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# CALCIUM-INDUCED INSOLUBILIZATION OF CALPAIN IN THE LENS

by

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# ABSTRACT

The broad objective of this research was to investigate the mechanism for activation of calpain, a calcium-activated neutral protease, in the lens during cataract formation. Previous data from our laboratory implicated calpain in cataract formation. However, in order for calpain to be active in the lens, it must overcome its requirement for high calcium concentrations and the endogenous inhibitor, calpastatin. Since phospholipids lower calpain's calcium requirement for activation, we hypothesized that calcium causes translocation of calpain from the cytosol to cellular structures which are insoluble to mild extraction. This may be an important event in the activation of calpain in the lens.

In support of this hypothesis, we found that calpain associated with several different fractions of the lens pellet, including a membrane-rich fraction, and that calcium increased this association. Calcium increased the specific activity, the ratio of enzyme activity to μg calpain antigen, and the ratio of calpain to calpastatin activity in the insoluble protein fraction. This suggested that the calcium-insolubilized calpain is more active than soluble calpain.

In order to further elucidate the mechanism for activation of calpain in cataractogenesis, specific inhibitors of calpain were tested in lens organ culture. These inhibitors were found to be effective in preventing lens opacity and proteolysis. Calpain inhibitors blocked the autolytic degradation of insoluble calpain and allowed the accumulation of enzyme in the insoluble fraction. Its accumulation in the insoluble fraction suggested that calpain had translocated from the cytosol to the insoluble fraction during the formation of cataracts.

The calcium-induced insolubilization of calpain is significant, because the insoluble fraction may be a site of activation for calpain and because the insoluble pellet may contain important calpain substrates. It has also been

hypothesized that calpain escapes inhibition by calpastatin by binding to the membrane. However, we found several possible forms of calpastatin in the lens and were able to measure insoluble calpastatin activity. Future studies are needed to determine the interaction between insoluble lens calpastatin and calpain.

# I. INTRODUCTION

## A. The Lens

The overall, broad objective of this present research was to investigate the mechanism for activation of calpain, a calcium-activated neutral protease, in the lens during cataract formation.

The lens functions to transmit and refract light reaching the eye. In a healthy lens this causes precise focusing of light rays onto the retina. As a result, the primary purpose of the metabolic processes in the lens is to maintain lens clarity necessary for its proper functioning.

The transparency of the lens is due to the short-range packing of the proteins within it. These high concentrations of structural proteins, called crystallins, make up 90% of the soluble lens proteins and account for 20% - 60% of the lens wet weight (Bloemendal, 1982). This high concentration of crystallins, combined with their highly organized arrangement, contributes to the high index of refraction necessary for focusing light. Three major classes of crystallins exist in mammalian lenses based on elution during molecular sieve chromatography (Bloemendal,1985). These are alpha, beta, and gamma crystallins. The beta-crystallins are most abundant in rats.

The lens is derived from a single cell type (epithelial) with older cells in the central nuclear region. The lens retains all the cells produced throughout life (Marshall et al., 1982). The lens is divided into three regions (Fig. 1): the epithelial layer, the cortex, and the nucleus (Koretz and Handelman, 1988). The epithelial cells form a single cuboidal cell layer on the anterior surface of the lens. The epithelial cells move near the lens equator differentiating into secondary fibers which form the second region, the cortex. Cortical fibers

elongate into adjacent fibers and are pushed inward by the continual laying down of new fibers. Fibers eventually lose their nuclei and most of their organelles as they become mature fiber cells and extend from pole to pole. The third region, the lens nucleus is the inner core of fibers containing the original embryonic cells. The rate of protein synthesis in the lens nucleus is very slow (Wannemacher and Spector, 1968).

The cell membranes in the lens serve to provide an extensive cell-cell communication network between fiber cells necessary for the functioning of the lens. This is accomplished by two types of structures found in the cell membranes; gap junctions and square arrays. The gap junctions in the inner fiber cells compose 50% - 60% of the area of the membrane, allowing for exchange of ions and metabolites between cells (Kistler et al., 1985; Louis et al., 1989). The primary gap junction proteins are approximately 26 kDa and 70 kDa (identified as MP 26 and MP 70). Extensive communication between cells is especially important because the lens is devoid of a blood or nerve supply.

Square arrays are another structure seen with the electron microscope at the cell membrane and are postulated to allow tight stacking of fiber cells. Square arrays are distributed more in the nucleus than in the cortex and are composed mostly of MP26 (Costello et al., 1989; Fitzgerald et al., 1985). The morphological changes occuring during differentiation and elongation, and the difference in distribution of membrane structures between the nucleus and cortex necessitates extensive reconstruction of the lens membrane. These changes are dependent on the turnover of certain proteins, implying a physiological role for regulated proteolytic activity in the lens. These events in the change of cell morphology may be similar to the limited proteolysis of the erythrocyte membrane skeleton by calcium-dependent proteinases during fusion of red blood cell membranes (Croall et al.,1986). However, uncontrolled proteolytic activity at the cell

membrane would adversely affect the functioning of the lens. A major aim of the studies in this thesis were directed towards studying the role of proteolysis in cataract formation.

## B. Cataracts

A cataract is an opacity in the lens of the eye. Sixty percent of Americans ages 65 to 74 show some sign of cataract. Testimony presented to the United States Congress on May 4, 1990, estimated that as much as one billion dollars could be saved if the onset of cataracts could be delayed by ten years (Weingeist, 1990). Cataracts are the third leading cause of blindness in the United States.

While many different initial insults lead to cataracts, the chemical processes involved in opacification may be similar. A rodent model of cataracts used in our laboratory is the selenite-overdose cataract. It is characterized by a severe nuclear opacity, a five-fold increase in calcium levels before the cataract formation and extensive proteolysis (David and Shearer, 1984; Shearer et al., 1992; Hightower et al., 1987; David and Shearer, 1986). The mechanism for the increased calcium concentration is not known. Proteolysis of normally soluble crystallin proteins has been postulated to result in their insolubilization, causing scattering of light (David et al., 1986). The proteolytic changes in the alpha and beta-crystallins can be mimicked <u>in vitro</u> by incubating isolated lens proteins with the calcium activiated protease, calpain II (David et al., 1986). The increase in calcium occurring in cataractogenesis was sufficient for calpain activation (Hightower et al., 1987). Calcium ionophore, selenite and xylose cataracts in cultured lenses were reduced by E64, an inhibitor of cysteine proteases such as calpain (Shearer et al, 1991; Azuma et al., 1990). In addition, purified calpain caused lens beta-crystallins to become turbid due to precipitation (David et al., in

press). The insoluble protein that was formed was also similar to insoluble protein formed in vivo during selenite cataracts. Thus, calpain has been implicated in cataractogenisis in the rat lens. An objective of this study was to confirm the role of calpain in cataract formation, and to elucidate possible mechanisms by which calpain is involved.

## C. Calpain

Calpain (EC 3.4.22.17) is a non-lysosomal protease, ubiquitously distributed in various tissues and cells. The exact role of calpain in physiological processes has not been definitively elucidated (Murachi, 1983). Calpain hydrolyzes proteins into large fragments by limited hydrolysis with no readily identified amino acid cleavage sites (Murachi, 1983). A list of specific calpain substrates taken from a review by Murachi (1983) is shown in Table I. Calpain, however, probably does not act as a general protease. In rat lens, alpha and beta-crystallins, but not gamma crystallins, undergo calcium-dependent proteolysis (David and Shearer, 1986). In addition to the role of calpain in cataractogenesis in rodent models, calpain has been implicated in diverse physiological functions such as: regulation of protein kinase C (Melloni, 1985), cytoskeletal modification during platelet activation (Hayashi et al., 1992), regulation of hippocampal glutamate receptor (Lynch and Baudry, 1984) and muscle protein degradation after injury (Bhattacharyya et al., 1991).

Calpain was first identified in the brain in 1964 by Guroff. Calpain has both an 80 kDa catalytic subunit and a 30 kDa regulatory subunit (Fig. 2). The 80 kDa subunit has 4 domains: a cysteine protease domain, a calmodulin-like calcium-binding domain, and 2 domains of unknown function (Suzuki, 1987). The calmodulin-like domain has 4 helix-loop-helix structures capable of binding

calcium. Calpain is normally inactive without calcuim and some investigators suggest the activity of the protease domain may be repressed by interaction with the other domains in the calpain molecule (Suzuki, 1987). After binding of calcium, calpain is believed to undergo a conformational change, exposing the active site. The 30 kDa subunit has a calmodulin-like domain at its carboxyl-terminus, which is highly homologous to the calmodulin-like domain of the 80 kDa subunit. The amino-terminus of the 30 kDa subunit is composed of a glycine-rich hydrophobic domain, thought to be capable of interacting with membranes due to a conformational change in the presence of calcium (Imajoh et al.,1986). This N-terminal hydrophobic region is separated from the rest of the 30 kDa subunit by a flexible region containing glycine and proline units (Emori et al., 1986).

D. Calpastatin

The endogenous inhibitor of calpain, called calpastatin, was first isolated in 1978 (Nishiura et al., 1978). Subsequently, calpastatin was found to be widely distributed and co-localized with calpain (Murachi, 1983; Kumamoto et al., 1992). Calpastatin does not share sequence homology with any other known proteins and is specific for calpain, having no or little effect on other proteases (Nakamura et al., 1984). The inhibitor is rich in hydrophilic residues but poor in aromatic residues (Nakamura et al., 1984; Takano et al., 1986; Emori et al., 1987). The inhibitory activity of calpastatin is resistant to heat treatment, and activity is retained even after SDS-polyacrylamide gel electrophoresis (Nakamura et al., 1984). Heating is often used as a purification step in isolating calpastatin from tissues.

predominant form of calpain in the lens, and has been assayed in several different species (Yoshida et al., 1985; David and Shearer, 1986).

In the presence of calcium, calpain II autolyzes very rapidly and the calcium requirement for activity is lowered into the micromolar range (DeMartino et al.,1986; Thompson et al., 1990). Suzuki et al. (1981) first demonstrated that calpain II from chicken skeletal muscle autolyzed rapidly with 0.5 mM $CaCl_2$. This resulted in decreased molecular weight of the large subunit from 82 kDa to 79 kDa. The calcium requirement for half-maximal activity of the 79 kDa form of calpain was decreased from 400 mM to 30 mM. Coolican et al. (1986) also reported autolysis of calpain II lowered the calcium requirement for activity. Autolysis can also produce extensive degradation resulting in loss of activity (Crawford et al., 1987).

## F. Regulation of Calpain Activity by Phospholipids

The overall concentration of free calcium in normal cells (300 nM - 1200 nM) is not high enough to activate calpain II (Cong. et al., 1989). Thus, in normal cells another means of activating calpain is necessary. Data from several laboratories have shown that the calcium activation requirement of calpain was reduced in the presence of specific phospholipids. Coolican and Hathaway (1984) reported that phosphatidylinositol and, to a lesser extent, dioleoylglycerol, reduced the calcium requirement for autolysis of calpain II from bovine aortic smooth muscle from 680 mM to 87 mM for half-maximal activity. Phosphatidyl-inositol, phosphatidylserine and dioleoylglycerol stimulated purified calpain II activity from bovine myelin by 193%, 89% and 78% respectively, while also causing a 10 - 20 fold reduction in the calcium requirement for calpain activation (Chakrabarti et al., 1990). Garret et al. (1988) reported evidence for binding

between calpain II and phosphatidylinositol, phosphatidylserine, and phosphatidylcholine vesicles independent of calcium. Phospholipid vesicles had a similar effect on the activation of erythrocyte calpain I (Pontremoli et al., 1985). Calpain may interact with the polar head of phosphatidylinositol or through a hydrophobic region. Using fragmented calpains, Crawford et al. (1987) found that the N-terminal hydrophobic region of the 30 kDa subunit was necessary for the phosphatidylinositol effect. The above results suggest calpain may interact with biological membranes.

Human erythrocyte calpain (calpain I) was shown by several investigators to bind erythrocyte membrane. Kuboki et al. (1987) added calpain and calcium to inside out membrane vesicles and found the intact enzyme (80 kDa) and the autolyzed form (76 kDa) associated with the membrane vesicles. This suggested a calcium-dependent binding of calpain to the plasma membrane where calpain then underwent autolysis. Upon further fragmentation to a 36 kDa fragment, it was released from the vesicle into the supernatant. Pontremoli et al. (1985) found similar binding of calpain to inside-out human erythrocyte vesicles. The above studies indicate binding of calpain to the membrane is a method for activation of calpain. Therefore, we felt it was important to investigate whether a similar method occurred in the lens.

## G. Distribution of Calpain

Calpain has been detected in the soluble fractions of almost every tissue (Murachi et al., 1981; Kawashima et al., 1988). Immunolocalization studies have shown that calpains are diffusely located in the cytosol of many tissues including human platelets (Taylor, 1991), porcine kidney (Yoshimura et al., 1984), human pancreatic beta-cells (Kitahara et al., 1985), rat cerebellum neuronal cells

(Hamakubo et al., 1986), and mature rat skeletal muscle cells (Kumamoto et al., 1992). However, some reports suggest that calpain interacts with the particulate fraction. Hatanaka et al. (1984) isolated 2% of total cellular calpain at the membrane in human red blood cells. Calpain II was detected in myelin membranes by ELISA but only with the inclusion of detergent, suggesting calpain was deeply imbedded in the membrane (Yanagisawa et al., 1988). Platelet membrane contained 10% of the total platelet calpain antigen and 24% of the total calpain activity (Schmaier et al., 1990). In neuronal tissues, an even higher percentage of calpain is distributed in the particulate fractions. Fifty percent of the calpain was distributed in myelin and 20% - 30% was in the cytosol (Chakrabarti et al., 1988). Calpain distributed along the membrane may be regulated differently than cytosolic calpain, and calpain may act on different substrates at the membrane. The differing subcellular distribution of calpain between tissues suggests its physiological role at the membrane may differ from the cytosolic calpain. Thus, another purpose of the present research was to determine the subcellular distribution of calpain in the lens to discover how calpain could become active.

Factors affecting the subcellular distribution of calpain may include increases in calcium and/or inhibition of calpain II by its endogenous inhibitor, calpastatin. The binding of exogenous calpain to rabbit liver plasma membrane with subsequent autolysis was calcium-dependent and reversible. Calpastatin prevented the binding (Suzuki et al., 1988). Calpain II, isolated from frog muscle, degraded proteins in skeletal muscle plasma membranes in a calcium dependent manner and the degradation was completely blocked by calpastatin (Zaidi and Narahara, 1989). In these experiments, calpain and calpastatin were preincubated before calpain could interact with the membrane. When tissues were homogenized directly in 50 μM $CaCl_2$, 30% - 60% of the cytosolic calpain II

activity was found in the particulate fraction (Gopalakrishna and Barsky, 1986). When extracted with EGTA, the majority of calpain II isolated from the calcium-treated particulate fraction showed optimal activity at low calcium concentrations, whereas there was no change in the calcium requirement for cytosolic calpain isolated from calcium-treated homogenates. In this same study excess calpastatin was added to crude homogenates and did not inhibit binding of calpain. However, if purified calpain and calpastatin were first incubated in calcium and then added to isolated particulate fractions, there was no detectable binding of calpain. The authors concluded prior interaction of calpain with the membrane in the absence of calcium prevents calpastatin from inhibiting the binding of calpain. Taken together these studies show calpain can bind membrane preperations and this binding is affected by calcium and calpastatin.

Studies specifically investigating calpain I, also demonstrated calpain binding to the membrane. Calpain crosslinked to other cytosolic proteins in cultured human erythrocytes (Sakai et al., 1989). However, when calcium ionophore was added to the medium, the amount of membrane proteins crosslinked to calpain increased. Stimulation of human neutrophils with phorbol myristate resulted in the translocation of 25% - 40% of the cellular calpain I activity to the plasma membrane (Pontremoli et al., 1989; Melloni et al., 1985). The calcium requirement for activity was also reduced. Presumably calpain was not prevented from translocating to the membrane by endogenous calpastatin. The above results confirm the effect of calcium on calpain binding to the membrane, and they suggest that endogenous calpatatin is not entirely effective in preventing calpain binding to the membrane. The possible effect of calcium on calpain distribution is an important question because of the reduced calcium requirement of calpain at the membrane. Thus, a purpose of the present

research was to determine the effect of calcium on calpain distribution in the lens.

Further evidence for activity of calpain at the membrane comes from proteolysis of cytoskeletal and membrane proteins. In vitro substrates of calpain include the cytoskeletal proteins; vimentin, spectrin, talin, ankyrin, microtubule associated proteins, and the membrane proteins: $Ca^{2+}$-ATPase, epidermal growth factor, receptor, and fodrin (Bolvin et al., 1990; Beckerle et al., 1986; Hall and Bennett, 1987; Fischer et al., 1991; Wang et al., 1989; Nelson and Traub, 1982; Baudry and Lynch, 1980; Cassel and Glaser, 1982).

The ability of calpain to cleave insoluble proteins has led investigators to study possible physiological roles for calpain. Calpain has been shown to be involved in mitosis during redistribution of calpain from the plasma membrane to the mitotic spindle during mitosis (Schollmeyer, 1988). Injection of calpain promoted the onset of mitosis. Calpain has been implicated in memory storage by degrading fodrin, and this exposes glutamate receptors in the forebrain synaptic membranes (Lynch and Baudry, 1984). Activation of calpain in erythrocytes and degradation of spectrin, band 3 and band 4.1, and morphological changes in cell shape were blocked by cBz-Leu-Leu-Leu-aldehyde, a specific inhibitor of calpain (Hayashi et al., 1992, 1991). The authors concluded calpain was essential for membrane fusion.

## H. Regulation of Calpain by Calpastatin

Calpain is not only regulated by calcium, and possibly by binding to the membrane, but also by its endogenous inhibitor calpastatin. The ratio of calpain to calpastatin differs between tissues suggesting that the ratio of calpain to calpastatin may be important in regulating calpain activity in different cellular

functions. In rat liver, skeletal muscle, cardiac muscle and in human erythrocytes, calpastatin activity exceeds calpain activity by several fold (Murachi, 1983). The reverse was true in the other tissues measured. It is conceivable that an alteration in the ratio of calpain to calpastatin is a mechanism for regulating calpain activity, either in a normal function or in a disease state. This is important because a mechanism is needed by which calpain can overcome inhibition by excess calpastatin.

The interaction of calpastatin with calpain may be complicated by the following factors:

1. Calpain can cleave calpastatin.
2. Different forms of calpastatin exist among tissues and species.
3. Calpastatin is co-localized with calpain.

An example of calpastatin acting as a substrate for calpain is found in bovine heart (Mellgren et al., 1986). Calpastatin fragments were detected when calcium was added to crude heart extracts. Likewise, calpain from rabbit skeletal muscle cleaved calpastatin (Nakamura et al., 1989). Cleavage of calpastatin occurred even though theoretically enough calpastatin activity was present to completely inhibit calpain activity. A 15 kDa fragment of calpastatin retained considerable activity. The physiological significance of the proteolysis of calpastatin by calpain is not known and its occurrence has yet to be proven in vivo. However, Salamino et al. (1991) reported decreased calpastain activity in erythrocytes of hypertensive patients. A similar decrease in inhibitory activity occurred when erythrocytes were incubated in calcium and calcium ionophore. While this suggests calcium dependent proteolysis had occurred in vivo, the authors did not investigate whether or not calpastatin breakdown products were

present. Fragmentation of calpastatin by calpain may or may not be a mechanism by which calpain overcomes inhibition by calpastatin.

A wide range of values are reported in the literature for the molecular weight of calpastatin. This is due to several factors. Calpastatin shows considerable molecular diversity both at the pre-translational and the post-translational stages. Thus, there are numerous possible interactions with calpain. The non-homologous N-terminal domain (domain L) of rat calpastatin contains multiple deletions, including a 38-residue deletion not found in other mammalian calpastatins (Lee et al., 1992; Ishida et al., 1991). Calpastatin from rat skeletal muscle eluted off DE32 chromatography at two different salt concentrations (Pontremoli et al., 1991). The later eluting peak was more inhibitory against calpain II than calpain I. In pig brain, two peaks of calpastatin activity were eluted off DEAE-cellulose at different salt concentrations. Results of this study differed from those of Pontremoli et al. in that the later eluting peak did not inhibit calpain II, but only calpain I (Takano et al., 1989). Evidence also supports the existence of multiple forms of human calpastatin. Differences in the amino-terminal domains of human calpastatin were detected by the reverse transcription polymerase chain reaction (Lee et al., 1992), and two different molecular forms of liver-type calpastatin, 102 kDa and 94 kDa, were found in various cell lines of the human hematopoietic system (Adachi et al., 1988).

Several different reasons may explain the molecular diversity of calpastatin at the post-translational stage. Calpastatin while heat stable is susceptible to proteolytic attack during purification, and may be similarly susceptible in vivo (Salamino et al., 1991). Purified calpastatin and calpastatin expressed in E. coli ran abnormally high on SDS-PAGE at a molecular weight approximately 1.5 times greater than the molecular weight deduced from the sequence (Takano et al., 1988; Emori et al., 1987). Monomers of calpastatin may

associate in the native state and can be isolated by gel filtration in their high molecular weight form. Human erythrocyte calpastatin was determined to be 280 kDa on a sephacryl G-200 column but 70 kDa by SDS-PAGE (Takano and Murachi, 1982). The above are examples of the molecular diversity of calpastatin. This diversity may enable specific inhibitory functions in different tissues.

Where calpain and calpastatin are localized in the cell may affect activity of calpain. A mechanism for activation of calpain may involve translocation of cytosolic calpain to the membrane in the presence of calcium. This may cause the calcium requirement for activation of calpain to be reduced (Mellgren, 1987; Suzuki et al., 1987) (Fig. 4). Using this mechanism, calpain at the membrane may escape the inhibition of the predominantly cytosolic calpastatin. Since calpastatin is a hydrophilic molecule it would remain in the cytosol. In support of this hypothesis, Gopalakrishna and Barsky (1986) homogenized rat tissues in calcium and found an increase in calpain activity associated with the particulate fraction but did not find calpastatin activity. The insoluble fraction may be a site where calpain could be active without interference from calpastatin.

Recent data, however, suggest that calpastatin is associated with the membrane in some tissues. Calpastatin was detected immunohistochemically at the cell membrane in rat central nervous system and in the sarcolemma of bovine heart myocytes (Kamakura et al., 1992; Lane et al, 1985). Another study isolated calpastatin from bovine myocardial sarcolemma preparations (Mellgren et al., 1987a) and reported that calpastatin inhibited the conversion of sarcolemmal protein kinase C to protein kinase M by calpain (Mellgren et al., 1987b). Purified myocardial and liver calpastatin bound acidic phospholipids, but erythrocyte calpastatin (missing the N-terminus) or calpastatin fragments did not bind acidic phospholipids (Mellgren, 1988; Mellgren et al., 1989). The N-terminal domain of calpastatin has an isoelectric point equal to 10.3 while the other 4

domains have isoelectric points between 4.3 and 4.9 (Lee et al., 1992). Therefore, the N-terminal domain may allow for interaction with acidic phospholipids at the membrane. Binding of calpastatin to membranes other than the sarcolemma has not been documented, and it remains to be determined if this is a more general phenomenon. Nonetheless, the hypothesis that calpain escapes inhibition from calpastatin by binding intracellular membranes is attractive and was a stimulus to experiments in this thesis.

## I. Hypothesis and Summary of Literature Review

Calpain is a calcium-activated protease abundant in the cytosol of every mammalian tissue examined, including the lens (Yoshida et al., 1985). Calpain is believed to be a major neutral protease in the lens and has been postulated to play a role in cataractogenesis (Shearer et al., 1992). Lens crystallins become insoluble when proteolyzed by calpain in vitro (David et al., in press). Similar proteolysis may occur in vivo causing opacity of lenses. However, the predominant type of calpain in the lens is calpain II, which requires high amounts of calcium for activation (Yoshida et al., 1985). Abundant amounts of calpastatin, an endogenous inhibitor of calpain, was also isolated from the cytosol of the lens, suggesting that if calpain were to be involved in cataractogenesis, mechanisms must exist for an increasing intracellular calcium and for allowing calpain to escape inhibition by calpastatin. This would be especially relevant to human lenses, in which calpastatin is in considerable excess to calpain. Truscott et al., (1989, 1990) reported calcium-induced breakdown of spectrin and vimentin in cultured rat lenses suggesting that calpain may be involved in rearrangement of the cytoskeleton. If calpain is to

function in normal physiological processes in the lens, such as cytoskeletal rearrangement during maturation of fiber cells, a mechanism must also exist to lower calpain's calcium requirement for activation.

Several pieces of data have led to the hypothesis that calpain may be activated by a calcium-induced translocation to the membrane (Fig. 3) (Pontremoli et al., 1985a). First, phospholipids lower the calcium requirement for activation of calpain (Garret et al., 1988) and secondly, calpain binds biological membranes in a calcium-dependent fashion (Gopalakrishna and Barsky, 1986). At the membrane, calpain may escape inhibition by cytosolic calpastatin and cleave specific membrane proteins. Calpain may also be released into the cytosol as an active enzyme.

While this hypothesis sounds attractive, it has not been met without controversy. Calpain in its autolyzed form was not found in DEAE elution profiles of cell extracts from bovine skeletal muscle, indicating bovine skeletal muscle may not normally contain autolyzed calpain (Edmunds et al., 1991). The calcium requirement for activity of calpain II in the presence of phospholipids was not lowered to a calcium concentration present in normal cells. These investigators proposed that autolysis of calpain may be involved in the turnover of calpain. In support of this hypothesis they reported that autolyzed calpain had less thermal stability than unautolyzed calpain.

In order to resolve some of this controversy, one purpose of the present studies was to determine the subcellular distribution of calpain in the lens and to determine the effect of calcium and calpastatin on this distribution. Another purpose was to determine if calpain undergoes a translocation-activation event during cataract formation in cultured lenses and if various calpain-specific inhibitors could prevent this event at the same time as preventing cataracts. Finally, since calpastatin is a diverse molecule, calpastatin in the lens was

characterized and possible differences amoung calpastatin in other tissues were explored. Differences in lens calpastatin may be a mechanism for regulating calpain in the lens.

The significance of such studies is that they would elucidate whether or not calpain was activated in the lens by calcium-induced translocation to the insoluble fraction and if this event was important for expression of activity in the presence of calpastatin.

