractive index.

During cataractogenesis there are extensive changes to the lens proteins (Table 6-1, Table 6-2). Chapter 4 examined the changes in both the WSF and USF within cataractous lenses from stages 0-6. As the cataract progressed there was increased truncation of both α- and β-crystallin.

Both αA and αB-crystallin were truncated at their C-termini. Removal of residues from the C-terminus of the α-crystallins has been shown to reduce their chaperone function. Thus it can be postulated that there is a decreased chaperone function within the ovine cataractous lens. Three β-crystallins, βB1, βA3 and βB3, truncated at their N-termini were observed at increased levels within the cataractous lens. Other research shows that truncation of the N-termini of the β-crystallins can lead to their insolubilisation and aggregation (David *et al.,* 1992). Therefore within the cataractous lens there is an increased level of truncated β-crystallin which becomes insoluble and aggregate. This aggregation cannot be prevented by α-crystallin as it has a reduced chaperone function due to increased C-terminal truncation. As a result of Chapter 4 the first hypotheses: "(i) the crystallin truncations seen in cataractogenesis are unique to this disease and different to the truncation seen with normal maturation of the lens" can be confirmed.

One of the questions that arises from this research is what is causing this increased truncation within the cataractous lens. Previous work within this area has shown an increase in Ca^{2+} within cataractous lenses and it was proposed that this could activate the calcium dependent cysteine proteases; the calpains.

The rest of this thesis dealt with the role that the three calpains found in the ovine lens have in the truncation of the crystallins. In Chapter 5, calpain 1, 2 and Lp82 purified from Chapter 3 were incubated in the presence of calcium with α and β-crystallin. It was found that all three calpains could proteolytically truncate the α-crystallins (Table 6-1). Unique truncations for calpain 2 and Lp82 were found on αA-crystallin ($\alpha A_{1\text{-}162}$ and $\alpha A_{1\text{-}168}$ respectively). Of the two unique truncations only $\alpha A_{1\text{-}168}$, the Lp82 specific truncation was found in the cataractous lens. The other truncations of αA observed in the cataractous lens could only be produced *in vitro* by calpain 1 and calpain 2 and were not found with Lp82 incubation. This may be due to the limited amount of Lp82 activity that could be incubated with the α-crystallins (10 times less than calpain 2). Researchers in other species have found that Lp82 can cause the equivalent truncations. Therefore it could be concluded that all three calpains could truncate αA-crystallin *in vivo* but that Lp82 is possibly the most important calpain isoform in this process. All three calpains cleaved αB-crystallin at the same location

($\alpha B_{1\text{-}170}$), therefore αB-crystallin truncation cannot be attributed to any single calpain isoform.

Crystallin	Maturation	Cataract	Calpain 1	Calpain 2	Lp82
αA	↓	↓			
αA+phos	↑	↓			
$\alpha A_{1\text{-}154}$	▬	▬	+	+	
$\alpha A_{1\text{-}157}$	▬		+	+	
$\alpha A_{1\text{-}151}$	▬	↑	+	+	
$\alpha A_{1\text{-}168}$		↑			+
$\alpha A_{1\text{-}163}$		↑	+	+	
$\alpha A_{1\text{-}162}$			+	+	
$\alpha A_{1\text{-}145}$			+	+	
αB	↓	↓			
αB+phos	↑	↓			
$\alpha B_{1\text{-}174}$	▬	↑			
$\alpha B_{1\text{-}170}$	↓	↑	+	+	+
$\alpha B_{1\text{-}163}$		↑	+	+	

Table 6-1 Summary of α-crystallin and truncations. Each crystallin species is listed on the left. Changes were assessed as either an increase (↑), decrease (↓) or no change (▬). For calpain incubations (+) indicates that the protein species was found after incubation. For maturation changes assessed as differences from fetal to 8 year old lens 2DE. For cataract the changes were assessed as differences from stage 0-6 cataractous lenses. No symbol within indicates that the protein species was not found.

Calpain 1, calpain 2 and Lp82 all truncated some of the β-crystallins (Table 6-2). βB1 was cleaved in a similar manner by all three calpains but the exact truncations could not be determined. βA3 was truncated by all three calpains and only calpain 2 truncated βB3. The same truncations of βB1 were found at increased levels during cataract formation and as all three calpains studied could truncate βB1 the isoform responsible for the *in vivo* truncation of βB1 can not be determined.