# II. MATERIALS AND METHODS

## A. Materials

### 1. Source of Lens Tissue

Lenses were obtained from several different species. Sprague-Dawley rats were purchased from Charles River laboratories (Wilmington, MA). The lenses were dissected from enucleated eyes, decapsulated and used either as whole lenses or further separated by dissection under a low power microscope into cortical and nuclear regions. Porcine and bovine lenses were collected from eyes purchased from Carlton Packing Company (Carlton, OR). Human donor eyes were obtained from the Lions Eye Bank of Oregon. Cortex and nucleus from human lenses were separated with a 6 mm trephine within 24 hours post-mortem.

### 2. Age of animals

Ages of rats ranged from 2 to 9 weeks old. Culturing experiments were done with lenses from 4 week old rats. Lenses from younger rats did not survive the dissection procedure well enough to be cultured. Lenses from various aged rats were used to investigate the effect of calcium on the distribution of calpain since the effect of calcium appeared to be the same regardless of the age of the rats. Calpastatin from different aged rats was isolated from the lens since calpastatin activity did not change with age (Varnum et al., 1989). Porcine eyes obtained from the slaughter house were over 6 months and bovine eyes were at least 1 year.

3. Reagents

The synthetic cysteine protease inhibitors, calpain inhibitor I and II and leupeptin, were commercially available from Boerhringer-Mannheim (Indianapolis, IN). CBz-ValPheH was a gift from Merrell-Dow (Cincinnati, OH). E64 was from Peptide Institute, Inc. (Osaka, Japan). Other reagents used were reagent grade.

B. Methods

1. Isolation of Calpain

Purified calpain was used as a standard for quantitating unknown amounts of calpain on immunoblots and to measure inhibitory activity in different samples.

Calpain was isolated from the hind leg muscles of six month old rats. Muscle tissue was homogenized at 100g /200 ml buffer A containing 20 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA, and 10 mM b-mercaptoethanol, plus 100 mM NaCl, and then centrifuged at 10,000 x g for 30 minutes. The supernatant was made 45% saturated in $(NH_4)_2SO_4$ and recentrifuged. The pellet was then suspended in buffer A, centrifuged at 10,000 x g for 30 minutes, filtered through Watman #2 filter paper, and applied to a Bio-Gel A DEAE (5 cm x 20 cm) at 0.5 ml sample/ml of gel volume. Calpain was eluted with a 0 - 500 mM linear NaCl gradient, and active fractions were concentrated, and subsequently applied to a Bio-Gel A 1.5 M gel filtration column (3 cm x 90 cm). Gel filtration fractions showing calpain activity were concentrated and applied to a Phenyl 5PW HPLC column (7.5 mm x 7.5 cm). The increase in specific activity of calpain by this procedure is shown in Table 2. Purified calpain was used as calpain standards for immunobloting or used to determine calpastatin activity of unknown samples.

The purification was probably several hundred fold over the crude homogenate. Calpain was similarly prepared from bovine or porcine heart freshly obtained from the slaughter house. Cardiac muscle was trimmed of vessels and connective tissue, passed through a meat grinder, and homogenized in a Waring blender and the above steps followed.

2. Isolation of Calpastatin

In order to generate a polyclonal antibody calpastatin was purified from bovine or porcine cardiac muscle by a modification of a method by Mellgren et al. (1988) . Hearts from animals killed on the same day were purchased from a local slaughter house and transported on ice to the laboratory. All procedures were carried out at 4°C. One hundred grams of trimmed and sectioned heart were homogenized in 250 mls of buffer A, then centrifuged at 10,000 x g for 15 minutes. The supernatant was filtered through glass wool and immediately heated to 95°C for 10 minutes, followed by centrifugation at 10,000 x g. The heat soluble proteins were precipitated with TCA at a final 15% weight to volume ratio. The precipitate was collected by centrifugation at 10,000 x g for 15 minutes. The supernatant was discarded, and the pellets were dissolved in 250 mM Tris (pH 8.0), 1 mM EGTA, 1 mM EDTA, and 10 mM β-mercaptoethanol. The pH of the suspended pellet was adjusted from 4.5 to 7.5. The solution was then dialyzed against buffer A overnight before being applied to a Bio-Gel A DEAE column. Proteins were eluted with a 0 - 400 mM linear NaCl gradient. Calpastatin activity eluting at 130 - 200 mM NaCl was pooled and applied to an Affi-gel blue column. Most of the protein did not bind to the Affi-gel column. However, calpastatin activity eluted off the column between 400 - 500 mM NaCl where no protein absorbance peak was detected. The increase in specific activity

during purification of calpastatin is shown in Table 3. Calpastatin at several fold excess molar ratio to calpain inhibited calpain greater than 80%.

3. Production of Calpastatin Polyclonal Antibody

Calpastatin antibody was used to detect changes in calpastatin molecule in the lens which would not be reflected in changes of its activity. Calpastatin purified through the Affi-gel step was further purified by electrophoresis, and the 130 kDa calpastatin band was sliced from the gel and electroeluted with 25 mM Tris, 192 mM glycine, and 0.1% SDS buffer. The electrophoretically purified calpastatin was concentrated (Amicon, Beverly, MA) and injected into rabbits for polyclonal antibody production. Three rabbits were each injected with 300 μg of electroeluted calpastatin in Freud's complete adjuvant. Two subsequent injections of 100 μg calpastatin in Freud's incomplete adjuvant were given at one month intervals. Bleeds were taken with each injection and at three later time points up to nine months. Calpastatin antiserum was affinity purified using CNBr-activated Sepharose 4B coupled to calpastatin. Calpastatin in 0.1 M NaHCO3, pH 8.3, containing 0.5 M NaCl was stirred overnight with CNBr-activated Sepharose (0.25 mg of calpastatin to 150 mg Sepharose in 1 ml volume). The residue active groups were blocked with 0.2 M glycine, pH 8.0. The excess adsorbed protein was washed away with sodium acetate buffer (0.1 M, pH 4). Antibody was eluted from the CNBr-activated Sepharose 4B with 0.1 M phosphoric acid in 10% isopropanol and the immunoreactive fractions detected with ELISA.

A 500-fold dilution of crude antiserum reacted strongly with 0.5 μg of calpastatin in an ELISA assay. There was no immunoreaction with preimmune serum even at only 10-fold dilution of the preimmune serum. The specificity of the antiserum to rat and porcine tissues was determined by immunoblotting.

Stock solution of 0.3 mg/ml affinity purified antiserum was diluted 30-fold in 1% geletin-Tris-buffered saline/ 0.05% Tween 20 (TTBS). The antibody reacted strongly to a 130 kDa band in heat soluble proteins from porcine heart (Fig. 4, lane 1) and not with any of the other bands that were seen on the Coomassie blue stained gel (Fig. 5). There was no immunoreaction with preimmune serum. Immunoreactive bands were detected in heat soluble proteins from rat liver but not leg muscle (Fig. 6, 7). A doublet at 100 kDa was seen in rat liver. Calpastatin from rat muscle previously purified on a Bio-Gel A column did not bind Affi-gel blue. This suggested that calpastatin in rat muscle underwent a post-translational modification, resulting in both a loss of antigenicity and Affi-gel blue binding, but no loss in inhibitory activity.

4. Isolation of Calpain in Subcellular Fractions

In order to test the hypothesis that calpain may be activated in the lens by interaction with the membrane, we first determined if calpain was present in the lens pellet which would include the membrane. Next, we determined how calcium and calpastatin affected the distribution of calpain between the soluble and insoluble fractions. This was accomplished by chemical extractions of the pellet.

a. Chemical fractions

Lenses from 24 day old rats were homogenized in buffer B containing 20 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM DTE. To buffer B 100 mM KCl and 2 mM phenylmethylsulfonylfluoride (PMSF) was added and samples were centrifuged at 10,000 x g for 30 minutes. The insoluble pellet was washed twice in the above buffer and then extracted in 7 M urea in 50 mM Tris and 1 mM EGTA (pH 7.4) (Russell, et al., 1981). The urea-insoluble pellet was resuspended in 0.1 M NaOH containing 10 mM β-mercaptoethanol. Following

centrifugation the remaining insoluble proteins were dissolved in 1% SDS. The above procedure was repeated with fifteen nine-week old rats in which one lens from each rat was pooled and treated as above and the other lens processed with 1 mM $CaCl_2$ in all the buffers. E64 was included in the initial homogenizing buffer to prevent rapid degradation of calpain in the high calcium buffer.

b. Sucrose gradient fractions.

Membrane-rich fractions were obtained using sucrose gradients. Thirteen day old rat lenses were homogenized in buffer C containing 5 mM Tris (pH 8.0), 5 mM β-mercaptoethanol, 1mM EGTA, 0.5 mM PMSF, and 100 μM E64 (Kistler, et al., 1986). The homogenate was split in half and 2 mM $CaCl_2$ was added to one of the halves. The insoluble pellet was isolated by centrifugation at 10,000 x g for 20 minutes and washed three times in the same buffer. The washed pellet containing 0.6 mg protein was suspended in 1 ml of 8% sucrose (w/w) in buffer C and layered onto 25%, 45%, 50%, and 55% sucrose solutions in a total volume of 5 mls. Ultracentrifugation was performed in a Beckman SW 50.1 rotor head (Beckman, Inc., San Francisco, CA.) for 90 minutes at 100,000 x g. The material at the interfaces was collected, diluted 4-fold in buffer C, and recentrifuged for 30 minutes at 100,000 x g. The pellets obtained from each interface were resuspended in buffer B and analyzed for protein, phosphate, calpain, and lens membrane protein MP26. Phosphate and MP26 were used as membrane markers. Protein was measured using the Bio Rad protein assay (BioRad, Richmond, CA.). Phosphate content was determined with the Fiske-Subbarow reagent according to manufacturer's directions (Sigma, St. Louis, MO.) and involved an organic extraction in chloroform: methanol (1:1) mixture followed by perchloric acid digestion. MP26 was identified by SDS-PAGE (12% gels) after

staining with Coomassie blue. Calpain was identified by immunoblotting described below.

5. Measurement of Calcium Dependency and Reversibility of Calpain Insolubilization

The calcium dependency for binding of calpain to the insoluble pellet was determined by homogenizing lenses from five week old rats in the presence of increasing amounts of added calcium (0 to 1 mM). The inhibitor peptide CbzValPheH was added at 0.24 mM concentration to prevent autolysis of calpain. (CbzValPheH was used because it was more readily available in our laboratory than E64 at the time this experiment was conducted.)

In order to determine if insolubilized calpain showed enzyme activity, the following experiment was performed. Calpain activity and immunoreactivity were measured in: water soluble; chelator extractable; and Triton-X 100 solublized lens proteins. Material was isolated from 200 lenses taken from 24 day old rats. Homogenization was carried out in 40 mls of 20 mM Tris (pH 7.5), 100 μM EGTA, 2 mM DTE, and 0.5 mM PMSF at 4°C. The homogenate was split in half and 200 μM $CaCl_2$ was added to one portion. This allowed measurement of both the EGTA-bound and calcium-bound calpain The pellets were rehomogenized three times in the appropriate buffer. Calpain was extracted from the total insoluble pellets by homogenizing three times in buffer B. This calpain was designated "chelator extractable". The remaining pellets were extracted overnight in 1% Triton X-100 followed by sonication. Triton X-100 was then removed using Pierce Extracti-gel (Pierce, Rockford, Il.) because Triton X-100 enhances calpain activity. All samples were applied to a TSK DEAE 5-PW column and proteins eluted with a linear gradient of 0.0-0.5 M NaCl in buffer B. Calpain activity was assayed using FITC-labeled caesin (David and Shearer,1986)

as described below. Enzyme linked immunosorbent assays (ELISA) on DEAE fractions, were preformed using calpain II antibody.

6. Lens Culture and Production of Cataracts In Vitro

The hypothesis that calpain may be regulated in the lens by interaction with insoluble components, and that this may be an event occurring during cataract formation was tested in cultured lenses.

Lenses were obtained from 4.5 week-old Sprague-Dawley rats by removing the posterior portion of the globe prior to dissecting away the suspensory apparatus. Lenses were kept at 37°C under 5% $CO_2$ in minimum essential medium (Gibco Laboratories, Grand Island, NY), supplemented with 10% fetal bovine serum (Gibco). The cataractogenic agents used to produce cataracts in vitro were either 10 μM calcium ionophore A23187 (Sigma) for 24 hours, or 25 μM sodium selenite in medium supplemented with 1 mM $CaCl_2$ for three days. When cataract inhibitors were tested, inhibitor was added to the culture medium for 12 hours prior to addition of the cataractogenic agent. The lenses were removed from the cataractogenic agent, placed in medium with inhibitor alone, and cultured an additional four to ten days with medium changes every two days. The inhibitor concentration used was based on the highest concentration not toxic to the lens when lenses were incubated in inhibitor alone. Inhibitor structures are shown in Table 4.

Following incubation, lenses were evaluated for clarity by densitometric analysis. The mean density of the nuclear region of the lens was determined by computer imaging and expressed as the average pixel density on a gray scale of 0-255 (Fig. 8). The density of the surrounding medium was subtracted as background. For each determination, lighting conditions were adjusted so that the average pixel density of a grey scale standard was the same.

Proteolysis of lens proteins was detected by electrophoresis of proteins from control lenses and cataractous lenses. Lenses were weighed and then homogenized in buffer B, (2 lenses/400 µl). Soluble and insoluble fractions were separated by centrifugation at 10,000 x g for 30 minutes. The insoluble fraction was further washed three times in the above buffer, dissolved in 2% SDS, and the lens proteins separated by SDS-PAGE (12% acrylamide/0.3% bis gel).

7. Lens Uptake of E64

The uptake of E64 into the lens was measured by reverse-phase HPLC. Lenses were homogenized in Milli Q purified water (Millipore, Bedford, MA.) at 2 lenses/ 500 µl, the particulate removed by centrifugation at 10,000 x g for 30 minutes, the soluble proteins precipitated by addition of trifluoroacetic acid (TFA) at a final 5% concentration, and centrifuged as above. The TFA soluble supernatant was concentrated to dryness by vacuum centrifugation. Samples were then dissolved in 100 µl HPLC reagent grade water and auto-injected onto a 4.6 x 150 mm Nova Pak C-18 column (Waters), maintained at 40°C. E64 was eluted with a linear 0-10% acetonitrile gradient in 1% phosphoric acid and the absorbance of E64 in the eluate measured at 210 nm. E64 eluted at 20 min, and the concentration was determined by integration of the eluate peak compared to E64 standards. No co-eluting peak was detected in normal lenses or in lenses incubated in cataract agent alone. Fifty-four to 64% of known amounts of E64 added directly to crude lens homogenates were recovered. Therefore, the concentration of E64 reported below was corrected for recovery and lens water content. The water content for cataractous lenses used for the calculations was 70%, and 60% for normal lenses (Azuma, et al., 1991).

8. Electrophoresis and Immunoblotting

Lens proteins were separated by polyacrylamide gel electrophoresis (Laemmli, 1970). Discontinuous gels (7 x 8 cm mini-gels, 0.75 mm thick) containing 12% acrylamide/0.3% bis separating gel, and a 4% acrylamide/0.1% bis stacking gel, were run for 150 volt-hours. The gels were stained with Coomassie blue. Proteins were transferred using the procedure of Towbin et al. (1979). Proteins from gels were transferred onto PVDF membrane (Millipore) between 25-45 minutes at 80 mAmp. After the transfer, the PVDF membrane was blocked with 3% gelatin in Tris-buffered saline (TBS), and then washed three times in TBS /0.05% Tween 20 (TTBS). PVDF membranes were incubated in primary antibody to calpain or to calpastatin. Calpain antibody, previously prepared in our laboratory, and affinity purified (0.5 mg/ml), was diluted 1:250 in 1% gelatin-TTBS. Affinity purified calpastatin antibody (0.3 mg/ml) was diluted 1:30 or crude antiserum against calpastatin was diluted 1:500. The PVDF membrane was washed three times with TTBS and then incubated with secondary antibody (goat anti-rabbit IgG) coupled to alkaline-phosphatase (Bio-Rad, Richmond, CA). The membrane was washed two times with TTBS and two times with TBS before the immunoreaction was detected with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Purified rat calpain II carried through an identical process was used as a standard. Molecular weight markers were biotinylated broad range standards from Bio-Rad containing lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), Carbonic anhydrase B (31.0 kDa), ovalbumin (45.0 kDa), serum albumin (66.2 kDa), phosphorylase B (97.4 kDa), β-galactosidase (116.3 kDa), and myosin (200.0 kDa). An avidin alkaline-phosphate conjugate (Bio-Rad) was included with the secondary antibody for detection of the molecular weight markers.

Calpain antibody was previously prepared in our laboratory by Dr. Larry David. The 80 kDa subunit of calpain stained heaviest and most rapidly on an immunoblot in a dose dependent manner (Fig. 9). There were no Coomassie blue staining bands in the region of the 80 kDa band. Calpain antibody also reacted but to a lesser degree with the 30 kDa subunit of calpain. Diffuse staining of some of the low molecular weight crystallins was seen. These proteins are present in the lens in high concentration and are clearly visible on Coomassie blue stained gel.

The relative density of both gels and immunoblots were estimated by scanning, using a Hoefer GS-300 densitometer (Hoefer Scientific Instruments, San Francisco, CA) in the transmittance mode for gels and the reflectance mode for immunoblots. Some gels and immunoblots were scanned using a video camera connected to a computer imaging program: Quick Image 24 (Mass Micro-systems, Inc.). Images were captured and then saved in an Image 1.31 format (Wayne Rasband, National Institute of Health), and the density of the bands was measured in pixels on a scale of 0-255. Linearity of staining of the 80 kDa band was established with increasing amounts of calpain standard (Fig. 9). Unknown amounts of calpain were quantitated using the linearity of the calpain standards. Proteins were quantified with dye binding reagent (Bio-Rad) with BSA as standard.

Calpain and calpastatin in DEAE fractions were also measured with enzyme linked immunosorbent assays (ELISA). Ninety-six well plates were coated overnight with antigen diluted 1:1 in 0.1M $NaHCO_3$ (pH 9.3). Wells were then suctioned and blocked with 1% bovine serum albumin in phosphate buffered saline (BSA-PBS). Wells were again suctioned and 50 µl of the first antibody ( 1:1000 dilution for calpain antibody (stock=0.5 mg/ml) and 1:100 dilution for calpastatin antibody (stock=0.3 mg/ml) was added for 1-2 hours.

Wells were washed three times with PBS and 50 µl of the secondary antibody (1:2500 of goat anti-rabbit alkaline phosphate conjugate) in 1% BSA-PBS added. Color reaction with nitrophenylphosphate was detected by change in absorbance at 405 nm according to the manufacturers specifications (Bio-Rad). Unknown amounts of calpain were quantitated against increasing amounts of calpain standards. The color detection of increasing amounts of calpain standards was linear.

9. Calpain Assays

The activity of calpain II purified from porcine heart was assayed by estimating the amount of TCA soluble FITC-labeled peptide released from FITC-labeled casein as substrate at 30°C, pH 7.5, with 2 mM $CaCl_2$ (David and Shearer, 1986). One unit of calpain activity released 1 µg FITC labeled, acid soluble fragments per minute. One unit of calpastatin activity inhibited one unit of calpain activity. The activity of calpain II purified from porcine heart was used to determine the amount of various commercially available calpain antagonists required to inhibit calpain activity by 50%.

10. Statistical Analysis

In all experiments, the significance of differences between the means of each test group and controls were evaluated by either the Student's t-test or ANOVA followed by the non-parametric Mann-Whitney U post-test for multiple mean comparisons. The level of significance was $p<0.05$.

# III. RESULTS

## A. Calpain Distribution in the Lens.

### 1. Calpain in the Insoluble Lens Pellet

The purpose of this experiment was to measure the distribution of calpain in particulate (insoluble) fraction of rat lens. Previous studies usually measured calpain in the cytosol because calpain is thought to be predominantly cytosolic due to its charged nature (David and Shearer, 1986). The total insoluble pellet was solubilized in urea to extract most of the crystallins and other proteins loosely associated with the lens membrane, and then further washed in NaOH to leave a membranous preparation (Russell, et al., 1986). Bands immunoreactive against calpain antibody and migrating as the 80 kDa catalytic subunit of calpain were quantitated by densitometry in the water soluble, urea soluble, NaOH soluble, and SDS soluble fractions.

As expected, the heaviest staining immunoreactive band in the four different isolated fractions was in the water soluble proteins (Fig. 10). However, calpain was not exclusively water soluble. Of the total calpain in lenses from twenty-four day old rats, 6% was insoluble, and the other 94% soluble calpain. Most of the insoluble calpain was extractable with urea, suggesting that calpain was loosely associated with the pellet. Calpain extracted from the pellet by urea was 5% of the total calpain. NaOH-soluble and SDS-solubile calpain contributed just 0.7% and 0.1%, respectively, of the total lens calpain. The total protein isolated from the same four fractions followed a similar distribution. Of the total protein in the lens, 92% of the protein was water soluble, 7% was urea soluble, 1% was NaOH soluble, and less than one percent remained in the pellet. In lenses from 9 week old rats, the total insoluble calpain increased to 9.5% of the

lens calpain (Table 5). As expected, total insoluble lens protein was also greater in older rats. Thus, the specific amount of calpain in the soluble and insoluble fractions (μg/mg protein) was similar between the young and old rats, indicating that insoluble calpain may be an important pool of calpain in the lens as it is in other tissues such as the brain (Chakrabarti et al., 1988).

2. Calcium-induced Insolubilization of Calpain.

Since calcium is a major intracellular regulator of calpain activity, the effect of calcium on calpain distribution was next determined. Calcium added to 9 week old rat lens homogenates caused a redistribution of calpain from the cytosol to all fractions of the insoluble pellet (Table 5). One millimolar $CaCl_2$ caused insoluble calpain to increase from 9.5% (normal) to 38.3% of the total lens calpain. The greatest increase (6-fold) occurred in the urea soluble fraction, with smaller increases in the NaOH and pellet fraction. These increases in the insoluble calpain were accompanied by a 29% decrease in the amount of soluble calpain.

The loss of calpain from the soluble fraction was due to specific calcium-induced translocation of calpain to the insoluble pellet. This was not due to a generalized insolubilization of lens proteins because there was no apparent difference in the amounts of total insoluble protein between control and lenses homogenized in calcium buffer. Insoluble calpain, however, increased from 3.1 μg to 7.9 μg. This indicated the calcium-induced effect was at least somewhat specific for calpain.

To obtain a more native membrane preparation, without the use of urea or NaOH, the insoluble pellet was separated on a discontinuous sucrose gradient. The major protein in the 25/45%, 45/50%, and 50/55% interfaces was found to be MP26, the lens transmembrane protein (Fig. 11, lanes 2,3,4). Phosphates, a

marker for phospholipids of the lipid bilayer, were also found at these interfaces (Table 6). Some MP26 and phosphates pelleted at the bottom of the gradient (Fig. 11, lane 5, and Table 6). Immunoblots showed that calpain co-migrated with MP26 (Fig. 12, lanes 2,3,4). Calpain was not present at the 8/25% interface (Fig. 12, lane 1). Adding calcium at a final concentration of 1 mM to the homogenizing buffer increased calpain found at the 25/45%, 45/50%, and 50/55% interfaces (Fig.12, lane 7,8,9). Calcium also increased the amount of calpain that became insoluble and pelleted (Fig. 12, lane 10). These data showed that calpain was associated with the membrane-rich fractions marked by MP26 and phosphates, and calcium increased this association. At the same time that calcium increased calpain association with the membrane, an absence of proteins above 31 kDa was observed (Fig.11, lane 7-9), with a decrease and shifting of some bands (Fig. 11, lane 10). This indicated that calcium may also have caused a general loss of high molecular weight proteins.

3. Calcium-Dependent Association of Calpain with the Insoluble Pellet.

Calcium increased insoluble calpain from 0-1000 μM calcium concentrations (Fig. 13a). Lenses from 5 week old rats were homogenized in buffers containing increasing amounts of calcium and the calpain inhibitor, cBz-ValPheH. With increasing calcium, more calpain became associated with the insoluble pellet. The amount of calpain associating with pellet started to plateau at approximately 500 μM, and resulted in a final 3 fold increase in insoluble calpain at 1000 μM calcium. The calcium-dependency curve showed the effect of calcium was greatest at the lower concentrations of calcium, which were the concentrations previously observed in cataractous lenses (Hightower et al.,1987).

Calcium caused a 3.8 fold increase in the amount of 30 kDa subunit associating with the pellet (Fig. 13b). The effect of calcium was approximately the

same for both subunits of calpain. The ratio of the 30 kDa to 80 kDa subunit was close to 1.4 over the entire range of calcium concentrations. The ratio of the subunits was not 1 for unknown reasons. The association of either the 30 or 80 kDa calpain subunits with the insoluble pellet does not give a classical binding curve and a binding constant could not be obtained.

4. Reversibility of Calpain Insolubilization.

Calcium-dependent association of calpain with the insoluble pellet was found to be reversible, and the re-solubilized enzyme remained active. This was determined by homogenizing lenses with and without 200 mM free $CaCl_2$, and the resulting insoluble pellets were then washed with buffer containing 1 mM EGTA and 1 mM EDTA. Lenses were homogenized at a calcium level chosen to increase insoluble calpain without causing extensive autolysis and degradation of calpain. Using immunoblot analysis, four times more calpain was extracted from the pellet isolated in the presence of calcium (cross-hatch bars) than from the control pellet (solid bars) (Fig. 14). Figure 14 is data from one experiment with 200 rat lenses. Two preliminary experiments showed similar results.

Calcium-insolubilized calpain was also found to retain enzyme activity (Fig. 15). The specific activity of the chelator-extractable calpain was 0.153 U/mg, which was greater than cytosolic calpain activity (0.011 U/mg). Also, the activity of the chelator-extracted calpain eluted from a DEAE-TSK column in 2 peaks. Both peaks were completely inhibited by 100 mM E64, a synthetic cysteine protease inhibitor, and both peaks were immunoreactive with calpain antibody in an ELISA assay. This indicated that the measurable calpain activity was due to calpain. The first peak of calpain activity eluted from the DEAE column at a salt concentration characteristic of calpain II (250 mM NaCl, David and Shearer, 1986). A second peak of calpain activity eluted earlier at 200 mM NaCl. The ratio

of calpain activity to mg of calpain was the highest in this fraction (Fig. 15). In three separate experiments, each using 100 rat lenses, 41-49% of the chelator solubilized calpain activity was in this earlier eluting peak, which contained only 14-20% of the chelator solubilized calpain antigen. Control lenses also contained chelator extractable calpain antigen. However, unlike calcium insolubilized calpain, this calpain had no enzymatic activity. In calcium treated samples, the earlier eluting calpain could be a more active form of the enzyme, or an altered form of calpain that is not as readily recognized by the antibody.

Some of the insolubilized calpain remained insoluble after several chelator washes and was only removed by Triton (Fig. 15). The enzymatic activity of this Triton-solubilized calpain was 0.11 U/mg. Unlike the EGTA extracted calpain, triton-extracted calpain from insoluble pellets of control lenses was also active (0.04 U/mg).

The activity of calpastatin in the samples from the above experiment was also determined. The specific activity of calpastatin in the water soluble proteins from control lenses was 0.59 U/mg, and the calpastatin activity in the calcium-treated lenses was 0.55 U/mg. Calpastatin activity in the insoluble fractions of the control lenses was 1.2 U/mg and 1.1 U/mg in the calcium treated lenses. Calcium did not increase insoluble calpastatin activity. However, chelator-extracted calpastatin from lenses homogenized in calcium did not elute off a DEAE column at the characteristic salt concentration (100 mM) but eluted at 150 mM NaCl, indicating a possible change in the overall charge of the calpastatin molecule. The ratio of calpain to calpastatin activity was highest in insoluble proteins from calcium-treated lenses, but was still less than 1 (Fig. 16). Taken together, these data provided some evidence to support the hypothesis that the expression of calpain activity could be most

favorable in the membrane associated (chelator-extractable) calpain from calcium-treated lenses.

5. Effect of Calpastatin on Insolubilization of Calpain

In order to more closely mimic the situation in humans where a greater excess of calpastatin to calpain exists than in the rat, the influence of exogenously added calpastatin on the calcium induced insolubilization of calpain was determined (Fig. 17). When no calpastatin was added, 100 mM calcium caused a statistically significant increase in calpain from 0.64 mg/mg pellet to 1.14 mg/mg pellet. When excess calpastatin was added, the usual calcium-induced increase in insoluble calpain was prevented. When BSA or trypsin inhibitor replaced calpastatin with an equal amount of protein, calcium still increased calpain binding to the insoluble pellet. These data suggested that an excess of calpastatin could prevent calcium-induced insolubilization of calpain.

Since protease inhibitors were included in this experiment, it is not known if calpain degraded calpastatin would still be able to prevent calcium-induced insolubiliation of calpain. However, based on our in vitro data we concluded that preincubating lens homogenates with exogenous calpastatin prevented calpain from associating with the pellet in the presence of calcium. Calpastatin may not only regulate calpain activity in the cytosol, but could also decrease calcium-dependent association of calpain with the lens pellet.

All of the above data in the section of "calpain distribution in the lens" is in press in Experimental Eye Research (Lampi et al., 1992).

B. Translocation of Calpain in Cultured Lenses

1. In Vitro Proteolysis in the Insoluble Pellet.

The role of calpain in the degradation of insoluble lens proteins was investigated (Fig. 18). High molecular weight insoluble proteins were proteolyzed when lenses were homogenized in buffer containing 2 mM calcium (lane 2). E64, a specific calpain inhibitor, inhibited this proteolysis (lane 1). However, when the insoluble proteins were first separated from the soluble proteins and then incubated in calcium, no proteolysis occurred (lane 3). This suggested that the insoluble calpain was not able to break down the insoluble proteins. If calpain were involved in proteolysis, the source of this calpain may be cytosolic calpain. This was further examined in cultured lenses below.

2. Calcium Induced Opacity in Cultured Lenses.

Lenses cultured in medium containing calcium ionophore A23187 became cataractous. The cataract was characterized by dense nuclear opacity, proteolysis of both the soluble and insoluble proteins, and loss of calpain. Several different inhibitors of calpain were able to ameliorate the effects of the calcium-induced cataract, implicating calpain involvement in the formation of the cataract.

First, different inhibitors were compared to determine which inhibitor was most effective in preventing cataract in cultured lenses. The ability of E64 to inhibit calpain in vitro was compared to that of leupeptin and three other aldehyde derivatives; cBz-ValPheH, and calpain inhibitors I and II whose structures are shown in Table 4. Standard amounts of calpain activity purified from porcine heart were titrated with increasing amounts of each inhibitor (Table 7). As can be seen, leupeptin, cBz-ValPheH, and inhibitor I had lower $IC_{50}$s in vitro than E64 or inhibitor II.

We then assessed the toxic effect of inhibitors on lenses in culture. Lens toxicity was estimated by the appearance of a diffuse haze over the entire lens that differs from the dense nuclear opacity associated with calcium-ionophore cataracts. Low concentrations of the cell permeable calpain inhibitors I and II caused lenses to become hazy (Fig. 19). Of the three uncharged calpain inhibitors, cBz-ValPheH appeared to be the least toxic, and the charged aldehyde-based inhibitor, leupeptin, was not toxic to the lens even at concentrations as high as 2 mM (data not shown). Five hundred micromolar E64 was not toxic to the lens. This indicated that the inhibitors themselves had varying degrees of toxicity to the lens.

The peptide inhibitors were next tested for their ability to prevent calcium ionophore-induced cataract in cultured lenses (Fig. 20). At non-toxic concentrations, only cBz-ValPheH and E64 were able to protect lenses against calcium-ionophore induced cataract. Five hundred micromolar cBz-ValPheH decreased the severity of the nuclear cataract by 69%, while at 100 μM it was ineffective. E64 at 5 μM and 1 mM decreased nuclear opacity by 29% and 60% respectively (Fig. 21). Calpain inhibitors I and II did not reduce nuclear opacity, neither did high concentrations of the charged aldehyde inhibitor, leupeptin. These data showed that specific inhibitors of calpain were able to ameliorate the effects of ionophore-induced cataract, implicating calpain in cataractogenesis.

To confirm the uptake of E64 into the lens, free E64 was measured by HPLC (Table 8). Only minimal levels of E64 were detected in lenses cultured in medium with E64 alone for 10 days. However, marked uptake of E64 was found in lenses cultured in cataract-inducing agents. The concentration of free E64 in lenses exposed to A23187 or selenite was 75% of the concentration of E64 initially added to the medium. This increased uptake of E64 was related to an increase in lens permeability as measured by water uptake, since A23187 caused lenses to

increase in wet weight (Table 9). While E64 reduced the uptake of water by cataract lens, it did not totally prevent this event. Nonetheless, E64 was present within the lens during formation of the cataract and would have been available to inhibit calpain.

The calpain inhibitors, E64 and cBz-ValPheH, were then used to investigate the role of calpain in cataractogenesis. Using accumulation of water as a measure of epithelial permeability, neither E64 nor cbz-ValPheH were able to completely block the damage occurring to the epithelial layer (Table 9). Proteolysis in lenses cultured in A23187 was shown by the loss of proteins at 31 and 20 kDa, and the accumulation of low molecular fragments below 20 kDa. The 31 kDa βB1-crystallin decreased to 15% of control value in the soluble fraction and 24% of control in the insoluble fraction of lenses cultured in A23187 (Fig. 22). CBz-ValPheH partially prevented the loss of βB1 in the soluble fraction, while blocking the loss of βB1 from the insoluble fraction. Five hundred micromolar E64 completely prevented the loss of βB1 from both the soluble and insoluble fractions. This inhibitory effect was also observed at 250-1000 μM E64 (Fig. 23, lanes 1-4 and 8-11). Lower concentrations of E64 (5 and 50 μM, Fig. 23, lanes 5, 6 and 12, 13) only partially prevented proteolysis. βB1-crystallin accumulated in the insoluble pellet of lenses cultured in A23187 plus E64. This may be due to a generalized effect of calcium-induced insolubilization of lens proteins. The other inhibitors tested were unable to inhibit proteolysis unless cytotoxic concentrations were used. Thus, calcium-induced proteolysis of both soluble and insoluble proteins was decreased by inhibitors specific for calpain.

Since Inomata et al. (1988) showed that calpain undergoes autolysis when activated in other tissues, we measured calpain activation in lens and its subsequent autolysis by loss of immunoreactivity of the 80 kDa subunit of calpain. In lenses cultured in A23187, a complete loss of the 80 kDa subunit of

calpain occurred in the water soluble fraction, and a 43% loss of calpain was noted in the pellet (Fig. 24). While cBz-ValPheH increased the concentration of calpain in the pellet derived from A23187-treated lenses, it did not prevent loss of calpain from the water soluble fraction. E64 partially prevented the loss of soluble calpain (Fig. 24 and Fig. 25, lanes 4-7) while allowing for accumulation of insoluble calpain (Fig. 25, lanes 10-13). Both E64 and cBz-ValPheH caused soluble calpain to decrease and insoluble calpain to increase. This indicated that during cataractogenesis calpain was translocated to the insoluble fraction from the soluble fraction where cBz-ValPheH and E64 prevented its autolysis and further degradation.

Parts of the above data in the section "calcium-induced opacity in cultured lens" is in press in Toxicology and Applied Pharmacology (Lampi et al., 1992).

## C. Characterization of Lens Calpastatin

### 1. Calpastatin Activity in the Lens.

Calpastatin activity was measured in human, bovine, and rat lens. Calpastatin activity in cortical and nuclear soluble proteins was measured in 7 pairs of human donor lenses at ages 1.5 months, 16, 27, 48, 50, and 66 years. Data from the 48 and 50 year old lenses were averaged, and data from two pair of 66 year old lenses were also averaged. At each age examined, calpastatin specific activity was greater in the nucleus than in the cortex, and calpastatin activity appeared constant with age with limited variation (Fig. 26). Calpastatin activity was in great excess over calpain activity at all ages, and this was most notable in the nucleus at the older ages (Fig. 27). These data has been published in part (David et al., 1989).

Calpastatin from 16 and 66 year old donor lenses was heat stable retaining 109% and 116% inhibitory activity respectively when heated for 15 minutes at 85°C. The slight increase in inhibitory activity may reflect heat inactivation of calpain I eluting off the DEAE column near calpastatin. In the 1.5 month donor lenses, a second peak of calpastatin activity was observed in the cortex. This second calpastatin peak was not seen in the nucleus, and was also observed in the cortical proteins of other 3 month and 1.5 year old donor lenses. This peak eluted at a broader salt concentration of 150-200 mM NaCl.

Calpastatin activity in 100 lenses from 1 month old rats was 0.6 U/mg, and in 120 lenses from 2.5-3 month old rats, calpastatin activity was 0.3 U/mg. In a representative experiment to determine calpastatin distribution in lenses from 2 month old rats, calpastatin activity was 0.6 U/mg in the cortex and 0.4 U/mg in the nucleus. This was 4-5 times greater than the corresponding calpain activity. Calpastatin activity in the epithelium was estimated to be in excess of 25 U/mg. No calpain activity was detectable in the epithelium. In 4 out of 4 experiments, calpastatin activity from the epithelium eluted at 240 mM NaCl rather than at the characteristic salt concentration. The epithelium consists of newly dividing cells unlike the terminally differentiated cortical and nuclear fibers, suggesting that the difference in elution pattern of calpastatin between cell regions may represent an alteration of calpastatin during maturation of fiber cells. This was also observed between young and old human lenses. Thus, calpastatin activity was found to be in excess of calpain activity in the water soluble fraction in both human and rat lenses, suggesting calpain activity may not be expressed in vivo unless some mechanism is present which allowed calpain to escape inhibition by calpastatin.

Calpastatin was next measured in subcellular fractions from 120 twelve-week old rat lenses. Calpastatin activity, eluting at the characteristic

salt concentration (100 mM), was found in the water soluble, urea soluble, and Triton X-100 soluble proteins (Fig.28). An experiment with cortical proteins from 20 bovine lenses gave similar results and is shown for comparison (Fig. 28). These data indicated that calpastatin is distributed in the insoluble fraction as well as in the soluble fraction. This was unexpected since calpastatin is a hydrophilic molecule, and thought to be distributed mostly in the cytosol.

2. Molecular Weight of Lens Calpastatin.

Different molecular weights have been reported for calpastatin in the literature (Adachi et al., 1988; Takano and Murachi, 1982; Takano et al., 1986). The molecular weight of calpastatin in the lens was investigated by immunobloting with a polyclonal antibody against purified bovine heart calpastatin prepared in our laboratory. The antibody was shown to react with calpastatin from porcine heart and certain rat tissues (liver, heart). A 130 kDa immunoreactive band and a less densely staining band just above this were detected in heat soluble proteins from the cortex of porcine lenses (Fig. 29A, lane 5). Calpastatin immunoreactivity was not detected in the nucleus (lane 4), nor in the pellet from either the cortex or nucleus (lanes 1 and 3). When the pellet from the cortex was further extracted with urea and then Triton-X 100, no distinct calpastatin bands were detected . This suggested that calpastatin lost antigenicity as cortical fibers matured into nuclear fibers. Also, activity measurements suggested that calpastatin is present in the insoluble proteins, but it is not recognized by the antibody.

The 130 kDa band in the soluble fraction disappeared from lens homogenates treated with 150 μM $CaCl_2$ (Fig. 29B, lane 8 and 9), with no appearance of a corresponding band or any fragments in the pellet (lanes 2-7).

However, when lenses were homogenized in 1 mM $CaCl_2$ plus 400 mM NaCl and the protease inhibitors, PMSF, leupeptin, pepstatin A; no loss of calpastatin was evident, indicating the calcium-induced loss of soluble calpastatin in the lens may be due to proteolysis by calpain (Fig. 30).

The molecular weight of rat lens calpastatin was investigated and found to be lower than porcine lens calpastatin. An immunoreactive doublet at 100 kDa was repeatedly seen in soluble rat lens proteins (Fig. 31). A calpastatin of similar molecular weight was detected in rat liver (Fig. 6). Calpastatin in the rat lens pellet was detected at 80 kDa by both a polyclonal and a monoclonal antibody, suggesting a possible difference between soluble and insoluble calpastatin (Fig. 32).

3. Molecular Weight of Lens Calpastatin as Determined by Gel Filtration.

Cortical proteins from porcine lenses were homogenized in 20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 1 mM DTE, centrifuged at 10,000 x g, and the supernatant heated to 95°C for 5 minutes, and again centrifuged. The soluble proteins were applied to DEAE-Sepharose column and the active calpastatin fractions concentrated and applied to a TSK-3000 column connected in series to a TSK-4000 gel filtration column. Peak calpastatin activity eluted at 79 kDa. A second peak of activity eluted at approximately 18 kDa. Both peaks were immunoreactive in an ELISA assay. The specific activity of the low molecular weight calpastatin (18 kDa) was several fold higher than the higher molecular weight calpastatin (79 kDa) (Table 10). Only one peak of calpastatin activity and immunoreactivity was detected in porcine heart at 210 kDa. This sample was prepared in parallel with the lens sample. The specific activity of heart calpastatin was considerably higher than in the lens, possibly reflecting the high structural protein content of the lens and the richness of calpastatin in the heart.

The molecular weight of lens calpastatin from 6 week old rats was also investigated and found to elute from a TSK-3000 column after cytochrome c at10 kDa. No other activity peaks were found. Calpastatin activity was not detected in 6 gel filtration runs of 6-20 human lenses from donors over 50 years old. These data indicated that with age, lens calpastatin may undergo degradation in vivo.

# IV. DISCUSSION

## A. Importance of Insoluble Calpain

The purpose of the present research was to test the hypothesis that calpain may be activated in the lens by association with the insoluble lens fraction. In support of this hypothesis, a major new finding of the present investigation was that calpain was associated with several different fractions of the insoluble pellet of lens, including a membrane-rich fraction, and that calcium increased this association. Calcium caused a specific association of calpain with various insoluble fractions. "Translocation" of calpain to the insoluble fraction is defined as specific association with various insoluble fractions as well as direct insolubilization of calpain by calcium.

The calcium concentration needed for translocation to occur was determined in this study to be lower than what would be required for soluble calpain II activity and lower than calcium concentrations in cataractous lenses. This suggests that the translocation of calpain to the membrane is a feasible event and would occur in vivo before calpain II was fully active. Also, as calcium concentrations rise during cataractogenesis, calpain would first associate with the membrane. Calcium not only causes translocation of calpain to the membrane but precipitation of calpain (Fig 12).

Insoluble calpain is important for two reasons. First, the insoluble fraction may be a site of activation for calpain. Although calpain was found to be predominantly cytosolic in the lens, cytosolic calpain may not be active under physiological conditions because of the possible inhibition from cytosolic calpastatin which exceeds the calpain activity in the lens. The membrane was first suggested to play a role in activation of calpain when phospholipids were

found to lower the calcium requirement for activation (Pontremoli et al., 1985b). The plasma membrane may also be a site of localized high concentrations of calcium ions (Yanagisawa et al., 1988; Peng, 1984; Hightower and Reddy, 1982). It has also been suggested that upon autolysis calpain may lose its regions of interaction with the membrane and be released into the cytosol as an active enzyme (Suzuki, 1987). Thus, calpain associated with the lens pellet may represent calpain with potential activity both at the membrane and in the cytosol.

Secondly, calpain in the insoluble pellet may be important because of the different substrates available to calpain in the pellet than in the soluble fraction. Calpain may be loosely associated with the membrane as suggested by the extraction of water insoluble calpain with urea. The urea soluble proteins are comprised of cytoskeletal proteins, many of which are known calpain substrates (Table 1). Spectrin and vimentin were degraded in lenses cultured in calcium suggesting involvement of calpain (Truscott et al. 1991). Calpain also was found in "native" membrane preparations obtained from a sucrose gradient (Fig. 12). Therefore, calpain is located near potential substrates in the lens membrane (David and Shearer, 1986; Roy et al., 1983; Ireland and Maisel, 1984: Kistler et al., 1986). This is significant because certain membrane proteins facilitate cell-cell communication important to the functioning of the lens.

Several pieces of evidence supported the notion that expression of calpain activity may be most favorable in the fraction of calpain that associates with the pellet. Insoluble calpain was active. When calpain from calcium-treated lenses was resolubilized with chelator buffer, this chelated calpain had high specific activity. This increase in specific activity of calpain in the pellet is due to an increase in the relative amount of calpain and may be due to the presence of autolytically activated calpain (Fig. 15). The presence of autolyzed calpain in the

calcium treated lenses was suggested by an increase in the ratio of enzyme activity per μg calpain antigen, and by a change in the salt concentration that calpain eluted from DEAE. Therefore, calpain activity released from the pellet through chelation implies calpain may be active at the membrane. Further tests are needed to confirm calpain is active while still associated with the membrane.

Endogenous calpastatin did not interfere with calcium-induced binding of calpain to the particulate fraction. Due to an increase in calpain activity, the ratio of calpain to calpastatin activity was highest in the calcium insolubilized proteins. Calcium did not cause a similar increase in calpastatin activity in the insoluble pellet. However, calcium did alter the salt concentration at which calpastatin eluted from a DEAE column, indicating a possible change in the structure of the molecule. It is possible that the calcium-induced insolubilization of calpain resulted in calpain activation followed by subsequent degradation of calpastatin (Nakaruma et al, 1989). This may be a mechanism by which calpain overcomes inhibition by calpastatin at the membrane.

Preincubating rat lens homogenates with large amounts of exogenous calpastatin blocked the calcium-induced translocation of calpain. This is particularly relevant to the situation in human lens, where a considerable excess of calpastatin over calpain exists (David et al., 1989). Other investigators have similarly shown that exogenously added calpastatin blocks the binding of calpain to the membrane (Pontremoli et al., 1985c; Zaidi and Narahara, 1989). However, this data must be interperted with care since the source of exogenous calpastatin added in the present study was from bovine heart and may have different properties than endogenous rat lens calpastatin.

Calcium-induced proteolysis of insoluble lens proteins occurred in crude homogenates, but not when calcium was added to the pellet isolated in the absence of calcium (Fig. 18). This suggested that cytosolic calpain translocated to

the insoluble fraction was necessary for degradation of insoluble lens proteins. Insoluble lens proteins include cytoskeletal proteins and gap-junction proteins as well as crystallins thightly associated with the membrane that may be important in the maintenance of lens transparency. This further supports the importance of calpain associating with the insoluble fraction.

B. Specific Calpain Inhibitors Decrease the Effects of Calcium-Ionophore Cataract

Previous data from our laboratory showed that E64, an inhibitor of calpain, ameliorated calcium-ionophore cataract (Shearer et al., 1991). We confirmed the previous data and further quantitated the effect of E64. In order to elucidate the mechanism for activation of calpain in cataractogenesis, other potentially more potent inhibitors of calpain were tested for their effectiveness in preventing cataracts in culture. We found that E64 and cBzValPheH were effective in decreasing calcium ionophore cataract but that leupeptin and calpain inhibitors I and II were not.

The effective concentration of cBz-ValPheH and E64 in culture was much higher than required to inhibit calpain in vitro. Thus, the effectiveness of these compounds is due not only to the calpain-inhibitor interaction in the cell, but also to permeability of lens to the inhibitor. The influx of inhibitor into the deep nuclear region of the lens, where the cataract forms, may have been minimal for leupeptin and calpain inhibitors I and II.

The effectiveness of E64 in preventing the formation of cataract was somewhat unexpected, since E64 is a charged compound and was not expected to penetrate well across lens fiber plasma membrane to inhibit cytosolic calpain. However, the present results demonstrated that E64 was taken up in the lens in high concentrations during the formation of the cataract. Penetration of E64 into

the lens was likely due to passive diffusion through lens membranes permeabilized by calcium ionophore. Lenses not exposed to ionophore did not take up E64. The importance of permeability was further supported by the observation that the ionophore treated lenses undergo an increase in hydration, indicating increased membrane permeability.

Several uncharged inhibitors of calpain were also tested for their ability to prevent calcium ionophore-induced cataract in lens culture, since permeability of the uncharged molecules was expected to be greater than E64. Of the uncharged aldehyde inhibitors tested, cBz-ValPheH exhibited the lowest toxicity and was able to decrease calcium-induced opacity in cultured lenses. This suggested that, while membrane permeability is essential, structural features of inhibitors are also important. CBzValPheH has a substituted carbonyl group, while calpain inhibitors I and II are N-acetylated tripeptides with C-terminal aldehydes. These uncharged aldehyde inhibitors are structurally similar to the protease inhibitor, leupeptin (N-acetyl-leucyl-leucyl-arginal).

Surprisingly, leupeptin was tested and found to be ineffective at preventing calcium-induced cataract. Like E64, leupeptin is charged and therefore would be expected to enter the lens only after the permeability of the lens membrane had increased. Once inside the lens, leupeptin would be expected to inhibit proteases. The ineffectiveness of leupeptin in preventing cataract may be related to differences in its structure and charge. In agreement with our data, Hayashi et al. (1991) reported varying effectiveness of different calpain inhibitors. When exogenous cBz-LeuLeuLeu-aldehyde was added to isolated erythrocytes, the activation of intracellular calpain was inhibited and the degradation of membrane proteins was prevented. However, leupeptin and a derivative of E64, E64d, were not effective.

The aldehyde inhibitors differ in their structure from E64. E64 contains an epoxide ring, which forms an irreversible thioether bond with the active site thiol in calpain (Mehdi, 1991). Aldehydes inhibit calpain by forming a stable tetrahedral adduct with the sulfhydryl at the active site (Wang, 1990; Mehdi et al., 1988). These differences in structure may partially explain differences in effectiveness between E64 and cBzValPheH.

The effectiveness of protease inhibitors in preventing cataracts further implicates calpain-induced proteolysis as a cause of cataracts in rat lens. E64 and cBz-ValPheH prevented proteolysis of soluble and insoluble β-crystallins. These data demonstrate possible role for calpain in cataract formation. Furthermore the data suggested the possible subcellular site where calpain is active.

For example, the inhibitor data supported the suggestion that calpain may be active in the insoluble fraction. Calpain inhibitors blocked the autolytic degradation of insoluble calpain and allowed for the accumulation of the enzyme in the insoluble fraction. The accumulation of insoluble calpain suggests that calpain had translocated from the cytosol to the insoluble fraction during the formation of cataracts. Hightower et al. (1989) previously found that calcium concentrations increase during cataractogenesis to 100 μM. We showed in the present studies that even 50 μM calcium caused insolubilization of cytosolic calpain. E64 partially also prevented the loss of soluble calpain. E64, a water soluble compound, may inhibit activation and autolysis of cytosolic calpain during cataractogenesis. Once inhibited by E64, a major portion of the cytosolic calpain may then become insoluble due to the presence of calcium.

In contrast to E64, cBz-ValPheH did not prevent the loss of soluble calpain. This implied that cBzValPheH and E64 may co-localize with calpain at different intracellular sites. CBzValPheH, expected from its structure to be hydrophobic, may penetrate cell membranes more readily but have more difficulty than E64 in

diffusing into the cytoplasm. Soluble calpain may have autolyzed before it became associated with the membrane and inactivated by cBzValPheH. The finding that cBzValPheH was still effective in preventing cataract supported the idea that the membrane may be an important site of the proteolysis which induces cataract. Whatever the mechanism, inhibition of opacity, proteolysis, and calpain degradation by calpain inhibitors are strong indicators that calpain is an important factor in cataract formation in rat lens.

C. Characterization of Calpastatin in the Lens and Possible Implications for Expression of Calpain Activity.

Calpastatin activity has previously been measured in the lens (Yoshida et al., 1985). However, the type of calpastatin which exists in the lens is not known. This is an important question since the high and low molecular weight forms of calpastatin may have different properties and localize differently in the cell (Mellgren, 1988). The purpose of experiments presented above was to characterize lens calpastatin. We found calpastatin activity in both the cytosol and the insoluble fractions of lens, but an immunoreactive band of calpastatin only in the soluble fraction at 120 kDa. A possible interpretation of this data is that insoluble calpastatin differs from soluble calpastatin so that the insoluble calpastatin is not recognized by our antibody. In support of this, a faint immunoreactive band at 80 kDa in the pellet of the rat lens was detected by both our polyclonal antibody and a monoclonal antibody (Fig. 32).

Calcium may also affect the distribution of calpastatin in the lens. We found that the addition of calcium to rat lens homogenates did not change the calpastatin activity in the soluble or chelator extracted proteins, but calcium did alter the salt concentration at which insoluble calpastatin eluted off a DEAE

column. We also found that calcium caused a loss of the 120 kDa calpastatin band from the soluble proteins of porcine lenses (Fig. 29B), suggesting that activity in calcium treated lenses was due to fragmented calpastatin not recognized by our antibody. In support of this, fragmented calpastatin has been reported to retain its activity (Nakamura, 1989).