Both calpain 2 and Lp82 were able to cleave the lens cytoskeletal proteins vimentin, spectrin and filensin. Proteolysis of lens cytoskeletal proteins is found in many models of cataract including the heritable ovine cataract. Chapter 5 also explored the effect truncation of the lens proteins had on their solubility. As mentioned previously

there is an increase in aggregated and insoluble protein during cataractogenesis. This research found that truncation of the lens crystallins by calpain 2 resulted in insolubilisation of some of the crystallin, demonstrating that crystallin truncation by calpain 2 could be responsible for the increase in insoluble protein in cataract lenses.

Crystallin	Maturation	Cataract	Calpain 1	Calpain 2	Lp82
βB1	↓	↓	↓	↓	↓
βB2	▬	▬	▬	▬	▬
βB3	↓	↓	▬	↓	▬
βA1	▬	▬	▬	▬	▬
βA2	▬	▬	▬	▬	▬
βA3	↓	↓	↓	↓	↓
βA4	↓	↓	▬	▬	▬
$\beta B1_{16-252}$	↑	↓	↓	↓	↓
$\beta B3_{10-210}$	↓	↑	▬	↑	▬
$\beta A3_{12-215}$	↓	↓	↑	↑	↑
$\beta A3_{23-215}$	↑	↓	▬	▬	▬
$\beta B1_{10-252}$		↑	↑	↑	↑
$\beta B1_{11-252}$		↑	↑	↑	↑
$\beta B1_{13-252}$		↑	↑	↑	↑
$\beta B2_{8-204}$		↑			

Table 6-2 Summary of β-crystallin and truncations. Each crystallin species is listed on the left. Changes were assessed as either an increase (↑), decrease (↓) or no change (▬). For maturation changes assessed as differences from fetal to 8 year old lens 2DE. For cataract the changes were assessed as differences from stage 0-6 cataractous lenses. For the incubations changes were assessed as differences before and after incubation. No symbol indicates that the protein species was not found.

As a result of this research the second hypothesis "(ii) That incubation of calpains 1, 2 and Lp82 with the crystallins will result in truncation of the crystallins in a similar manner to that seen in cataractogenesis" can be confirmed. However which calpain isoform is the causing the truncation *in vivo* will require further investigation.