Calpastatin was also characterized at different ages. Calpastatin activity did not change with age (Fig. 25) in human and rat lenses (Varnum et al., 1989). However, in young human donor lenses, an additional peak of calpastatin activity eluted from DEAE chromatography of cortical proteins. This peak was not seen in older lenses or in the older nuclear proteins. A similar peak was present in rat epithelium, but not in rat cortex or nucleus. Two peaks of calpastatin were detected in pig brain and rat skeletal muscle and may correspond to the calpastatin peaks we measured (Takano et al, 1986; Pontremoli et al, 1991). Different isoforms of calpastatin may have different specificities towards calpain. Therefore, the changes noted in the lens with age may be an important way to alter calpain activity.

The molecular weight of calpastatin from porcine lens (80 kDa) differed from the molecular weight of cardiac muscle calpastatin (210 kDa) when isolated by the same method. The high molecular weight determined for cardiac calpastatin by gel filtration may reflect association of monomers of calpastatin not seen with lens calpastatin. The most striking observation about the molecular weight of lens calpastatin was a peak of inhibitory activity at 18 kDa. It appears that lens calpastatin has been degraded. This degradation may have occurred in vivo and may not necessarily be the result of artifacts in tissue preparation. Of course, it is possible that lens calpastatin may be more susceptible to degradation during homogenization than cardiac muscle calpastatin.

We concluded that lens contains several different forms of calpastatin depending on the subcellular distribution, anatomical region, and possibly on the level of proteolytic activity in the lens. It is important to determine if the different forms of calpastatin differ in their specificities towards calpain, since we postulate that these different forms of calpastatin could be important regulator devices in control of lens calpain.

## V. SUMMARY

The overall contribution of these studies to our understanding of the biochemistry of the lens was that calcium-induced translocation of calpain to the insoluble fractions may be important for activation of calpain in the lens. This idea was supported by four major findings.

1. Calpain was located in the insoluble fractions of the lens including a membrane-rich fraction and was markedly increased in the insoluble fractions by the addition of calcium.

2. The calpain associated with the pellet in calcium-treated lens homogenates was active and had a different elution profile on an ion exchange column than soluble calpain. These data suggest that calcium-insolubilized calpain may be modified.

3. Calcium did not cause an increase in insoluble calpastatin activity, therefore calpastatin is not translocating to the pellet. This results in a higher calpain/calpastatin ratio in the particulate fraction than the soluble fraction. The increased ratio may favor calpain activity in the insoluble fraction.

4. Specific calpain inhibitors prevented cataracts implicating calpain-induced proteolysis as a cause of cataracts. E64 and cBz-ValPheH blocked the autolytic degradation of insoluble calpain and allowed for the accumulation of the enzyme in the insoluble fraction. It is expected that cBz-ValPheH would remain at the membrane, therefore the fact that it is also effective suggests that insolubilization of calpain may occur during formation of cataracts.

The purpose of elucidating the mechanism of calpain activation in the lens and determining the properties of lens calpastatin, was to understand how calpain overcomes its requirement for high concentrations of calcium and is able to escape inhibition from calpastatin. Our results are summerized in Figure 33. Calcium even at low concentrations caused calpain to translocate from the cytosol to a membrane-rich fraction in the lens. This calpain was resolubilized from the pellet with chelator and had a higher ratio of calpain activity to mg calpain than cytosolic calpain implying calpain may have been autolyzed at the membrane. Further tests are needed to confirm autolysis of translocated calpain. Calcium did not cause translocation of calpastatin activity suggesting that calpain may partially escape calpastatin by binding the membrane.

During calcium-ionophore cataract formation cBz-ValPheH was able to prevent the loss of insoluble calpain and allowed for its accumulation. CBz-ValPheH is a neutral molecule and would not be expected to penetrate into the cytosol. The fact that it is an effective anti-cataract agent suggests that activation of calpain at the membrane is an important event in cataract formation. Indeed proteolysis of both soluble and insoluble crystallins occurs in cataracts. Since E64 is a charge molecule it may be acting to inhibit autolysis of both the membrane-associated calpain and the cytosolic calpain which would then precipitate in the presence of calcium. We conclude that calcium causes calpain to associate with the lens pellet <u>in vitro</u> and that this may be an important event in formation of cataract in culture. Insolubilized calpain may be the active form of calpain in the lens and therefore is significant.

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## Table 1

## In Vitro Substrates of Calpain

1. ENZYMES
   phosphorylase b kinase, pyruvate kinase, protein kinase C, tyrosine hydroxylase, transglutaminase

2. CYTOSKELETAL PROTEINS
   neurofilament, vimentin, myofibrillar protein, microtubule associating proteins, fordin

3. RECEPTOR PROTEINS
   estrogen receptor, epidermal growth factor receptor, alpha-adrenalin receptor

## Table 2

### Purification of Calpain from Bovine Heart

| Step | Total Protein (mg) | Total Activity (Units) | Specific Activity[a] (Units/ mg) |
|---|---|---|---|
| Crude homogenate | 33200 | n.d. | n.d. |
| DEAE Bio-gel A | 86 | 695 | 8 |
| Gel Filtration | 17 | 270 | 16 |
| Phenyl 5PW | 0.2 | 162 | 698 |

n.d. = not determined

[a]Calpain activity was assayed by estimating the amount of TCA soluble FITC-labeled peptide released from FITC-labeled casein as substrate. Units of calpain activity were divided by the amount of soluble protein at each purification step.

## Table 3

### Purification of Calpastatin from Bovine Heart

| Step | Total Protein (mg) | Total Activity (Units X $10^3$) | Specific Activity[a] (Units/ mg) | Fold Purification |
|---|---|---|---|---|
| 12,000 g supernate | 112000 | 251 | 2 | 1 |
| Heated supernate | 12000 | 209 | 17 | 9 |
| TCA concentrate | 1700 | 137 | 82 | 41 |
| DEAE-Sepharose | 106 | 24 | 229 | 115 |
| Affi-gel blue | 16 | 5 | 741 | 371 |

[a]Calpastatin specific activity equals the amount of unknown sample that inhibits one unit of calpain II activity divided by the amount of soluble protein at each step.

## Table 4

### Compounds used to Inhibit Calpain

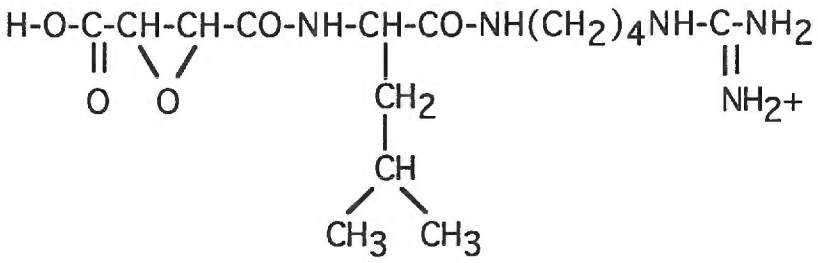
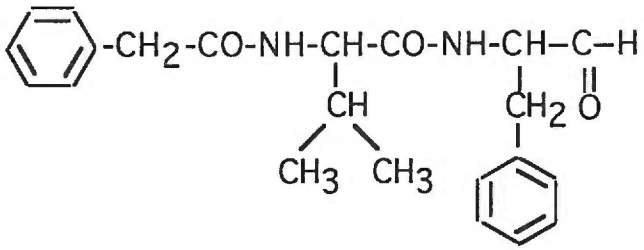
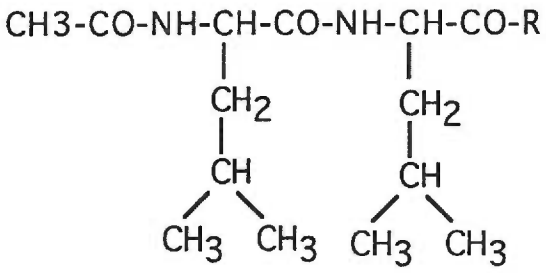
| | |
|---|---|
| **E64** |  |
| **cBz-ValPheH** |  |
| **Leupeptin, R=Arg** |  |
| **Calpain Inhibitor I, R=nLeu** | |
| **Calpain Inhibitor II, R=nMet** | |

Table 5

**Effect of calcium on association of calpain with various lens fractions from 9 week old rats.**

| Subcellular Fraction | Homogenization in EGTA | | Homogenization in 1 mM $CaCl_2$ | |
|---|---|---|---|---|
| | µg Calpain / mg protein | % of total Calpain | µg Calpain / mg protein | % of total Calpain |
| Water soluble | 1.00 ± 0.05[a] | 90.5 | 0.42 ± 0.06[a] | 61.7 |
| Urea soluble | 0.59 ± 0.07[b] | 3.2 | 2.09 ± 0.22[b] | 19.6 |
| NaOH soluble | 0.42 ± 0.10[b] | 5.4 | 0.97 ± 0.09[b] | 15.9 |
| Pellet | 0.93 ± 0.20 | 0.9 | 1.90 ± 0.10[b] | 2.8 |
| | Total insoluble | 9.5 | Total insoluble | 38.3 |

[a] Mean ± S.D. (n=5)

[b] Significantly different from water soluble at $p < 0.01$.

**Table 6**

**Distribution of phosphates in discontinuous sucrose gradient.**

| Interface | Homogenization in EGTA<br>nmol phosphates<br>µg protein | Homogenization in 1mM $CaCl_2$<br>nmol phosphates<br>µg protein |
|---|---|---|
| 8/25% | 0.0 | 0.0 |
| 25/45% | 0.17 | 0.67 |
| 45/50% | 0.72 | 0.76 |
| 50/55% | 0.72 | 0.13 |
| pellet | 0.11 | 0.23 |

## Table 7

## In Vitro Inhibition of Calpain II[a]

| Inhibitor | $IC_{50}$ (μM) |
|---|---|
| Leupeptin | 0.09 ± 0.02[b] |
| cBz-ValPheH | 0.10 ± 0.04 |
| Inhibitor I | 0.10 ± 0.02 |
| Inhibitor II | 0.24 ± 0.04 |
| E64 | 0.26 ± 0.07 |

[a] Inhibition of calpain did not follow classical kinetics so the $IC_{50}$ was estimated as 50% inhibition of caesinolytic activity of 0.5 units of porcine heart calpain II.

[b] Mean ± SD, N= 4-5

## Table 8

### E64 Uptake in Lenses [a]

| Days in E64 | Cataractogenic Agent | E64 (μM) |
|---|---|---|
| 0 | none | n.d. |
| 0.5 | none | n.d. |
| 5 | none | 33 ± 7 [b] |
| 10 | none | 72 ± 10 |
| 3 | A23187 | 327 ± 57 [c] |
| 5 | A23187 | 378 ± 59 [c] |
| 10 | Selenite | 375 ± 66 [d] |

[a] Free E64 in TFA soluble fraction of lenses cultured in medium containing 500 μM E64.

[b] Mean ± SD for 3-5 pools of lenses (2 lenses/ pool).

[c] Significantly different ($P \leq 0.03$) from mean of lenses cultured for 5 days without cataractogenic agent.

[d] Significantly different ($P \leq 0.03$) from mean of lenses cultured for 10 days without cataractogenic agent.

n.d. = not detectable

## Table 9

### Wet Weights of Cultured Lenses [a]

| Inhibitor | Medium [b] | | |
|---|---|---|---|
| | A | B | C |
| cBz-ValPheH, 500 µM | 25 ± 1 | 33 ± 3[c] | 29 ± 3 [c] |
| E64, 500 µM | 22 ± 1 | 31 ± 1[c] | 28 ± 1 [d] |

[a] Mean mg/ lens ± SD , N = 5

[b] Lenses were cultured in A) basic medium, or B) basic medium with 10 µM A23187, or C) basic medium with A23187 plus inhibitor.

[c] Significantly different from means of lenses in group A at $p < 0.05$.

d Significantly different from means of lenses in group A and B at $p < 0.05$.

## Table 10

### Calpastatin Activity (Units/mg)

| | Crude Homogenate | DEAE | Gel Filtration Peak 1 | Peak 2 |
|---|---|---|---|---|
| Heart | 1.9 | 109 | 485 (210 kDa) | |
| Lens | 0.3 | 5 | 1 (79 kDa) | 9 (18kDa) |

Tissues were homogenized in buffer containing 20 mM TRIS (pH 7.5), 1mM EGTA, 1 mM EDTA, and 1mM DTE, centrifuged, and immediately heated to 95 C. The heat soluble proteins were separated by DEAE Sepharose and the active calpastatin fractions applied to a 7.5 X 300 mm TSK G 3000 gel filtration column connected to a G 4000 column equilibrated with 50 mM NaCl in the above buffer. Calpastatin activity for the lens is the average of two experiments. Activity in peak 1 eluted at 210 kDa for heart and 79 kDa for lens. Activity in peak 2 eluted at 18 kDa for lens. Only 1 peak of calpastatin activity eluted from the heart. Gel-filtration standards were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa).

Figure 1:

Drawing of lens showing different regions. Adapted from Kortez and Handelman, 1988.

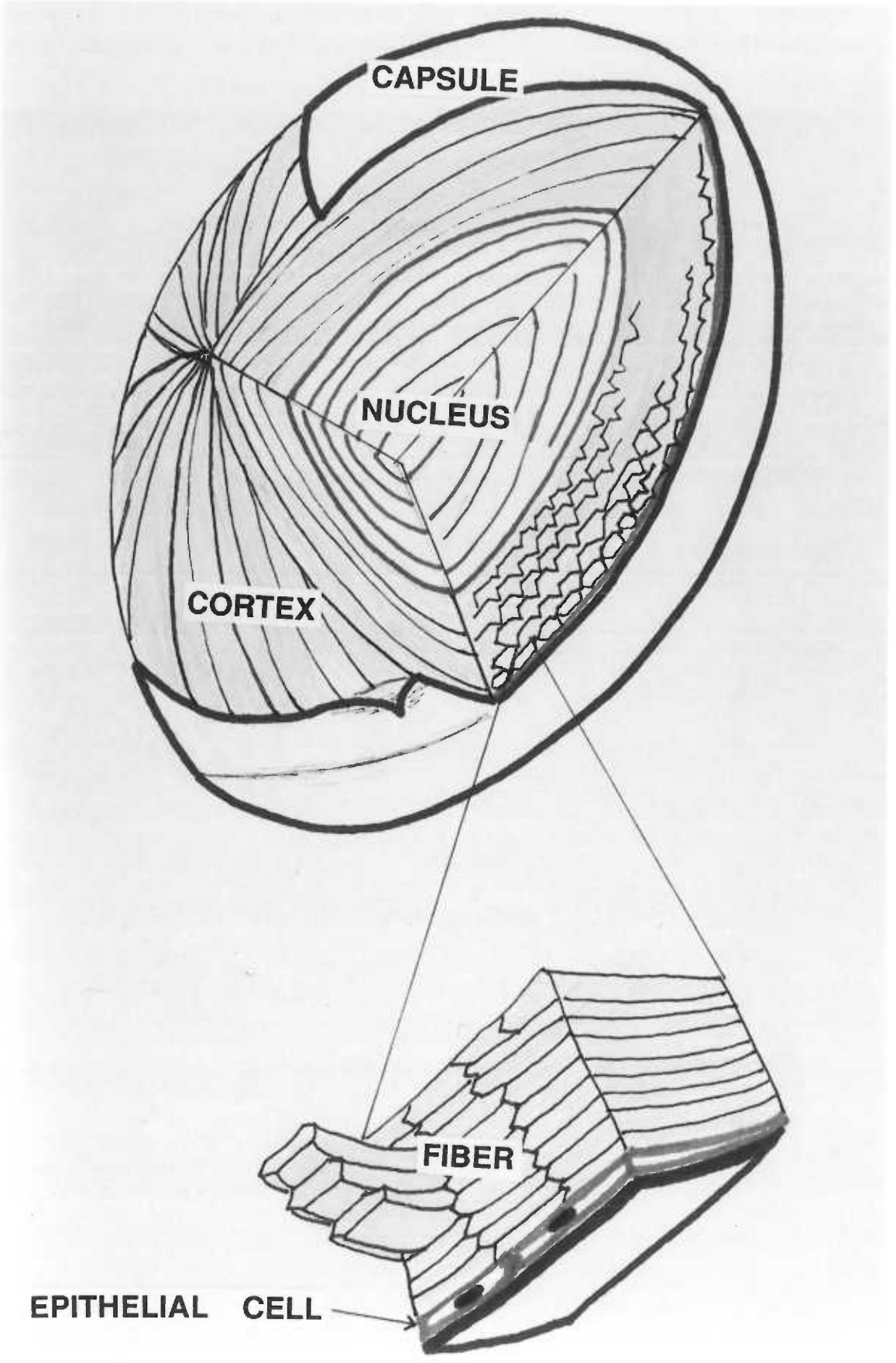
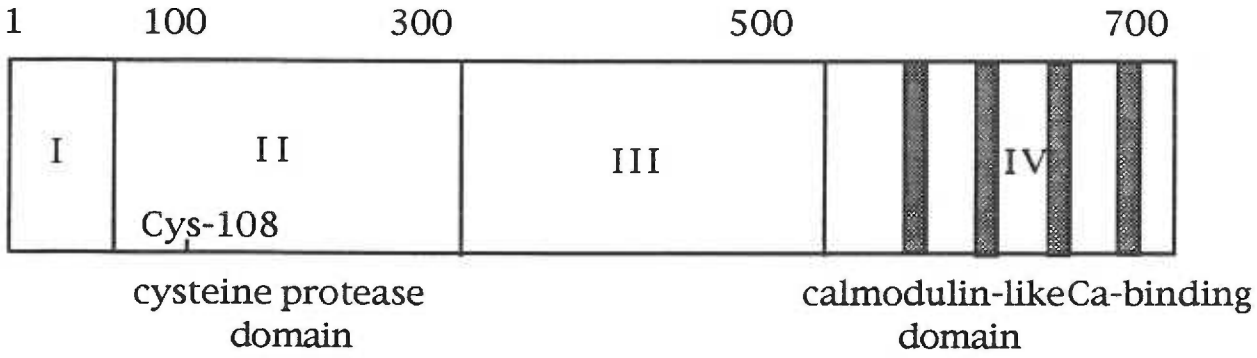

CAPSULE
NUCLEUS
CORTEX
FIBER
EPITHELIAL CELL

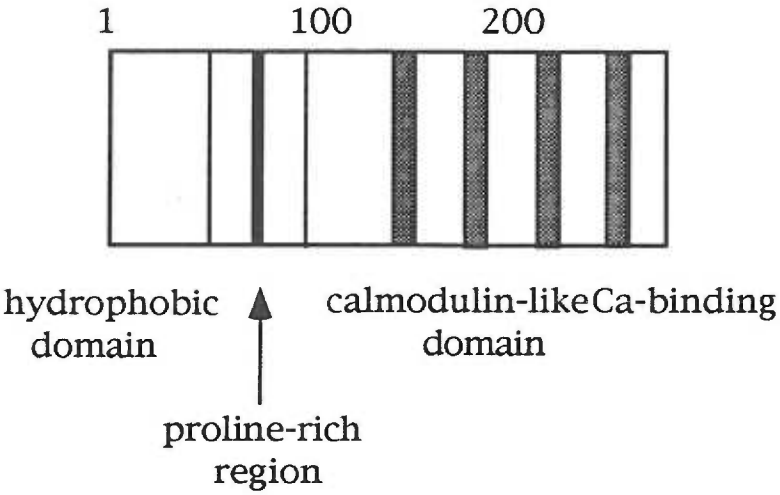
Figure 2:

Structure of translation products of calpain and calpastatin adapted from Suzuki et al, 1987.

## CALPAIN 80 K Subunit




## 30 K Subunit




## CALPASTATIN

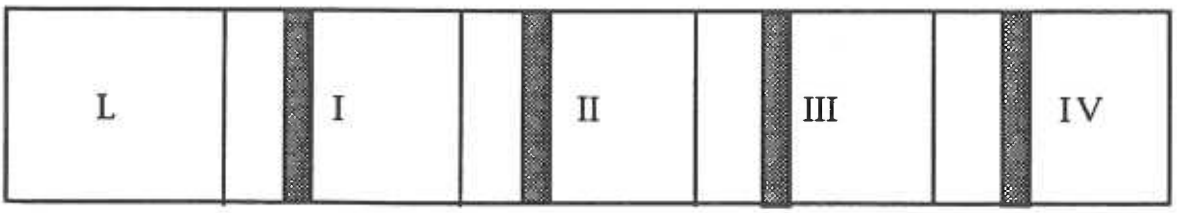


Figure 3:

Model to be tested of the translocation - activation hypothesis for activation of calpain. Calpain is translocated from the cytosol to the membrane during an increase in intracellular calcium. Once at the membrane, sensitivity of calpain to calcium is increased and calpain is autolysized. Calpain may then be able to degrade membrane proteins. Translocation of calpain may also enable calpain to escape inhibition of cytosolic calpastatin.

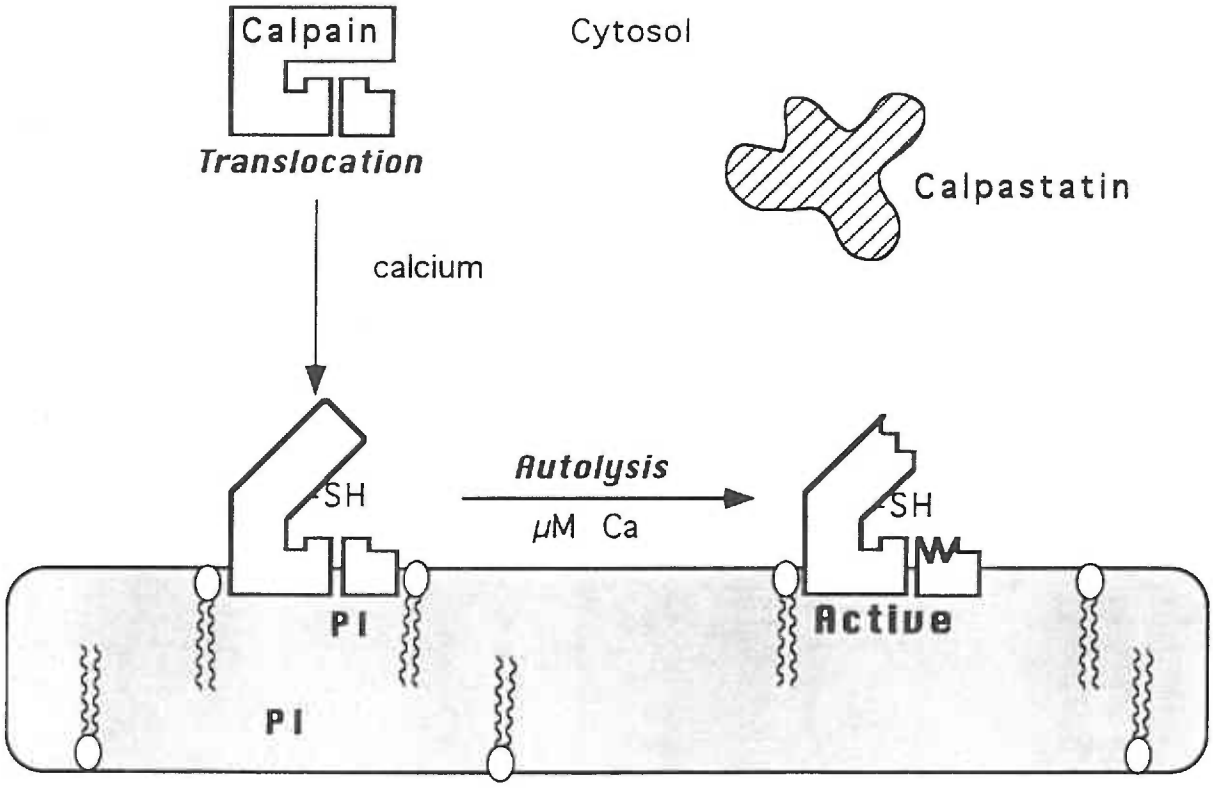

Calpain
Cytosol
Translocation
Calpastatin
calcium
Autolysis
μM Ca
SH
SH
PI
PI
Active
Membrane

Figure 4:

Immunoblot of pocine heart calpastatin reacted against polyclonal calpastatin antibody on left or preimmune serum on the right.

Lanes above 1: 10 μg of heat and water soluble proteins from porcine heart with molecular weight markers in the left lane.

Lanes above 2: 50 ng of purified porcine calpastatin. Preimmune serum did not recognize the dark staining band of calpastatin at 130 kDa.

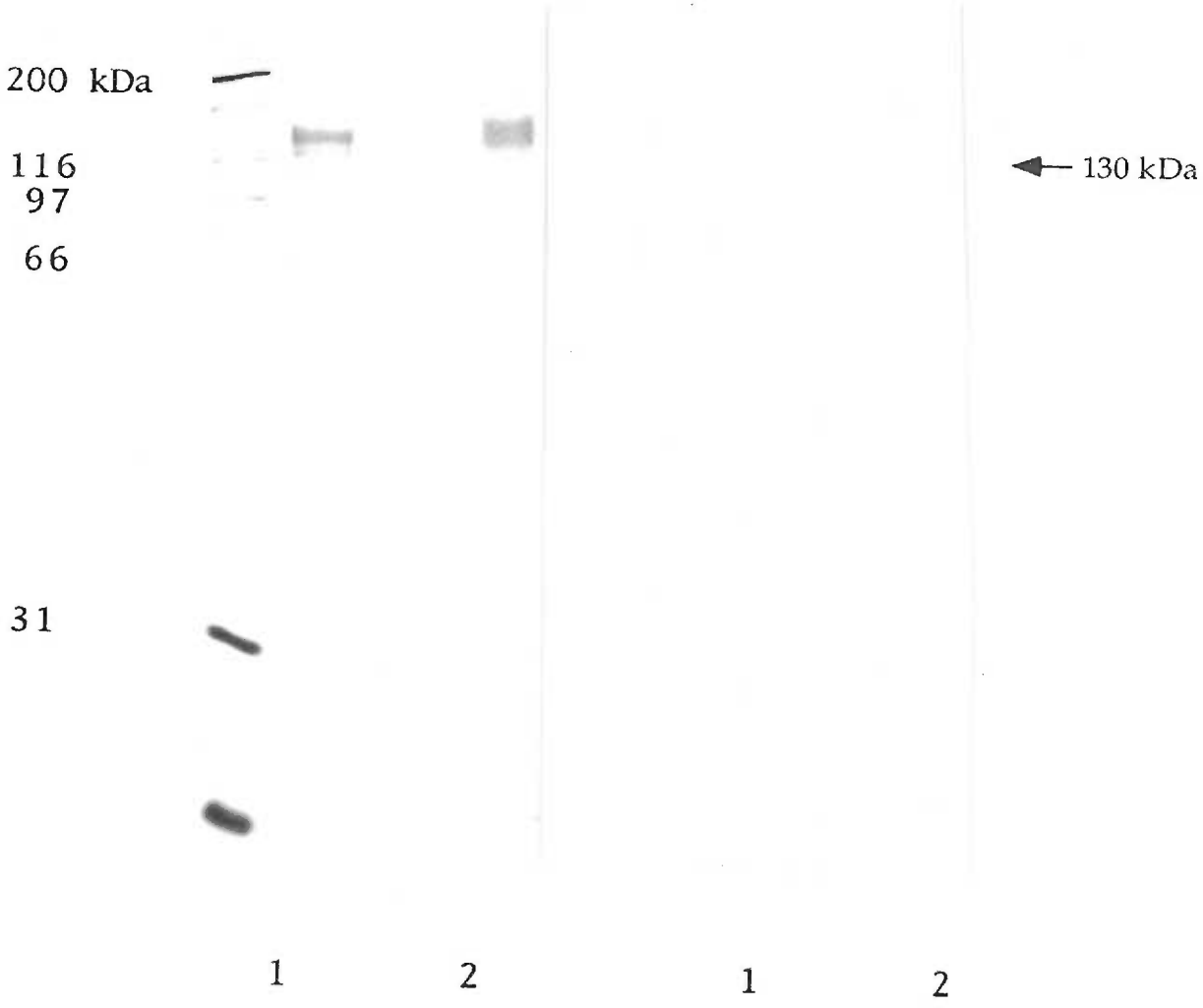

200 kDa
116
97
66
31
130 kDa
1
2
1
2

Figure 5:

SDS -PAGE of Coomassie blue stained gel of 10 μg of heat and water soluble proteins from porcine heart.

Molecular weight markers are in the left lane.

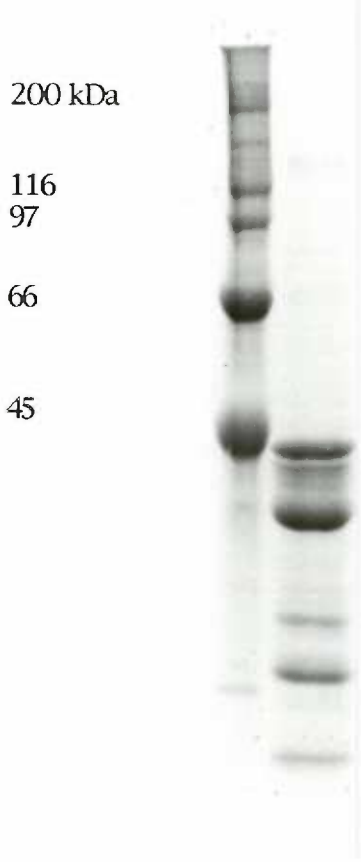

200 kDa
116
97
66
45

Figure 6:

Immunoblots of rat liver (lane 1) and sketetal muscle (lane 2) using polyclonal calpastatin antibody showing an immunoreactive doublet at 110 kDa in heat and water soluble proteins from liver but not muscle.

Molecular weight standards in kDa are on the left.

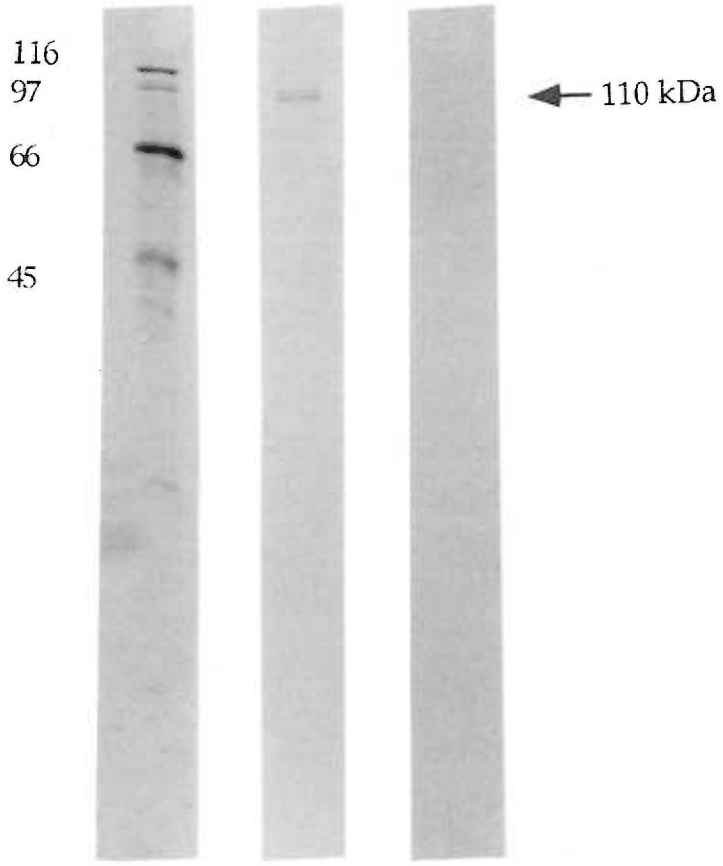

116
97
66
45
110 kDa
1
2

Figure 7:

SDS-PAGE of Coomassie blue stained gel of heat and water soluble proteins in rat liver (lane 2) and rat skeletal muscle (lane 3) corresponding to the same samples in Fig. 6. Lane 1 contains 200 ng of purified calpastatin from bovine heart with molecular weight markers on the left.

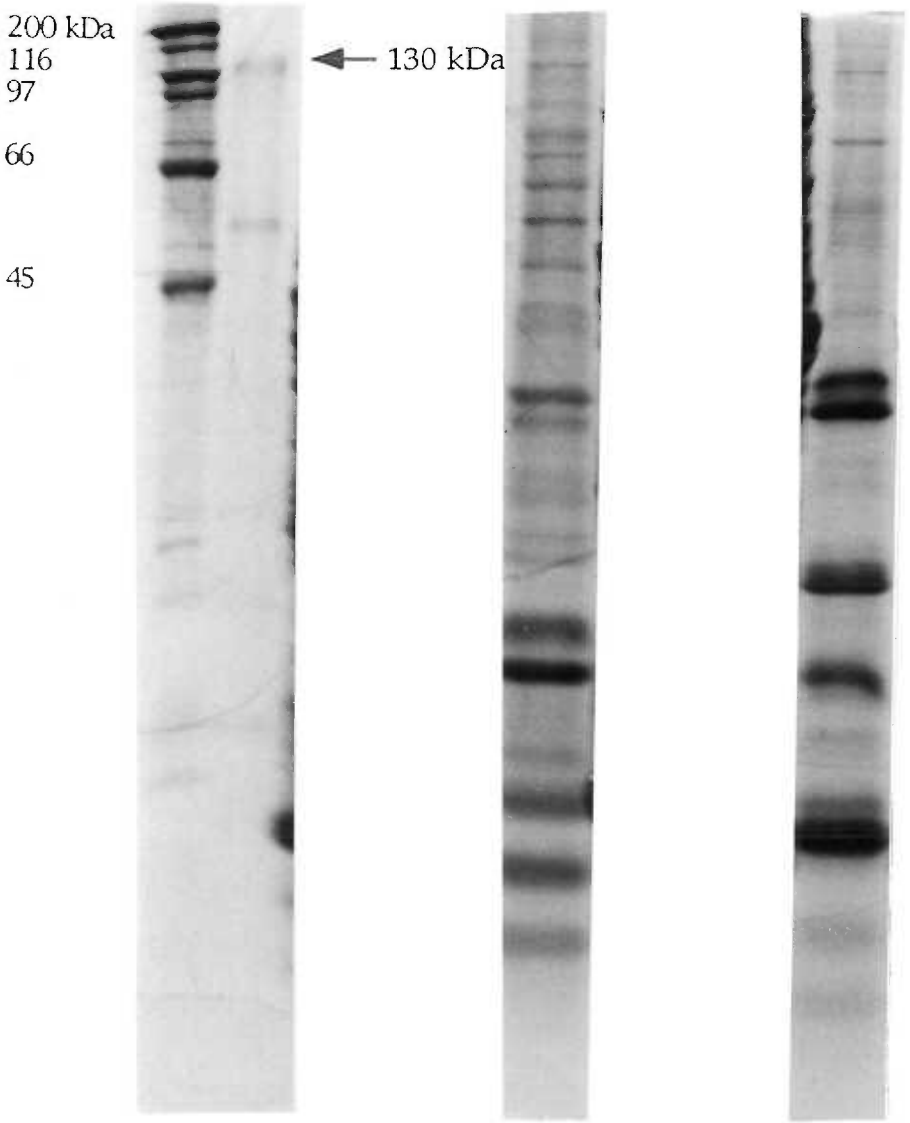
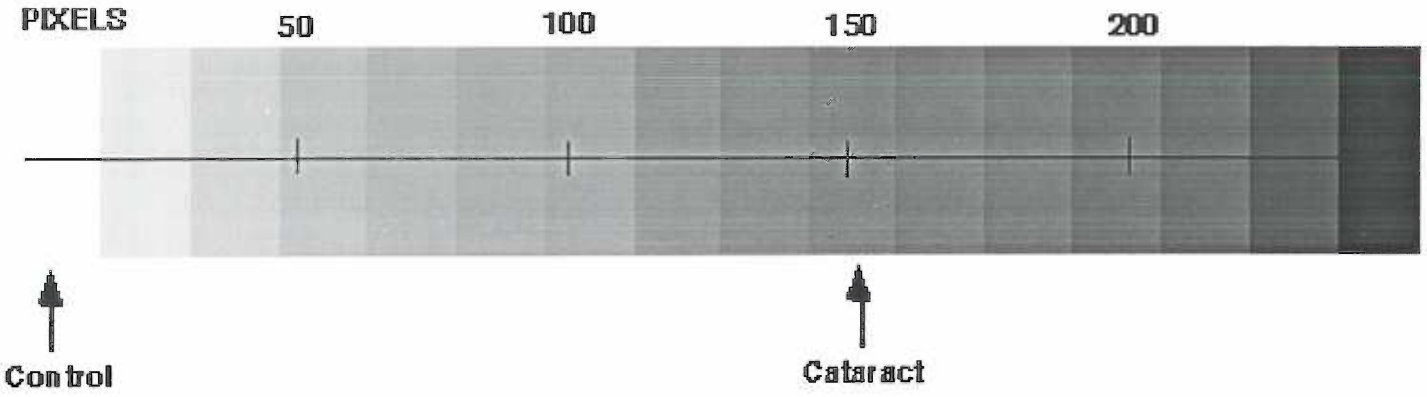
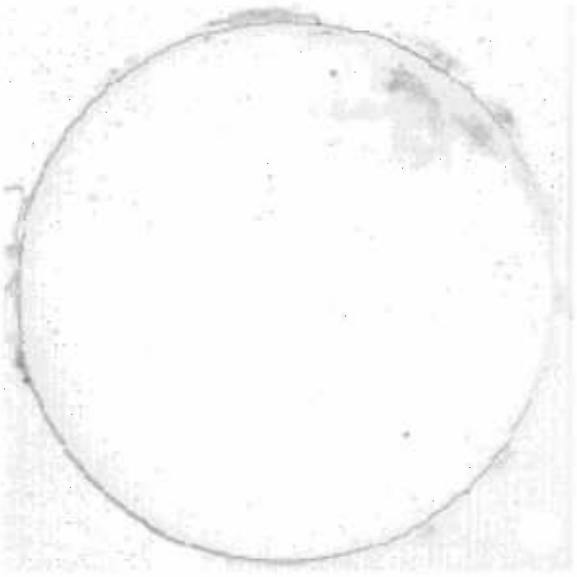

200 kDa
116
97
66
45
130 kDa
1
2
3

Figure 8:

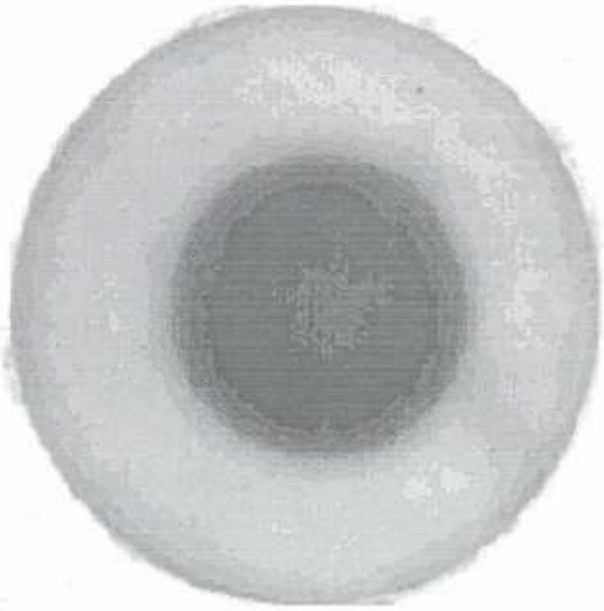
Greyscale used to quantitate nuclear opacity. Pixel values range from 0 - 255. Representative control and cataractous rat lenses captured on Quick Image (Mass Micro Systems Inc.) are shown with pixel values. Cataract was induced by calcium ionophore A23187.

## Grey Scale Used to Quantitate Nuclear Opacity



Control



Cataract

Figure 9:

Representative curve showing the linearity of the density of the 80 kDa calpain band on the immunoblots with increasing amounts of calpain standards. Calpain standards were purified from rat hind leg muscle as described in methods. $R^2$= regression analysis of the line of best fit.

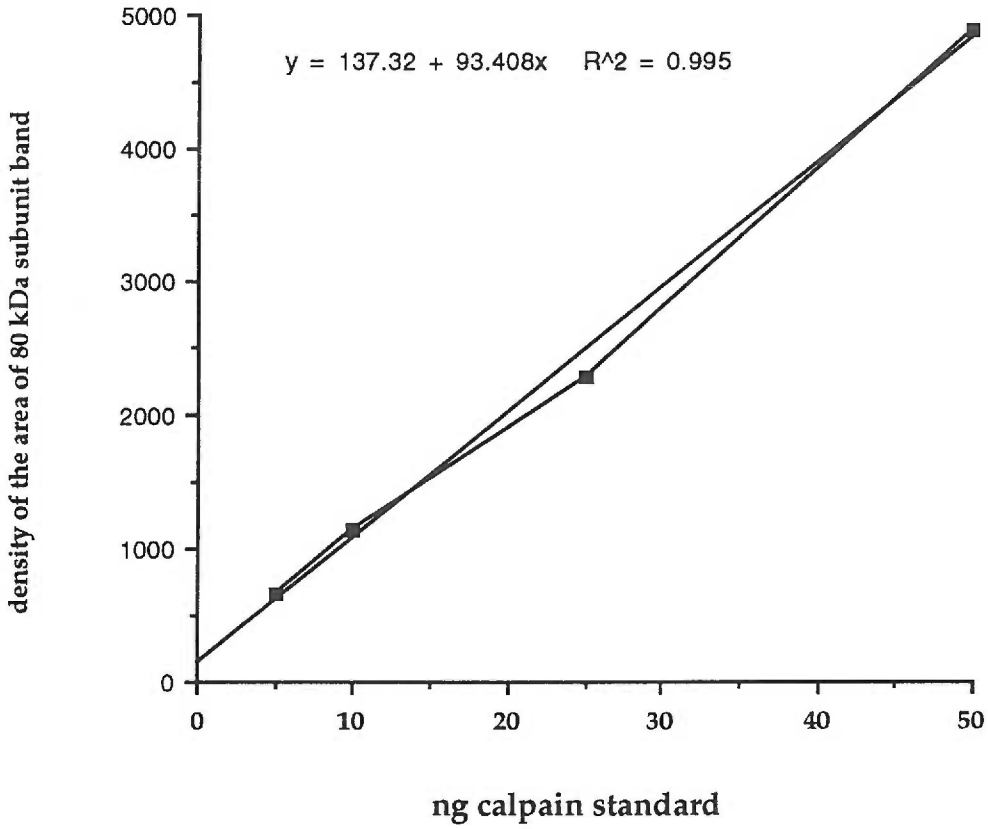

y = 137.32 + 93.408x R^2 = 0.995
density of the area of 80 kDa subunit band
ng calpain standard
5000
4000
3000
2000
1000
0
0
10
20
30
40
50

Figure 10:

Immunoblot for calpain in various fractions of lenses from 24 day old rats. Arrows indicate the 80 kDa catalytic subunit and the 30 kDa regulatory subunit of calpain.

Lane 1: molecular weight standards at the designated Mr shown to the left.

Lane 2: total water soluble protein.

Lane 3: 7 M urea wash of insoluble pellet.

Lane 4: 0.1 M NaOH wash of urea insoluble pellet.

Lane 5: remaining insoluble protein.

All lanes contained 50 μg protein. Lanes 6-10 contain 2.5, 5, 10, 25, and 50 ng calpain standards.

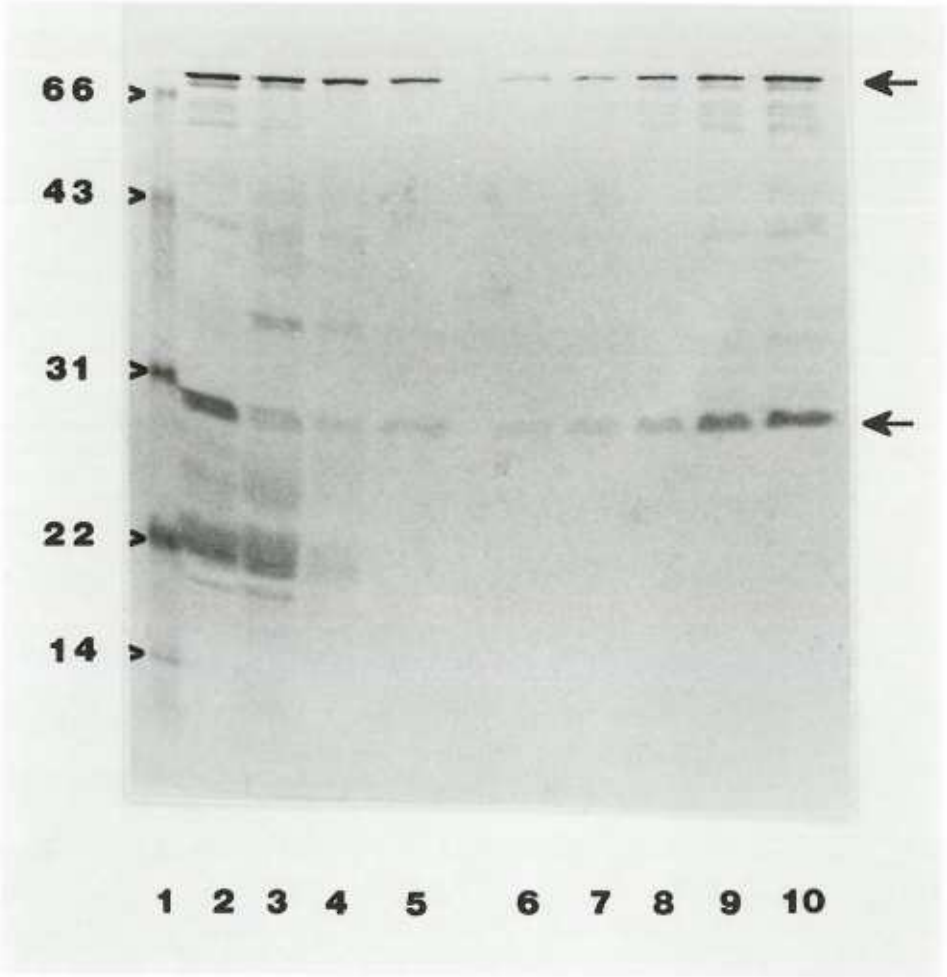

66
43
31
22
14
1 2 3 4 5 6 7 8 9 10

Figure 11:

SDS-PAGE of gel of 13 day old rat lens insoluble pellet stained with Commassie blue showing MP26 at the interfaces from a discontinuous sucrose gradient. The same volume (10 μl) was applied to each lane from identically treated samples. Lanes 1, 2, 3, and 4 are the 8/25%, 25/45%, 45/50%, and 50/55% sucrose interfaces, respectively. Lanes 6, 7, 8, and 9 are the same interfaces from a sucrose gradient in which 2mM calcium was added to all the buffers, including the initial homogenate. Lanes 5 and 10 are from the pellets of the above gradients.

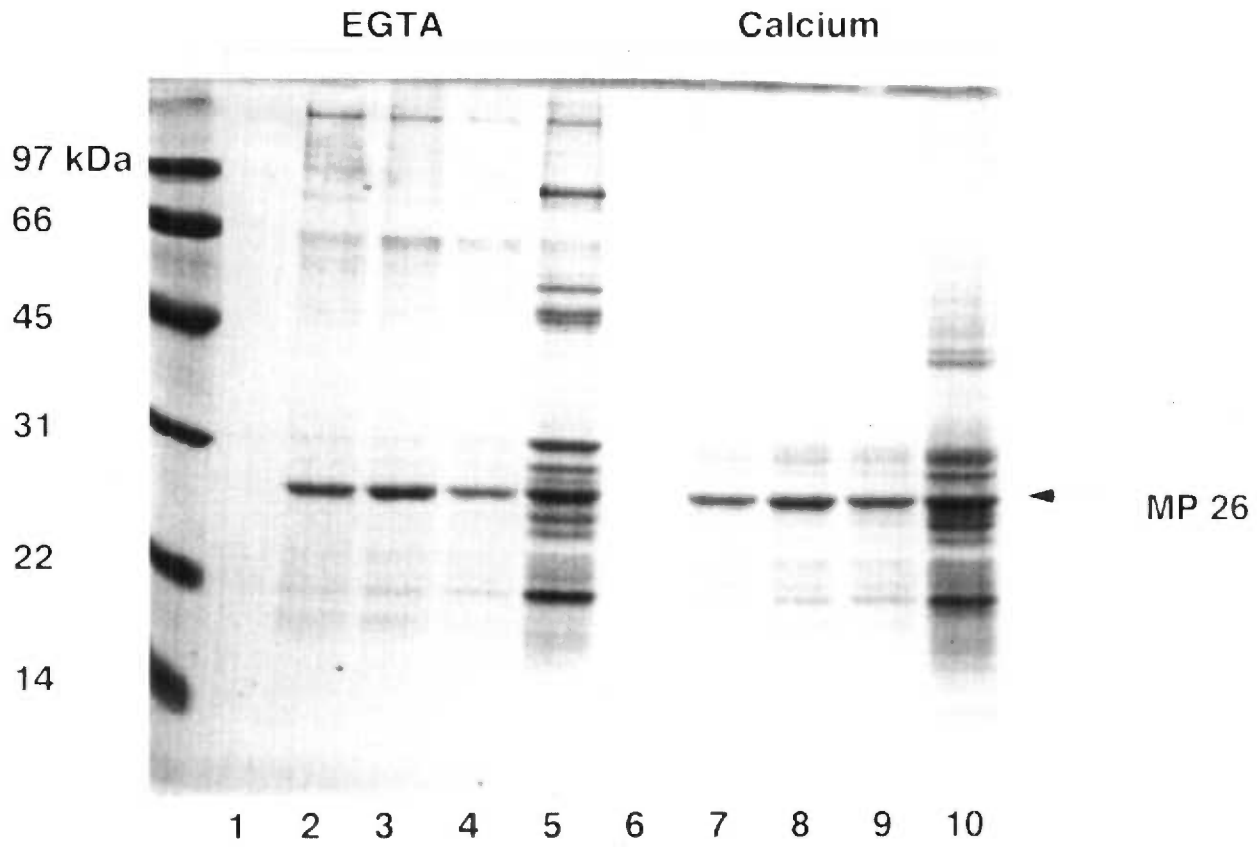

EGTA
Calcium
97 kDa
66
45
31
22
14
MP 26
1 2 3 4 5 6 7 8 9 10

Figure 12:

Immunoblot for calpain in 13 day old rat lens insoluble pellet from the interfaces of a discontinuous sucrose gradient. Lanes 1, 2, 3, and 4 are the 8/25%, 25/45%, 45/50%, and 50/55% sucrose interfaces, respectively. Lanes 6, 7, 8, and 9 are the same interfaces from a sucrose gradient in which 2mM calcium was added to all the buffers, including the initial homogenate. Lanes 5 and 10 are from the pellets of the above gradients. Lanes 11, 12, 13, and 14 are 2.5, 5, 15, and 30 ng of calpain standards, respectively.