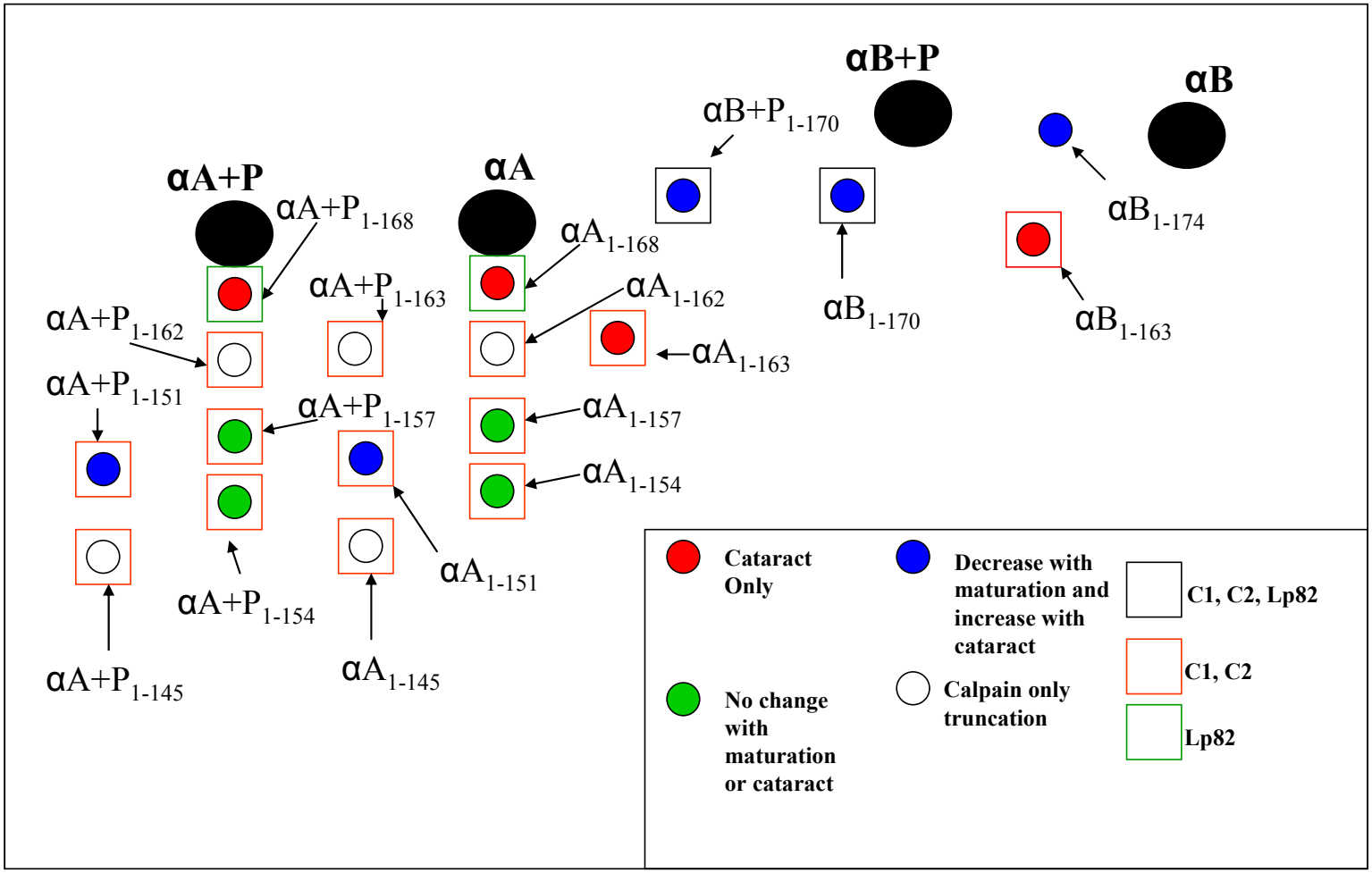

Figure 6-1 Schematic 2DE of the changes to α-crystallin during maturation, cataractogenesis and after incubation with the calpains. For a table of the results see (Table 6-1). Spots are coloured to indicate how species changes with maturation and cataractogenesis. Boxes indicate those species present after incubation with calpains.