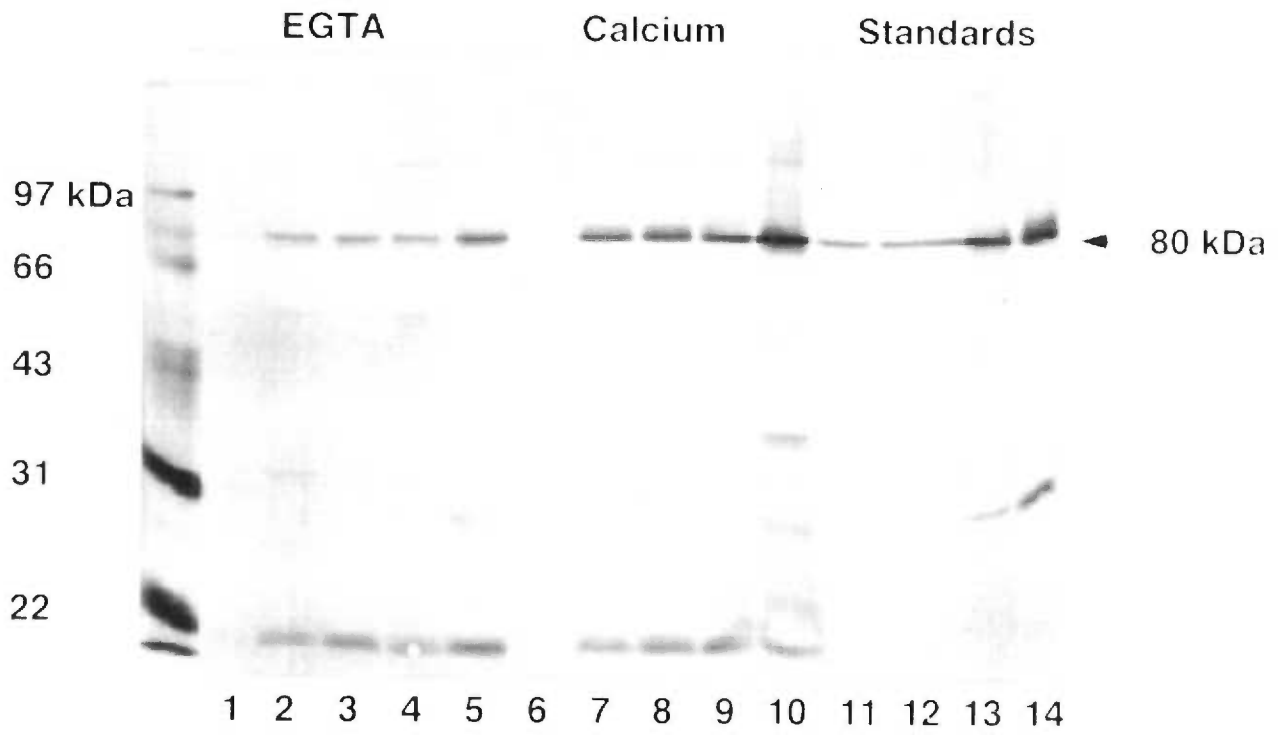

EGTA
Calcium
Standards
97 kDa
66
43
31
22
80 kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14

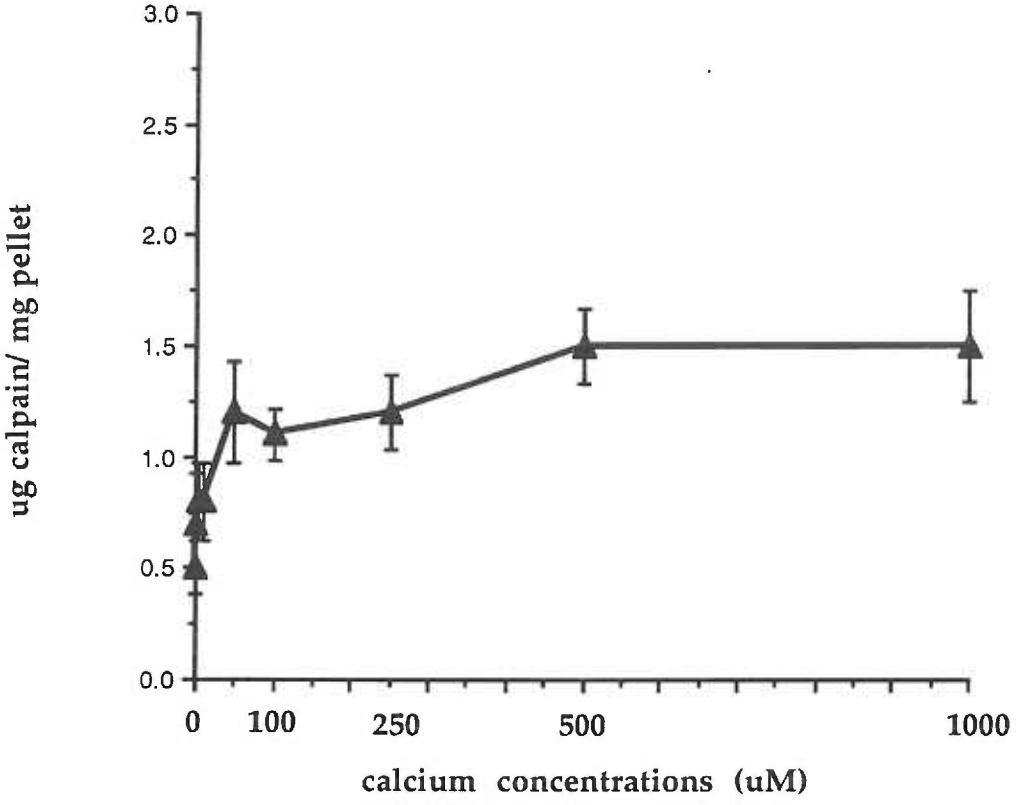
Figure 13:

Calcuim dependency curve for binding of calpain to lens total insoluble pellet from 5 week old rats.

A. Amount of 80 kDa subunit associating with the pellet. Data does not follow classical binding curve. However, the calcium concentration at which 50% of the calpain bound was estimated to be 25 μM.
B. Amount of 30 kDa subunit associating with the pellet. Data do not follow classical binding curve. However, the calcium concentration at which 50% of the calpain bound was estimated to be 50 μM.

Each value is an average of 4 determinations, with standard error of the mean. Significant differences were observed at 500 μM $CaCl_2$ ($p < 0.05$).

A. 80 kDa subunit




B. 30 kDa subunit

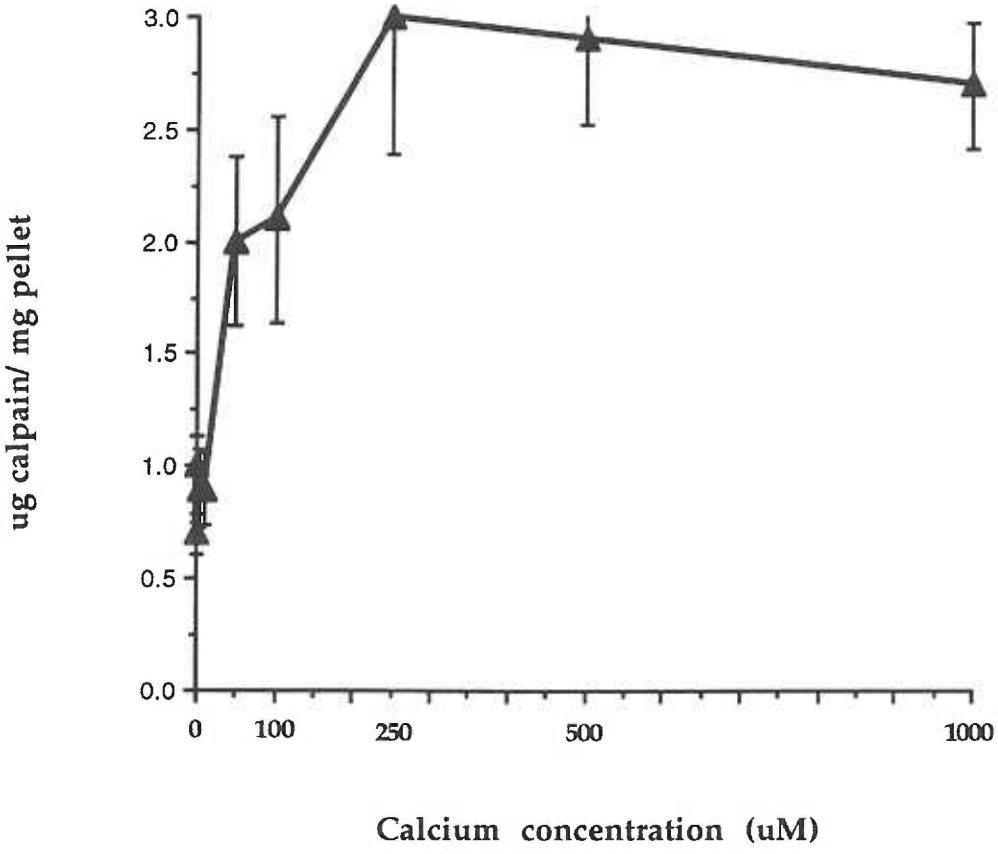


Figure 14:

Specific amount of calpain in chelator and Triton X-100 washes of the insoluble pellet compared to water soluble calpain.

Lenses were homogenized with low chelator buffer (100 μM EGTA). The homogenatewas split in half and 200 μM $CaCl_2$ was added to one of the halves. Homogenates were kept on ice for 15 minutes and then centrifuged at 10,000 x g. The total insoluble pellet was washed 3 times and then extracted with high chelator (1 mM EGTA and EDTA) followed by 1% Triton X-100. Lenses were from 24 day old rats.

Cross-hatched bars = control values with no added calcium. Stippled bars = values from lenses homogenized in 200 μM $CaCl_2$.

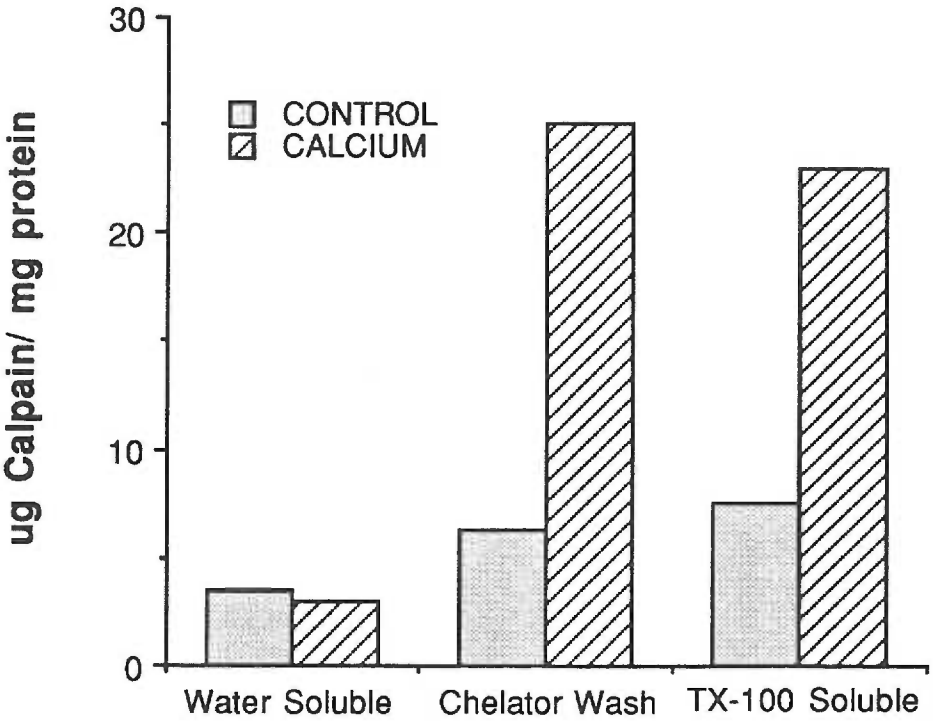

30
20
10
0
ug Calpain/ mg protein
CONTROL
CALCIUM
Water Soluble
Chelator Wash
TX-100 Soluble

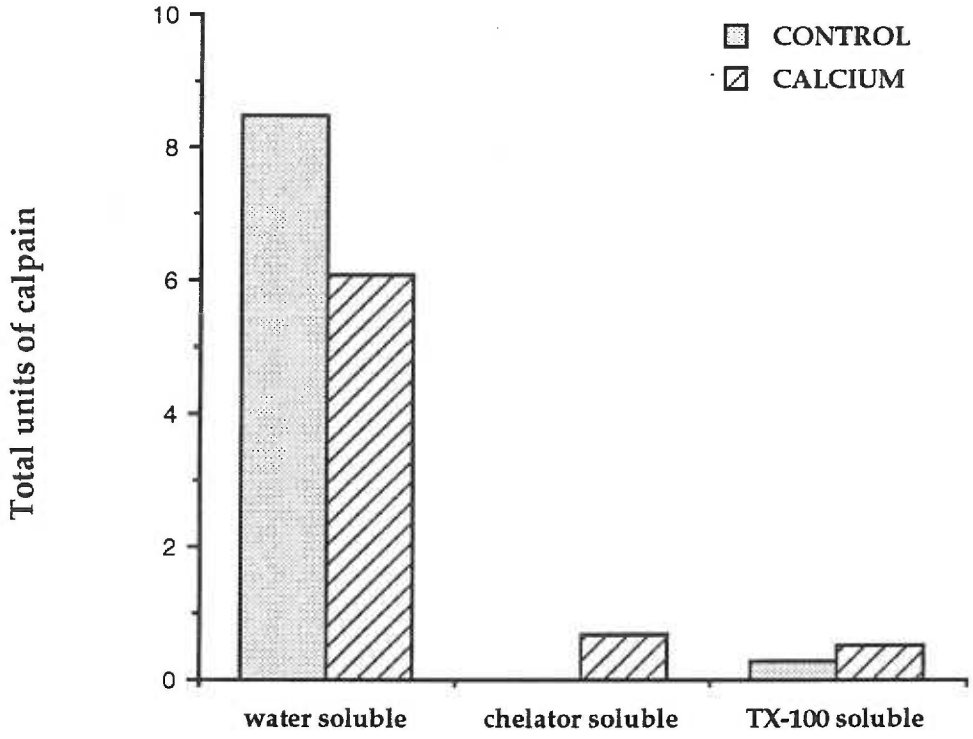
Figure 15:

Total activity of calpain (A) and units activity/mg calpain antigen (B) in chelator and Triton X-100 soluble compared to water soluble proteins.

Lenses were homogenized with low chelator buffer (100μM EGTA). The homogenate was split in half and 200 μM $CaCl_2$ was added to one half. Homogenates were kept on ice for 15 minutes and then centrifuged at 10,000 x g. The total insoluble pellet was washed 3 times and then extracted with high chelator (1 mM EGTA and EDTA) followed by 1% Triton X-100. Lenses were from 24 day old rats. Two peaks of calpain activity (200 and 250 mM NaCl) eluted from DEAE of chelator-extracted proteins from calcium-treated lenses. Calpain normally elutes at 250 mM NaCl.

Cross-hatched bars = control values with no added calcium. Stippled bars = values from lenses homogenized in 200 μM $CaCl_2$.

A.


Total units of calpain
10
8
6
4
2
0
CONTROL
CALCIUM
water soluble
chelator soluble
TX-100 soluble

B.

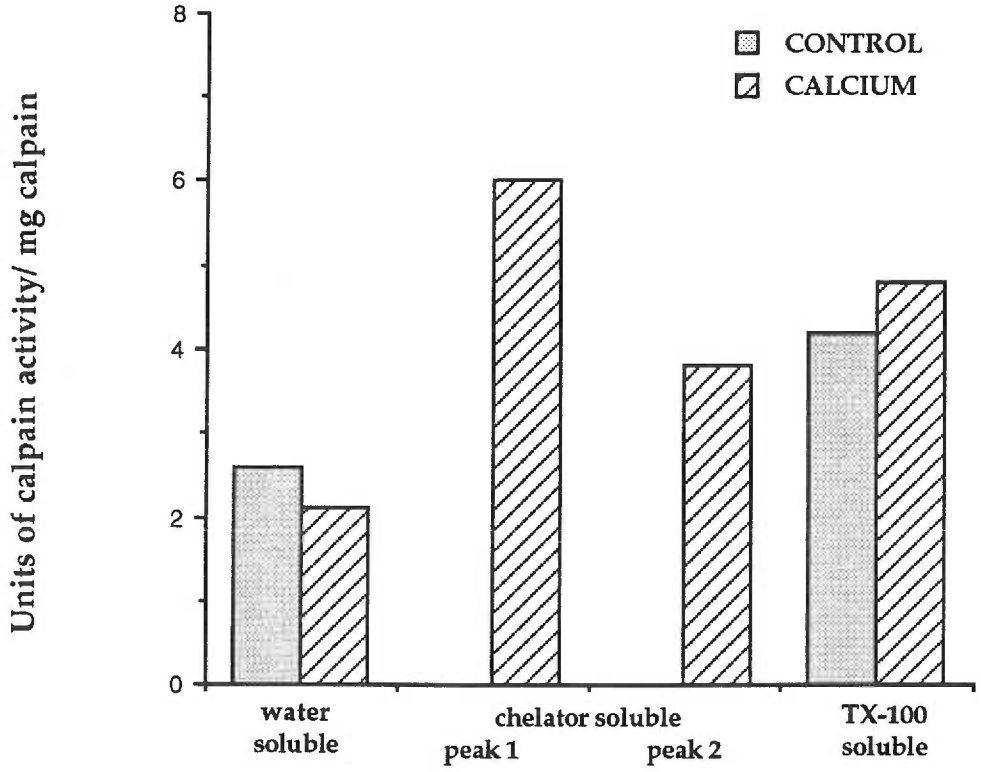

Units of calpain activity/ mg calpain
8
6
4
2
0
CONTROL
CALCIUM
water soluble
chelator soluble peak 1
peak 2
TX-100 soluble

Figure 16:

The ratio of calpain (units/mg) to calpastatin (units/mg) in water soluble and insoluble lens protein extracted with 1 mM EGTA or 1% Triton X-100. Rat lenses were homogenized in 20 mM Tris (pH 7.5), 100 mM EGTA, 1 mM DTE, and 100 mM KCl with or without 200 μM $CaCl_2$.

n.d. = not detected

## Ratio of Calpain to Calpastatin Activity

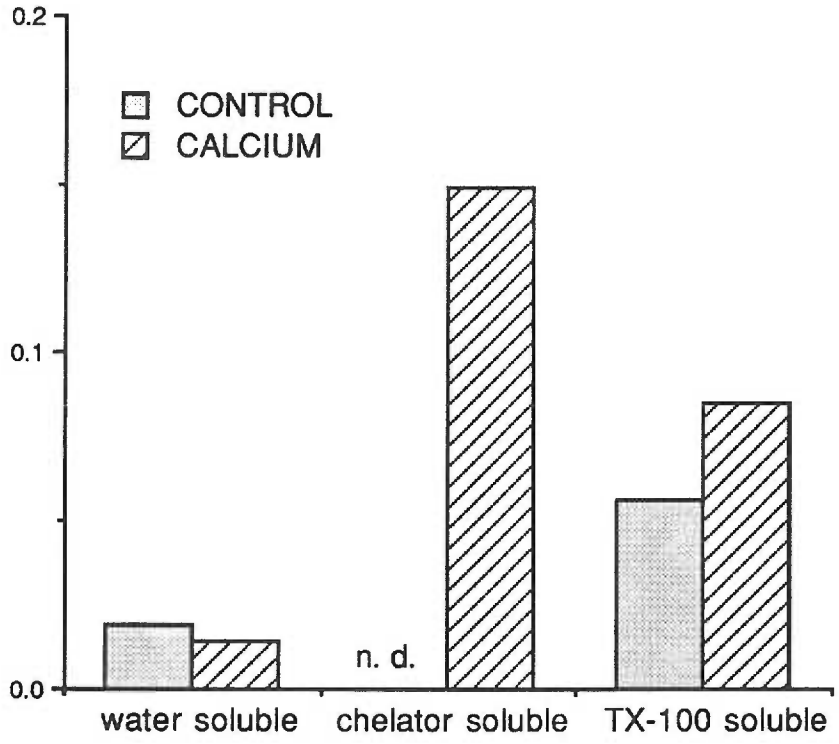


Figure 17:

Effect of calpastatin and other proteins on calcium-induced association of calpain with insoluble fraction of rat lens. Lenses from 5 week old rats were homogenized in EGTA buffer (lane 1) or 100 μM $CaCl_2$ (lane 2). Bovine serum albumin (BSA, lane 3), or trypsin inhibitor (TI, lane 4), or exogenous calpastatin (lane 5) were added to lenses homogenized in calcium.

Error bars are standard error of the mean for n = 5.

Asterick (*) indicates statistically different from lanes 1 and 5 at $p<0.001$.

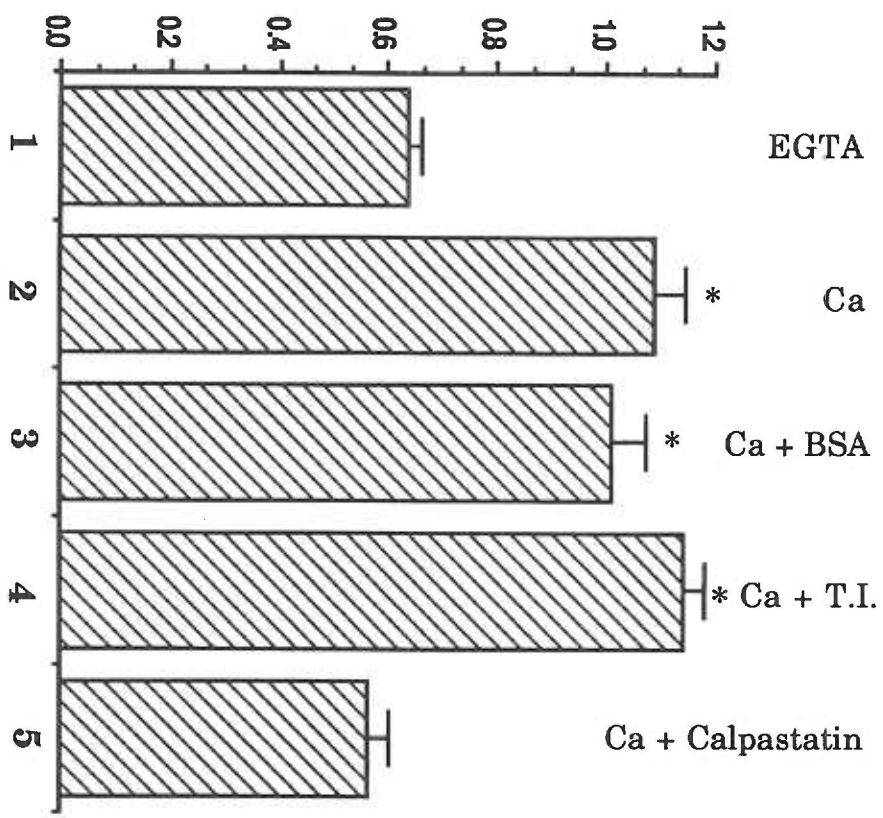

µg Calpain/ mg pellet
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1
2
3
4
5
EGTA
Ca
Ca + BSA
Ca + T.I.
Ca + Calpastatin
*
*
*

Figure 18:

Coomassie blue stained gel showing calcium - dependent proteolysis. Lenses from 21 day old rats were homogenized in buffer containing 20 mM Tris (pH 7.5), 100 μM EGTA, 1 mM DTE and 1 mM $CaCl_2$. Lenses were homogenized with 500 μM E64 (lane 1) or without E64 (lane 2). One mM $CaCl_2$ was added to the washed pellet separated from the soluble proteins. This sample differs from samples in lane 1 and 2 which contained both the soluble and insoluble fractions.

Arrows on right identify hydrolyzed proteins.

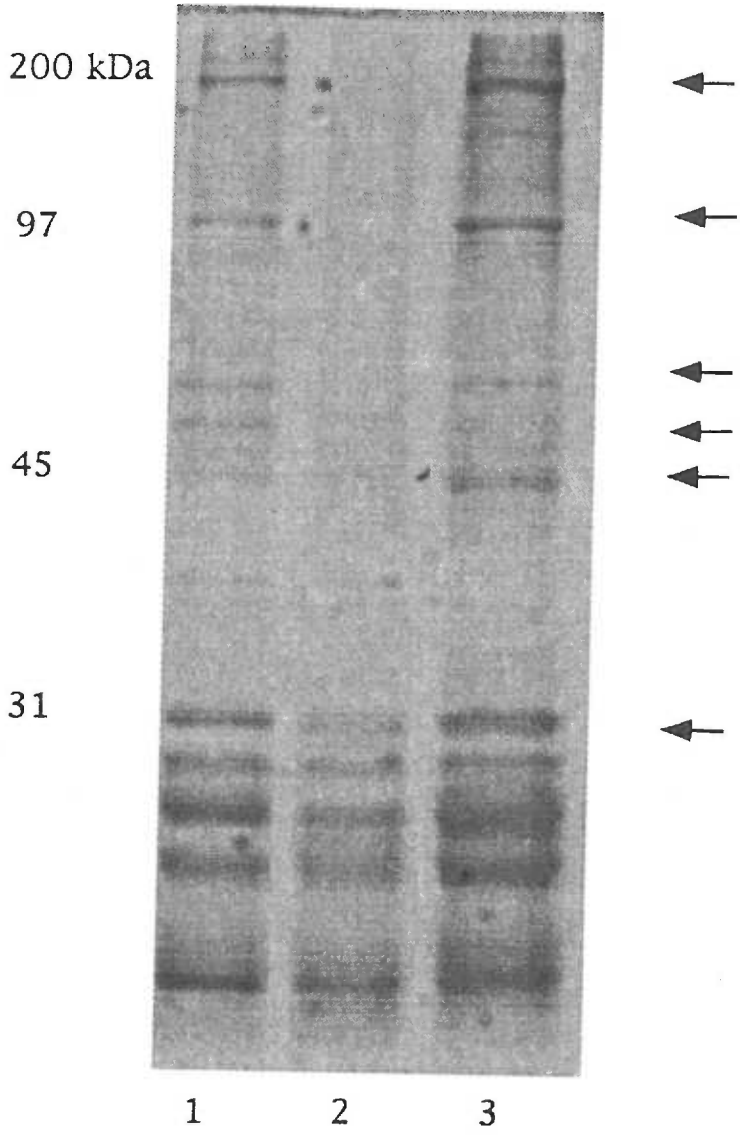

200 kDa
97
45
31
1
2
3

Figure 19:

Density of lens nucleus in lenses cultured in increasing inhibitor concentrations. Rat lenses were cultured in inhibitor in basic medium alone for 5 days. At the end of the experiment the density of the lens was quantitated with a computer imaging program.