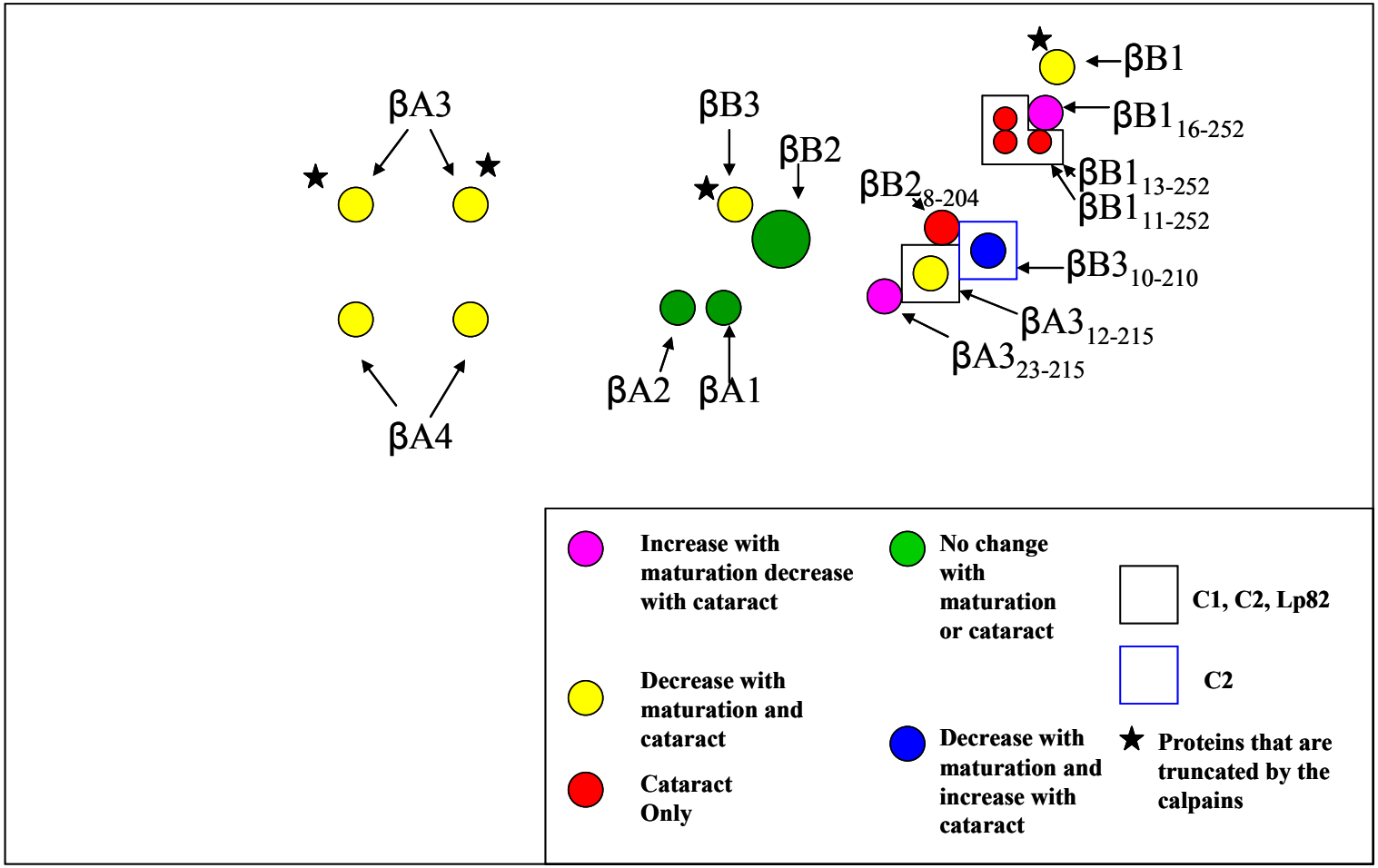

Figure 6-2 Schematic 2DE of the changes to β-crystallin during maturation, cataractogenesis and after incubation with the calpains. For a table of the results see (Table 6-1). Spots are coloured to indicate how species changes with maturation and cataractogenesis. Boxes indicate those species present after incubation with calpains. Stars represent β-crystallins that are truncated by the calpains.

Analysis of the relative amounts of each calpain and their location may give an insight as to which calpain is truncating the crystallins *in vivo*. Robertson (2005) studied the levels of all three calpains in sheep lenses. It was found that all three calpains were present in the soluble cortex (where the cataract occurs) fraction up to 7 weeks of age. Calpain 2 was found to be the most abundant of the calpains. By 13 weeks of age only calpain 1 and calpain 2 were found in the soluble cortex fraction with calpain 2 being the most dominant isoform and only a small amount of calpain 1 being found. In the present research Lp82 was able to be extracted from lenses up to 16 weeks of age. By 24 weeks of age only calpain 2 was found in the soluble cortex. Therefore all three calpains are present during early cataractogenesis and only calpain 2 is present in late cataractogenesis.

Although calpain 2 is the most prominent calpain within the lens this does not necessarily mean that calpain 2 is the most active *in vivo*, as the calpains within the lens all have different calcium concentrations required for activation. Calpain 1 requires 3-50μm, calpain 2 400-800μM and Lp82 20μM Ca^{2+} for half maximal activation *in vitro*. During cataractogenesis the calcium within the lens rises 10 fold from 0.42mM (stage 0) to 4.9mM (stage 6). Therefore all three calpains could be active within a stage 6 cataract. It would be logical to assume that as the calcium level within the lens rises first Lp82 and calpain 1 would become active followed by calpain 2 once the calcium rose to a sufficient concentration for activation. This could explain why there is more of the Lp82 specific truncation of αA-crystallin ($\alpha A_{1\text{-}168}$) within the ovine lens and why this truncation appears first in cataractogenesis.

In conclusion this research has documented the changes in lens crystallin with maturation. It has also studied the progression of crystallin truncation through stages 0-6 in the ovine cataract. Calpain 1, 2 and Lp82 were all shown to truncate the crystallins *in vitro*. Some of the truncations seen *in vitro* were the same as those in cataract lenses. Therefore it appears that uncontrolled proteolysis of the crystallins by a combination of all three calpains contribute to cataract formation in the ovine cataract.

6.2 Future Directions

The most obvious research for the future would be to carry out further mass spectromic analysis to determine all of the truncations that are seen during ageing, cataractogenesis and incubation with the calpains. This would involve refining of the 2DE protein extraction and mass spectromic protocols. Conversely, the samples could be analysed directly with mass spectromic techniques without prior separation on 2DE. This would allow greater sensitivity and would give masses for many of the less abundant protein species. However one of the limitations of the direct approach is that quantifying the protein species is difficult. Determining the protein sequence for the ovine β and γ-crystallins would also be beneficial for this analysis.