Data represents the mean ± SD, N = 4-10

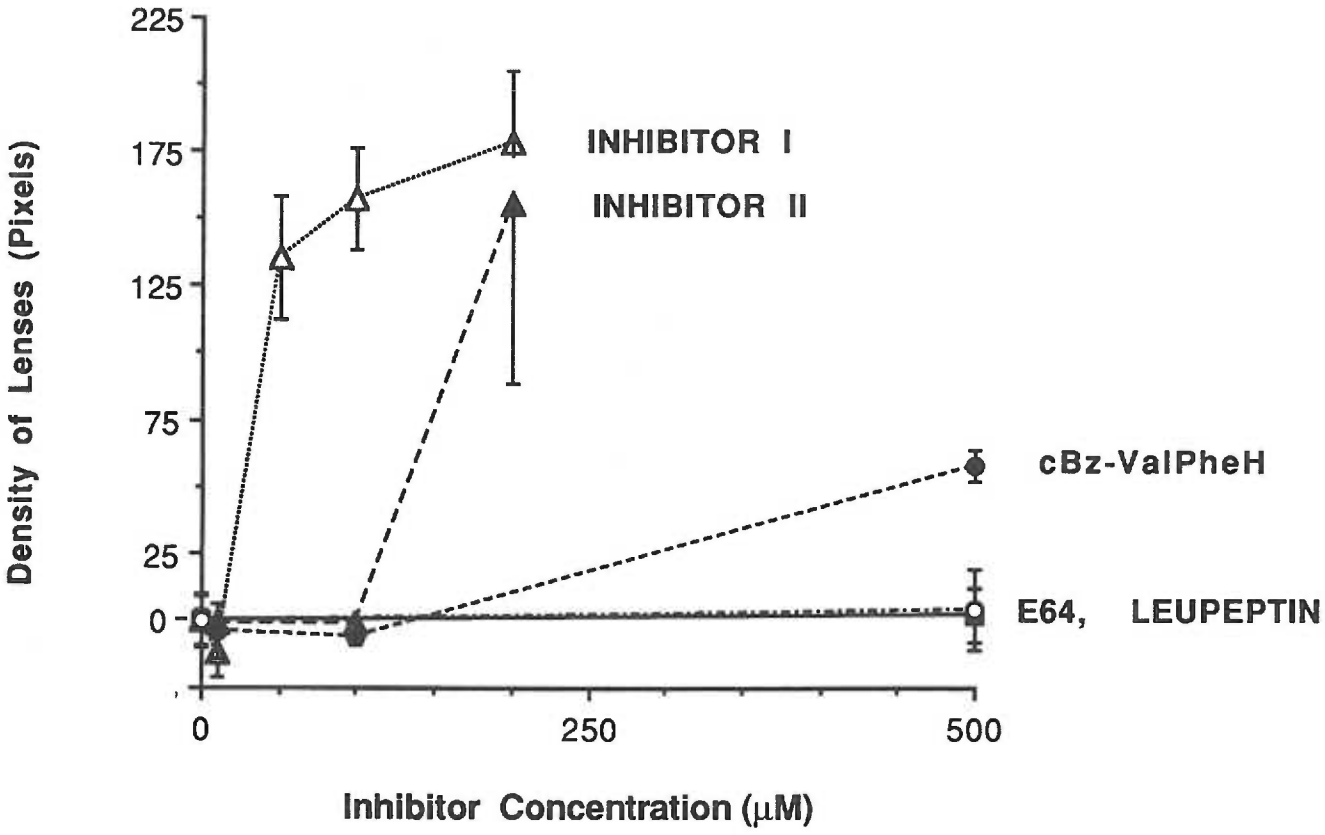

225
175
125
75
25
0
Density of Lenses (Pixels)
INHIBITOR I
INHIBITOR II
cBz-ValPheH
E64, LEUPEPTIN
0
250
500
Inhibitor Concentration (μM)

Figure 20:

Density of lenses cultured in calcium ionophore A23187 alone (solid column) or cultured in A23187 plus inhibitor (open columns). Inhibitor concentrations were 10 μM Inhibitor I, 200 μM Inhibitor II, 2 mM leupeptin, 500 μM cBz-ValPheH, and 50 μM E64.

Data represents mean ± SD, N=4-10.

*$p<0.05$, significantly different from lenses cultured in A23187 alone.

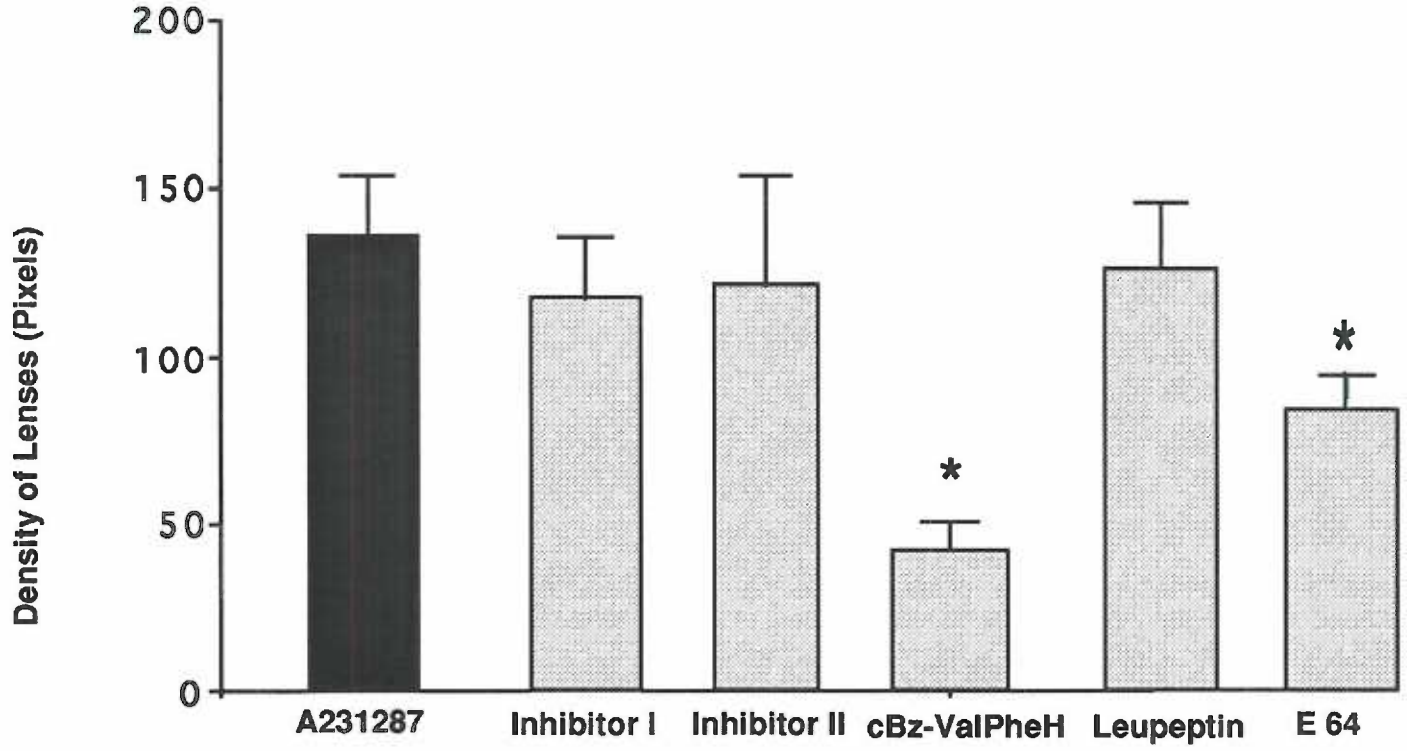

Density of Lenses (Pixels)
200
150
100
50
0
*
*
A231287
Inhibitor I
Inhibitor II
cBz-ValPheH
Leupeptin
E 64

Figure 21:

Density of lenses cultured in calcium ionophore plus increasing E64 concentrations. Rat lenses were preincubated in E64 for 12 hours and then 10 μM A23187 added for 24 hours. Lenses were cultured for an additional 4 days in E64. Because of the irreversible and tight binding of E64 to calpain, a true $IC_{50}$ determination is not possible. However, 50% of the maximum effect of E64 was approximated at 25 μM $CaC_2$.

Error bars are mean ± SD, N=4-6.

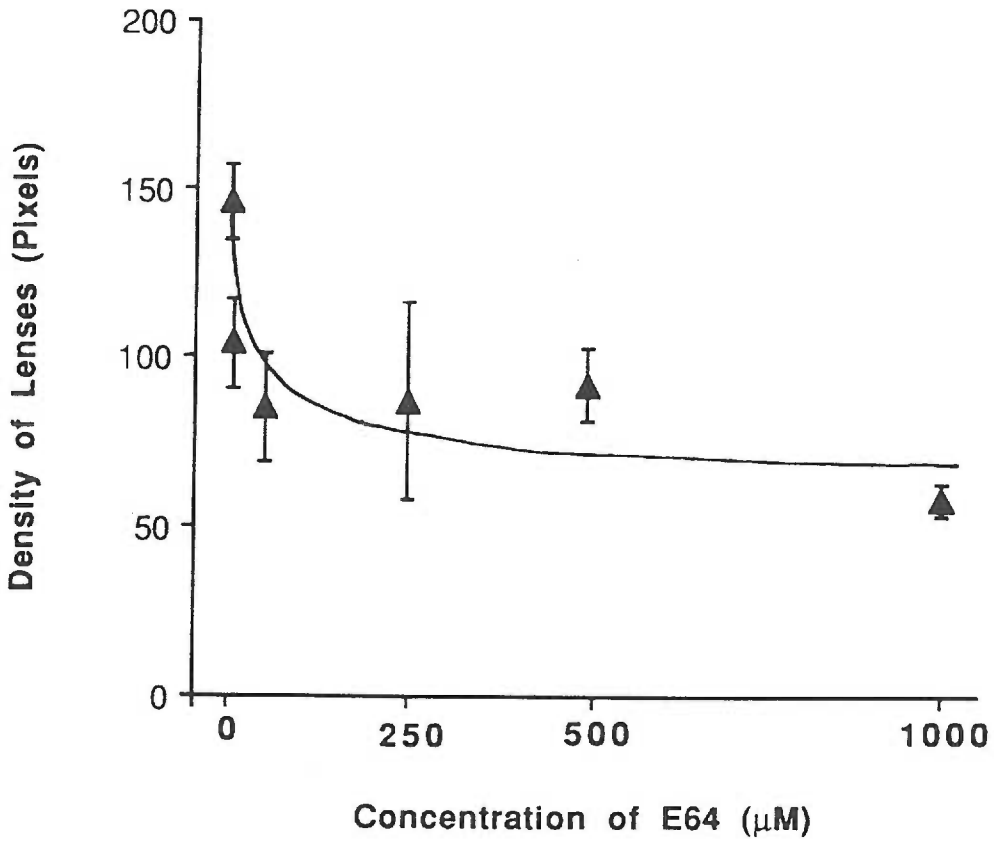

200
150
100
50
0
Density of Lenses (Pixels)
0
250
500
1000
Concentration of E64 (μM)

Figure 22:

Amounts of βB1-crystallin in the soluble and insoluble proteins of cultured lenses expressed as percent of control. Amounts were determined by densitometric scanning of SDS-PAGE gels. Inhibitor concentration was 500 μM.

Data are mean ±SD for 3-5 pools of lenses. Each pool contained 2 lenses.

*Significantly different from lenses cultured in A23187 alone at $p<0.01$.

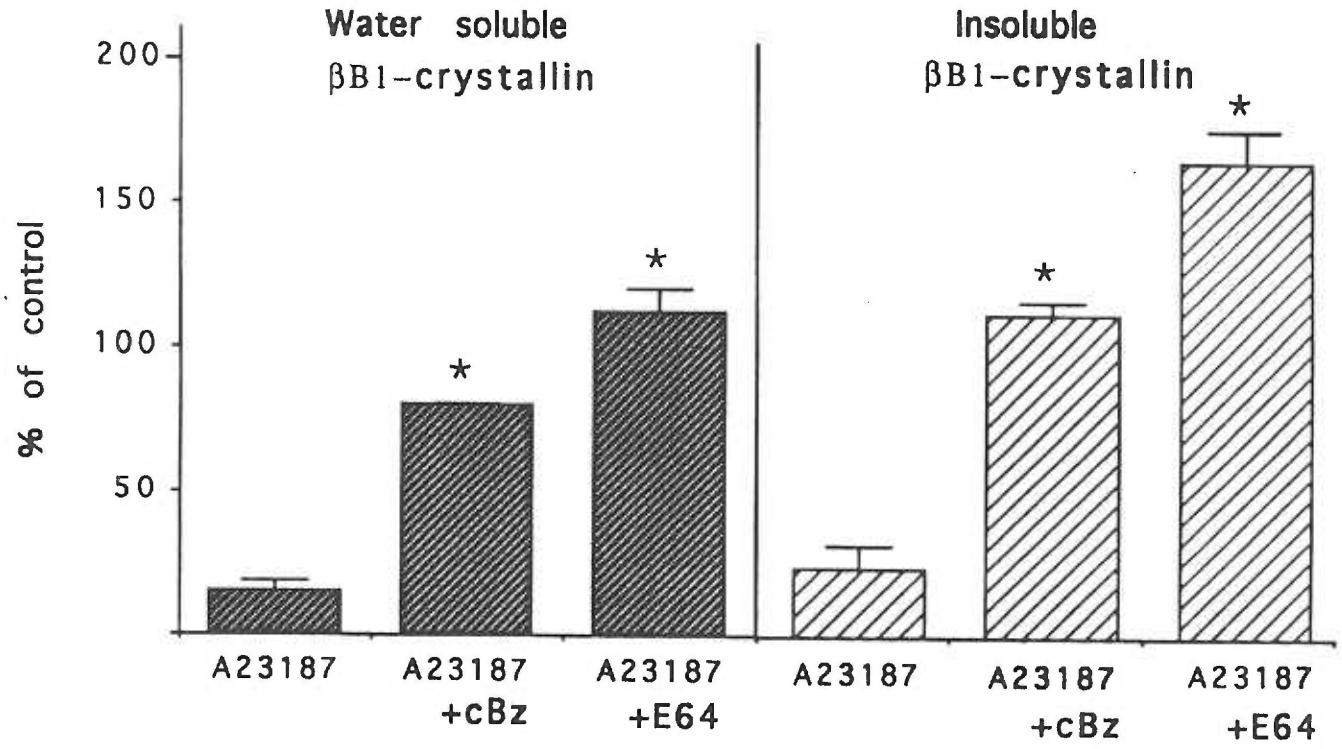

Water soluble
βB1-crystallin
Insoluble
βB1-crystallin
% of control
200
150
100
50
*
*
*
*
A23187
A23187 +cBz
A23187 +E64
A23187
A23187 +cBz
A23187 +E64

Figure 23:

SDS-PAGE of soluble proteins (lanes 1-7) and insoluble proteins (lanes 8-14) from lenses cultured in A23187 plus increasing E64 concentrations. Arrow indicates the 31 kDa band for βB1-polypeptide.

Lanes 1, 8: control proteins; lanes 2, 9: A23187 plus 1 mM E64; lanes 3, 10: A23187 plus 500 μM E64; lanes 4, 11: A23187 plus 250 μM E64; lanes 5, 12: A23187 plus 50 μM E64; lanes 6, 13: A23187 plus 5 μM E64; Lanes 7, 14: A23187 only.

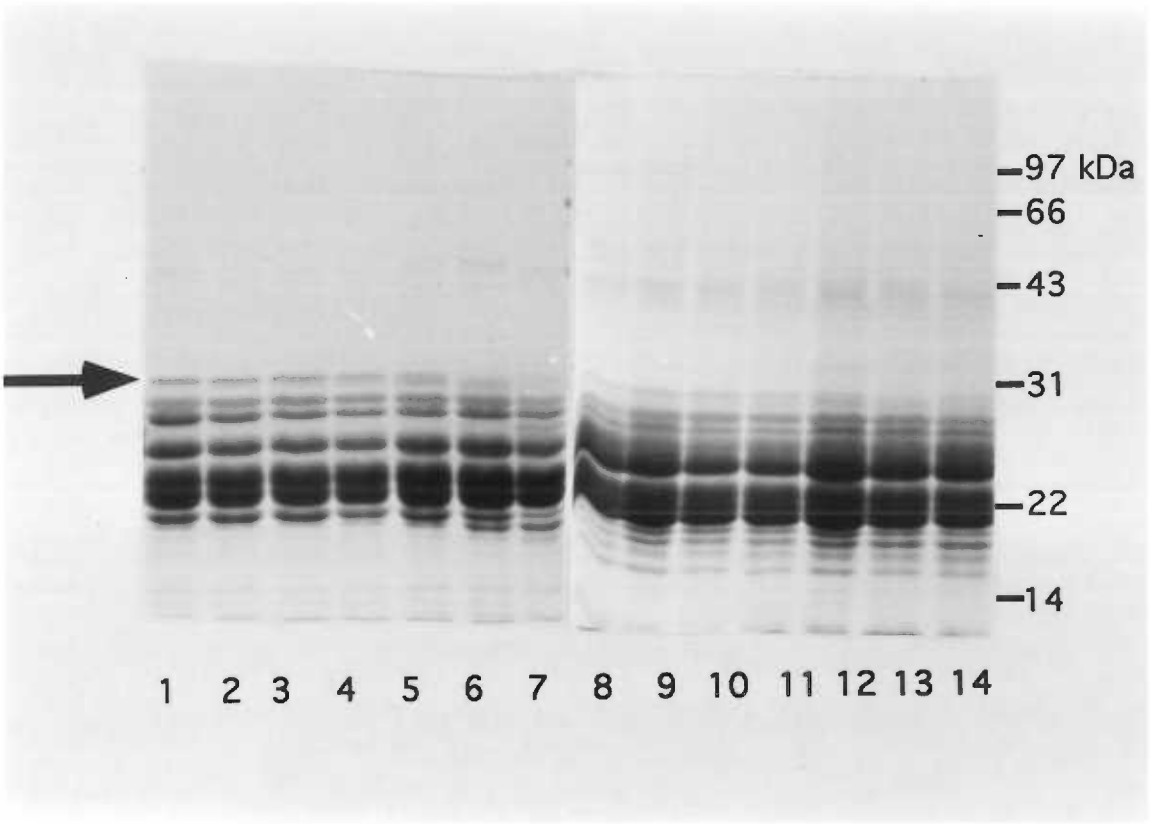

97 kDa
66
43
31
22
14
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 24:

Amounts of calpain determined by immunoblotting the soluble and insoluble proteins from lenses cultured in basic medium (control), in medium plus A23187, or in medium plus A23187 and either 500 μM cBz-ValPheH or E64.

Data are the mean ±SD of 3-5 pools of lenses.

*Significantly different from lenses cultured in A23187 alone at $p<0.05$.

n.d. = not detectable

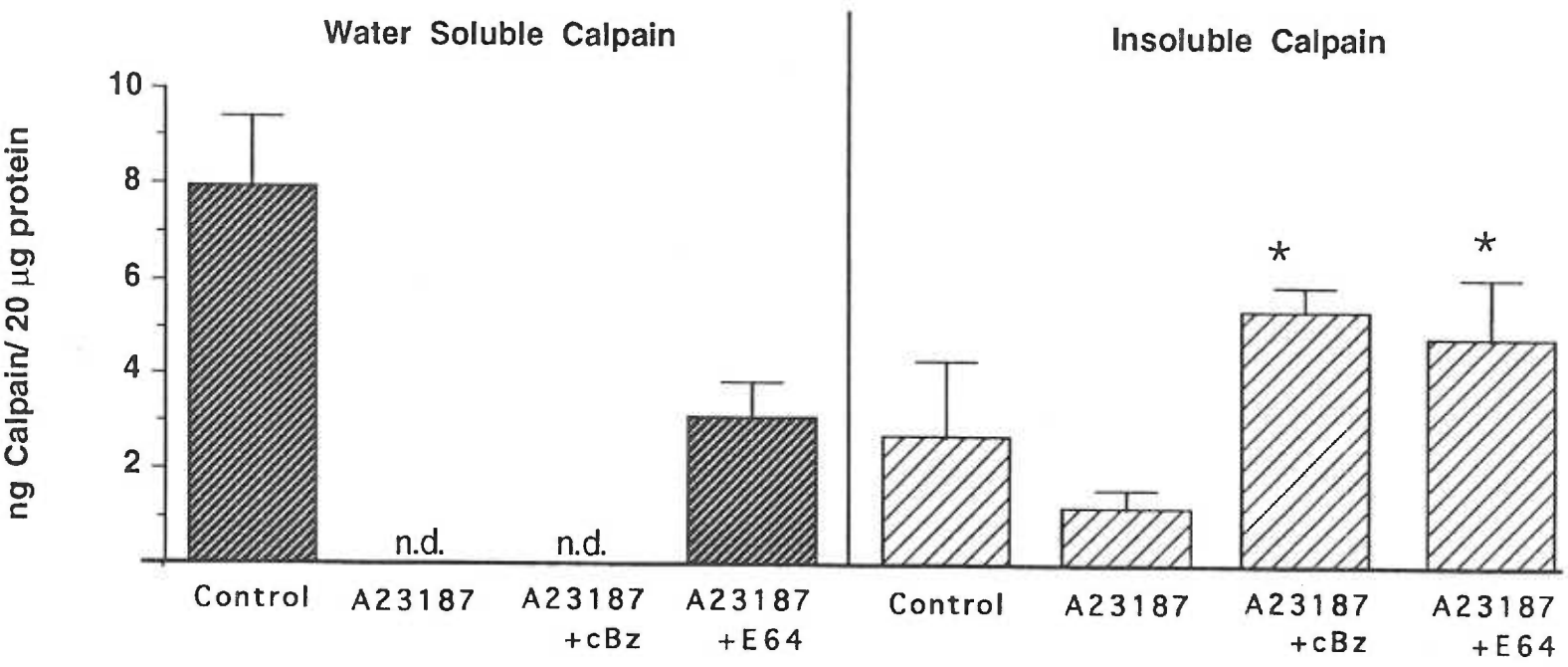

Water Soluble Calpain
Insoluble Calpain
ng Calpain/ 20 µg protein
10
8
6
4
2
n.d.
n.d.
*
*
Control
A23187
A23187 +cBz
A23187 +E64
Control
A23187
A23187 +cBz
A23187 +E64

Figure 25:

Immunoblot for calpain in the proteins from lenses cultured in A23187 plus increasing amounts of E64.

Lane 1 is 5 ng of purified calpain standard. Lanes 2-7 are soluble proteins and lanes 8-13 are insoluble proteins.

Lanes 2, 8: control; lanes 3, 9: A23187 plus 1 mM E64; lanes 4, 10: A23187 plus 500 μM E64; lanes 5, 11: A23187 plus 250 μM E64; lanes 6, 12: A23187 plus 50 μM E64; lanes 7, 13: A23187 only.

1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 26:

Calpastatin activity in the cortex (light cross-hatched bars) or nucleus (dark cross-hatched bars) of 1-2 pairs human lenses from different age donors. Lenses were homogenized in 20 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA, and 1 mM DTE, and the water soluble proteins were fractionated on a TSK-DEAE 5-PW column.

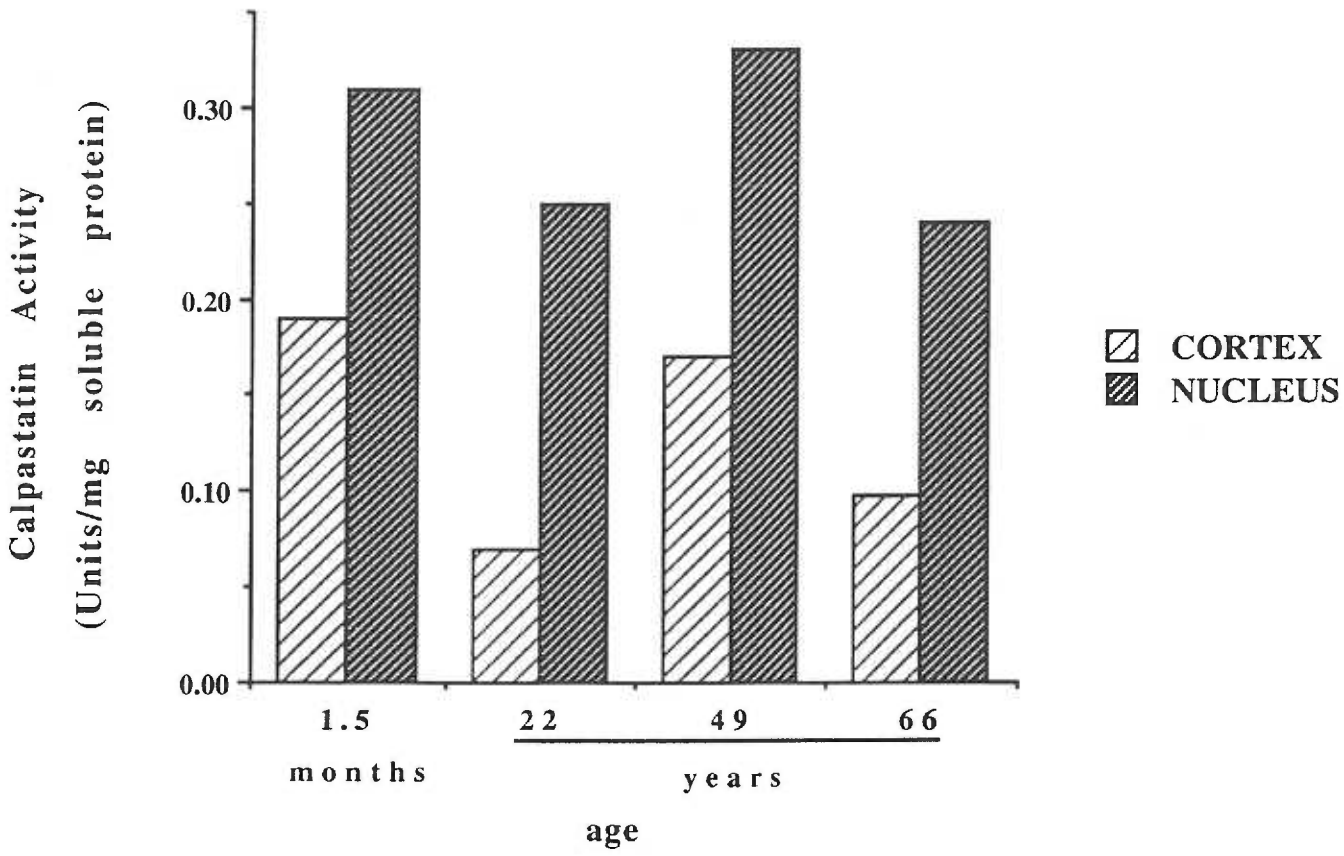

Calpastatin Activity
(Units/mg soluble protein)
0.30
0.20
0.10
0.00
1.5
22
49
66
months
years
age
CORTEX
NUCLEUS

Figure 27:

Ratio of calpastatin (units/mg) to calpain (units/mg) in water soluble protein from cortex (light cross-hatched bars) and nucleus (dark cross-hatched bars) of human lenses.