In the ovine cataract there are extensive changes to the α and β-crystallins, however little research has been carried out on the γ-crystallins and the changes with them during ageing and cataractogenesis. Therefore optimising the 2DE for γ-crystallins would provide an insight into any changes that occur with the γ-crystallins during ageing and cataractogenesis. Although in other species the calpains do not proteolyses the γ-crystallin it would be beneficial to test this with ovine γ-crystallins.

The theory of cataract formation presented in this thesis suggests that C-terminal truncation of α-crystallin results in loss of the chaperone function of the α-crystallin *in vivo*. An obvious extension of this research would be to isolate the α-crystallin from cataractous lens and compare its chaperone ability to that of α-crystallin isolated from normal lenses. This could then be compared to the chaperone function of α-crystallin after truncation with the calpains.

Mutating specific amino acids within the C-terminal extension of α-crystallin by site directed mutagenesis would allow the determination of which amino acids are important for calpain in choosing truncation position. This would also help in establishing why Lp82 has a different truncation site than calpains 1 and 2.

At present only three calpains have been discovered within the ovine lens. There is evidence in other species for a fourth calpain, calpain 10 within the lens. Determining

if this calpain is present within the ovine lens could provide further evidence for the calpain theory of cataractogenesis. Biochemical analysis, in particular determination of the calcium concentration requirements of the three calpains studied here would provide further insight as to their activation within the ovine lens.

At present the only way to determine if a sheep within the cataract flock has cataracts is by visual confirmation. Development of a genetic test which could predict which lambs will develop cataract would be a vital tool for further cataract research. It would mean that any changes with in the lens before clinical manifestation of the disease could by researched, in particular changes in the crystallins or other lens proteins that precedes cataract formation.

This research showed that the calpains could truncate the cytoskeletal proteins. A logical step from this would be to analyse the cytoskeletal proteins from the ovine lens using 2DE and mass spectrometry.

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Publications and Presentations arising from this Thesis

Papers

ROBERTSON, L.J.G.; DAVID, L.L.; RIVIERE, M.A.; WILMARTH, P.A.; MUIR, M.S.; MORTON, J.D. **(2008)**. Susceptibility of ovine lens α- and β-crystallins to proteolytic cleavage during formation of hereditary cataract. I*nvestigative Ophthalmology and Visual Science* 49: 1016-1022.

Poster Presentations

MUIR, M. S.; ROBERTSON, L. J. G.; DAVID, L. L. ; GATELY, K.; WOOD, J.; MORTON J.D. **(2007)**. Purification and Characterisation of Ovine Lp82 and Its Role in Cataractogenesis. *Investigative Ophthalmology and Visual Science*, 48: E-2048.

MUIR, M.S.; MORTON, J.D., ROBERTSON, L.J.G.. **(2005)**. Calpain II induces immobilisation of ovine lens crystallins in vitro and these insoluble proteins may induce cataract in vivo. *Investigative Ophthalmology and Visual Science*, 46: E-3882.

JAMES D. MORTON, J.D.; ROBERTSON, L.J.G.,LEE, H. Y.Y.; BICKERSTAFFE, R.; MCDERMOTT, J.D.; MUIR, M.S. AND WRIGHT, A. **(2007)** The inherited ovine cataract as a model of calpain action in human cataract. *Fifth General Meeting of the International Proteolysis Society*, Patras, 20-24 October 2007.

MCDERMOTT, J.D. MUIR, M.S., LEE H.Y.Y., ROBERTSON L.J.G., BICKERSTAFFE, R. AND MORTON J.D. **(2007)**. Calpain proteolysis of sheep lens cytoskeletal proteins. *FASEB SRC conference, Biology of the Calpains in Health and Disease*, Colorado, July 2007.

MORTON, J.D.; MUIR, M.; ROBERTSON, L.J.G.; HANCOCK, A.J. **(2004)** Proteolysis of ovine lens crystallins. *FASEB SRC conference Biology of the Calpains in Health and Disease,* Tucson, Arizona.

Oral Presentations

MUIR, M.S. **(2007**) Purification of Ovine Lp82 and its role in cataractogenesis. *New Zealand Society for Biochemistry and Molecular Biology, Canterbury Branch Meeting*. September 2007.

MUIR, M.S. **(2006)** Baa Baa Blind Sheep: Have you any sight? *Lincoln University Postgraduate Conference*. August 2006

MORTON, J.D.; ROBERTSON, L.J.G.; MUIR M.; DAVID L.L, **(2005)** The inherited ovine cataract as a model for the role of calpain in cataract. *Combio conference, Adelaide, Australia*. September 2005.

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