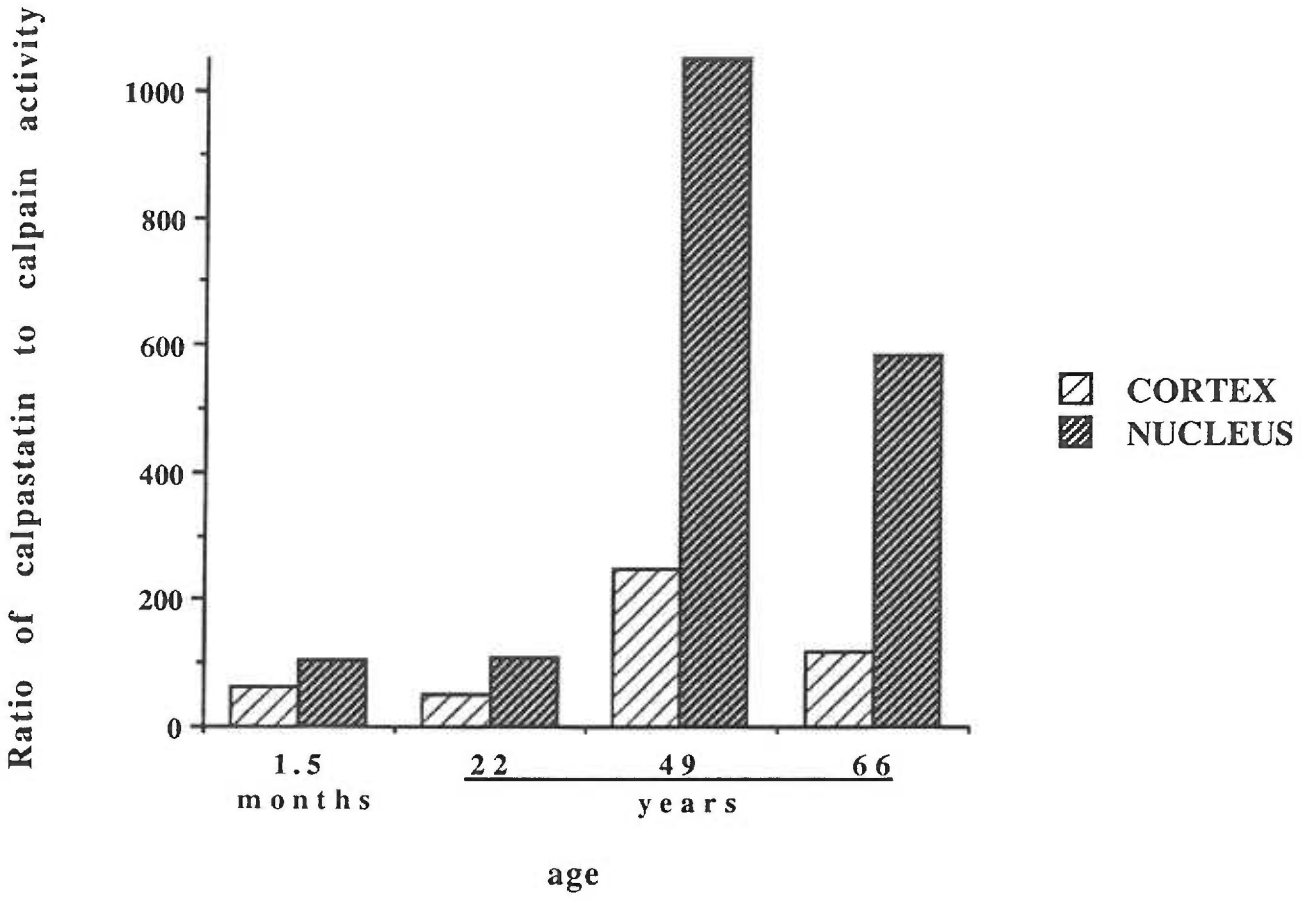

Ratio of calpastatin to calpain activity
1000
800
600
400
200
0
1.5
months
22
49
66
years
age
CORTEX
NUCLEUS

Figure 28:

Calpastatin activity in water soluble proteins and insoluble proteins extracted with 7M urea and then with 1% Trition X-100 from 120 rat (cross-hatched) or 20 bovine (stippled) lenses.

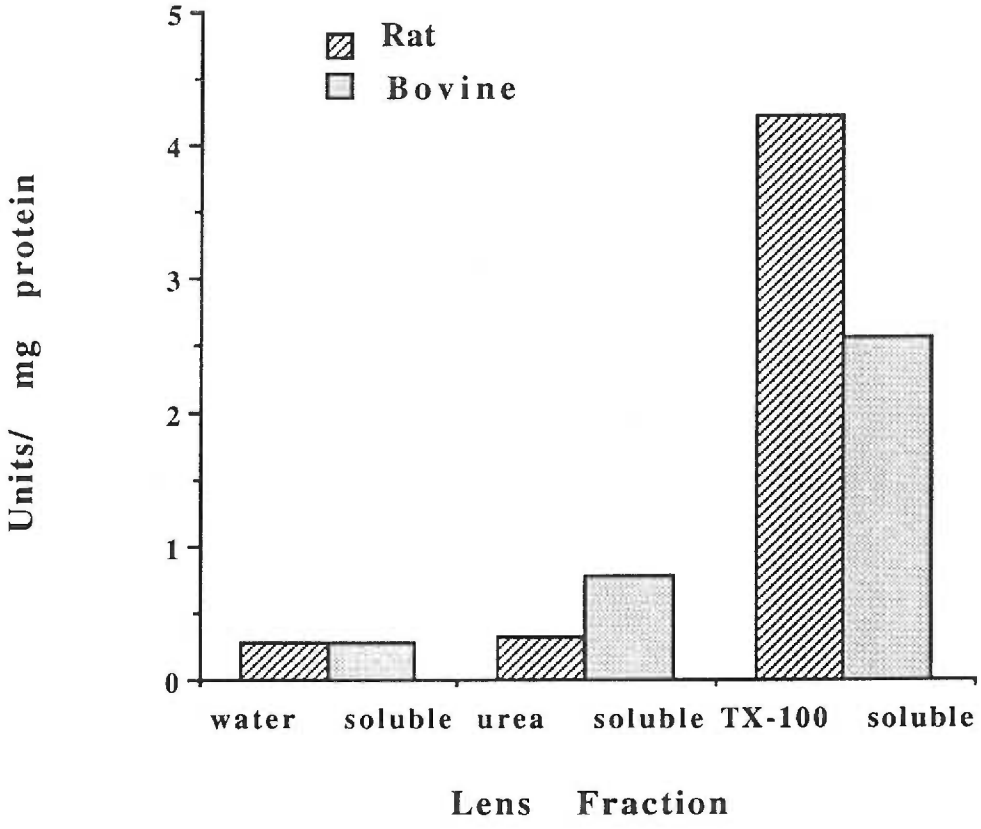

Rat
Bovine
5
4
3
2
1
0
Units/ mg protein
water soluble
urea soluble
TX-100 soluble
Lens Fraction

Figure 29:

Immunblots for calpastatin in porcine lenses.

A) Calpastatin in Lens Cortex and Nucleus

Lenses were homogenized in 20 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA, and 1mM DTE, plus 0.25 mM PMSF and 0.4 mg/ml pepstatin A, separated on 12% SDS-PAGE, transferred to immobilon-P membrane (Millipore), and reacted with 1:30 dilution of affinity-purified polyclonal calpastatin antibody.

Lane 1 = pellet from nucleus; lane 2 = pellet from cortex; lane 3 = heat soluble proteins from nucleus; lane 4 = heat soluble proteins from cortex. All lanes contain 10 μg of protein.

Molecular weight markers are shown on the right.

B) Effect of Calcium on Calpastatin in Lens Cortex

Porcine lenses were homogenized in 20 mM Tris (pH 7.5), 100 μM EGTA, 1 mM DTE plus 0.25 mM PMSF and heated. The soluble proteins were separated from the pellet and the pellet extracted sequentially with EGTA, 4 M urea, and 1% Triton X-100.

Lane 1 contains 50 ng purified calpastatin standard from porcine heart. Lanes 2 and 3 are Triton X-100 soluble proteins. Lanes 4 and 5 are urea soluble proteins. Lanes 6 and 7 are chelator extracted proteins. Lanes 8 and 9 are water soluble proteins. Lanes 2, 4, 6, and 8 are proteins from lenses homogenized in 150 μM $CaCl_2$. Each lane contains 10 μg of protein.

Arrow denotes immunoreactive calpastatin standard.

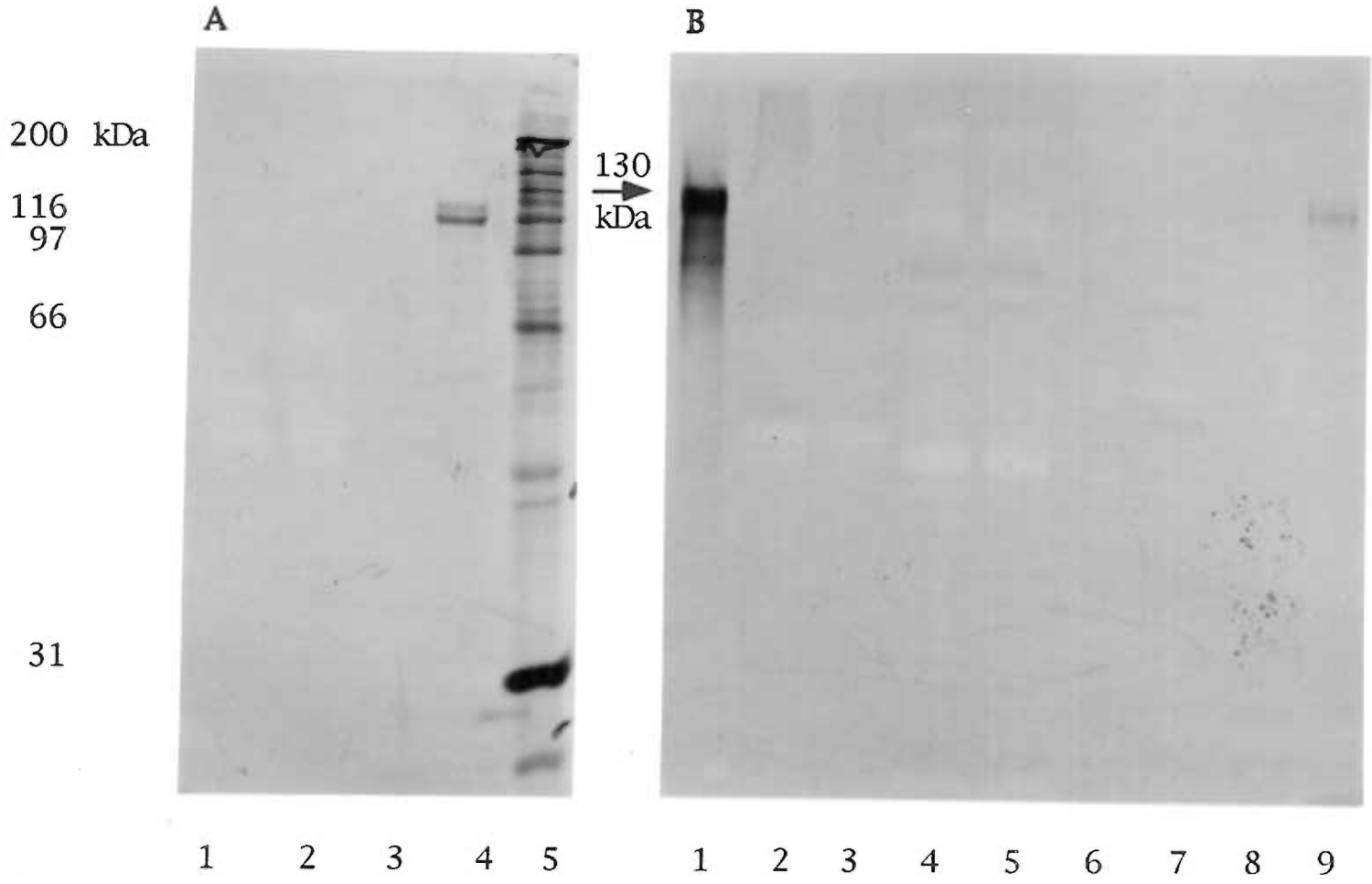

A
B
200 kDa
116
97
66
31
130
kDa
1 2 3 4 5
1 2 3 4 5 6 7 8 9

Figure 30:

Calpastatin in subcellular fractions of cortical proteins from porcine lenses. Lenses were homogenized in the same buffer as in Figure 29B plus 400 mM NaCl and the protease inhibitors, leupeptin (200 mM), and pepstatin A (0.4 mg/ml), with or without 1 mM $CaCl_2$. the insoluble pellet was extracted in 1 mM EGTA and EDTA, followed by 4 M urea and then 1% Triton X-100.

Lanes 2 and 7: chelator extracted proteins; lanes 3 an 8: urea soluble proteins; lanes 4 and 9: TX-100 soluble proteins; lanes 5 and 10: the remaining pellet. Each lane contains 14 μg of protein. Lane 11 contains purified calpastatin from porcine heart. Lanes 6-10 are plus 1 mM $CaCl_2$.

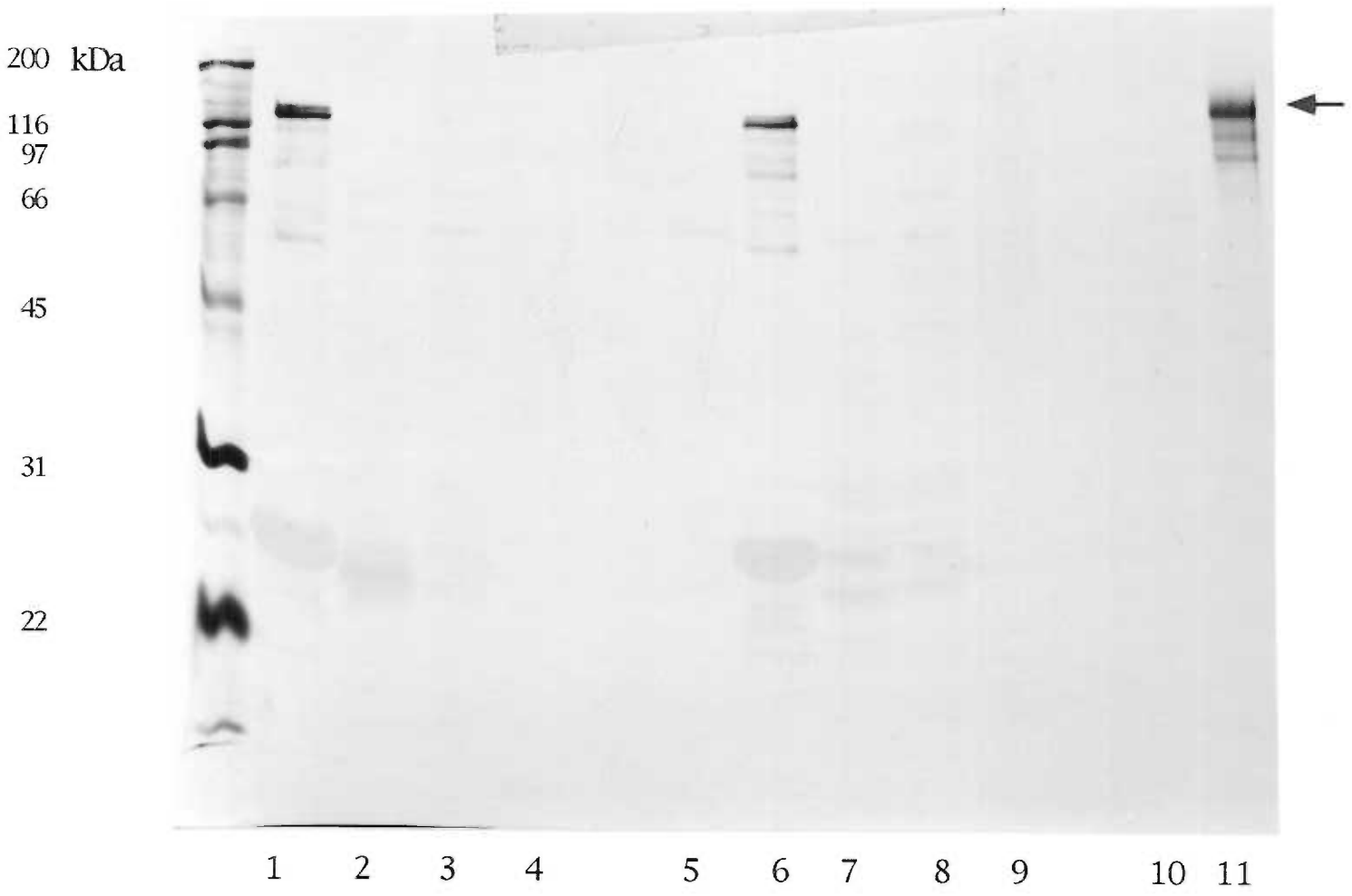

200 kDa
116
97
66
45
31
22
1 2 3 4 5 6 7 8 9 10 11

Figure 31:

Immunoblot of calpastatin in 12 day old rat lens. Lenses were homogenized in buffer containing 20 mM Tris (pH 7.5), 1 mM EGTA, and 1 mM EDTA plus 0.75 mM PMSF, centrifuged, and the water soluble proteins heated.

Lane 1: molecular weight standards in kDa.

Lane 2: 5 μg of heat soluble proteins were run on SDS-PAGE, electroblotted to PVDF and reacted with 1:250 dilution of crude calpastatin antibody.

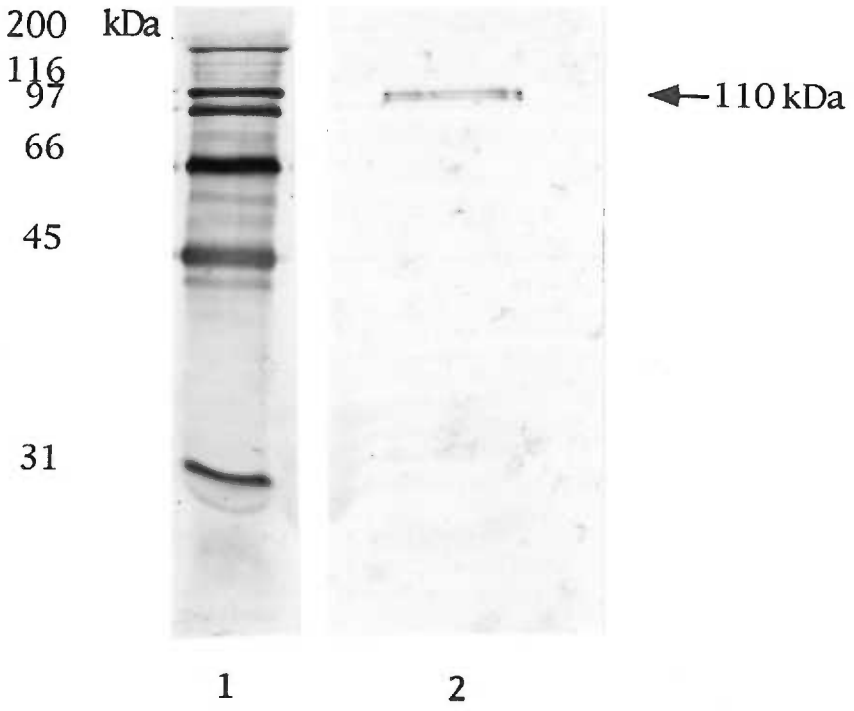

200
kDa
116
97
66
45
31
110 kDa
1
2

Figure 32:

Immunoblot of calpastatin in pellet of 14 day old rat lens reacted with 1:30 dilution of polyclonal calpastatin antibody (lane 2) or 1:500 dilution of a monoclonal antibody to calpastatin purchased from Pierce (lane 1). Purified calpastatin standard from porcine heart is shown for camparison (lane 3).

Arrow indicates the immunoreactive 80 kDa calpastatin, which did not photograph well, but which was repeatedly demonstrated on immunoblots.

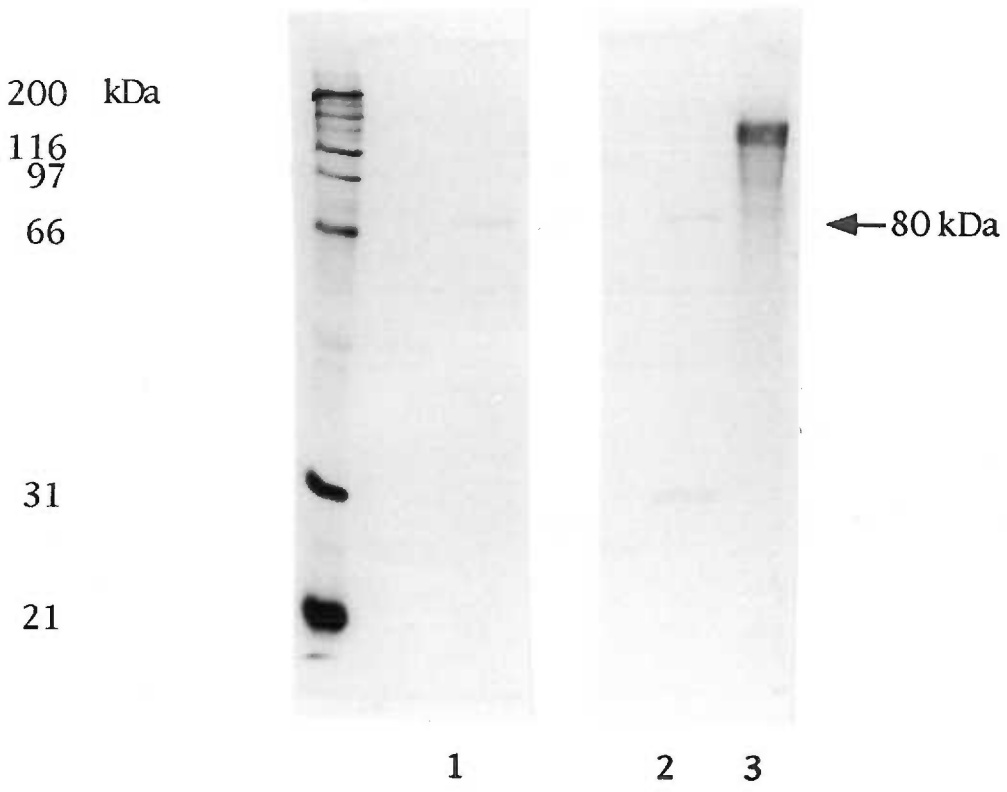
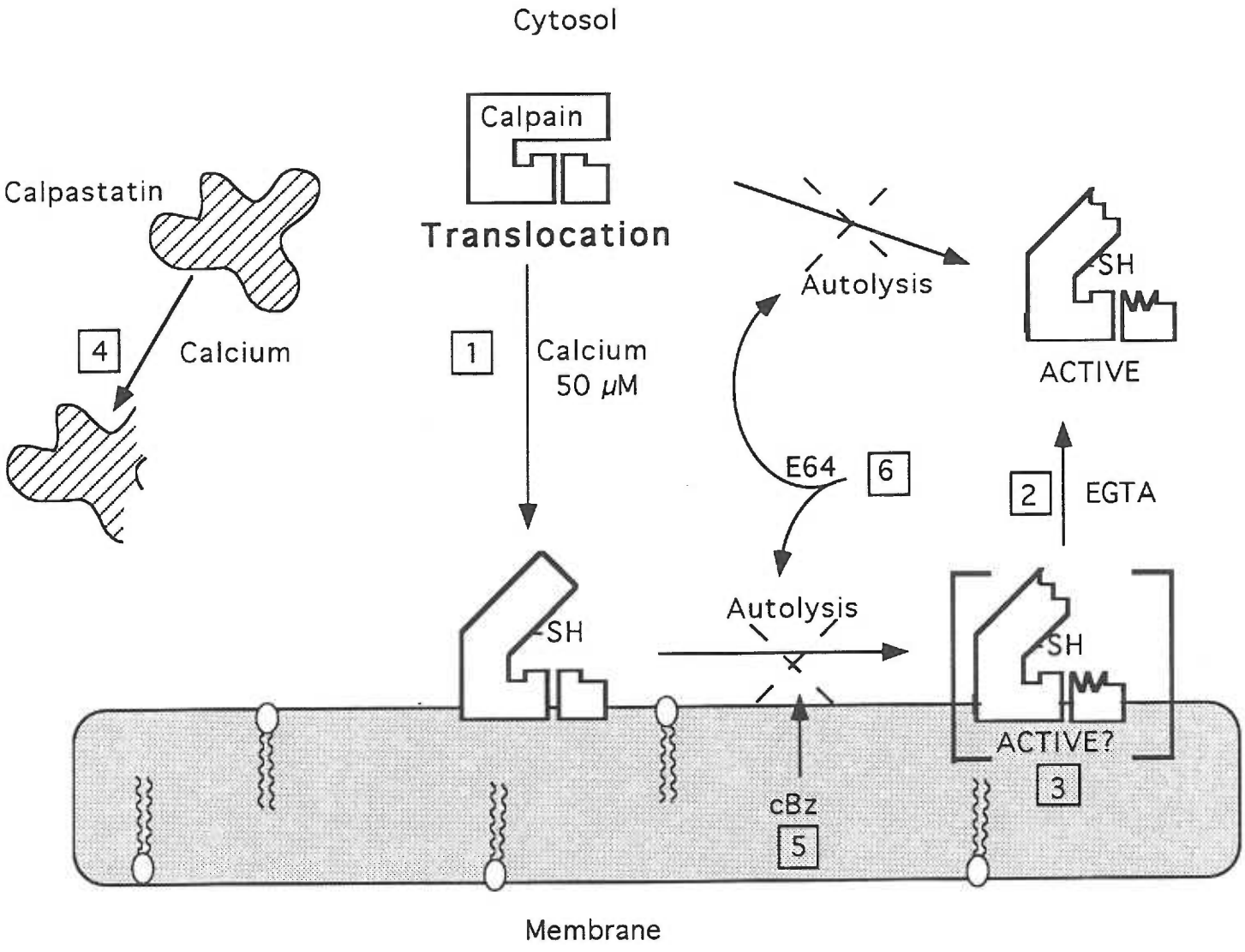

200 kDa
116
97
66
31
21
80 kDa
1
2
3

Figure 33:

Summary Model

Calcium even at low concentrations caused calpain to translocate from the cytosol to a membrane-rich fraction in the lens (1). This calpain was resolubilized from the pellet with chelator (2) and had a higher ratio of calpain activity to μg calpain than cytosolic calpain implying that calpain may have been autolyzed at the membrane (3). Further tests are needed to confirm autolysis of translocated calpain. Calcium did not cause translocation of calpastatin activity (4) suggesting that calpain may partially escape calpastatin by binding the membrane.

During calcium-ionophore cataract formation, cBz-ValPheH was able to prevent the loss of insoluble calpain and allowed for its accumulation (5). CBz-ValPheH is a neutral molecule and would not be expected to penetrate into the cytosol. The fact that it is an effective anti-cataract agent suggests that activation of calpain at the membrane is an important event in cataract formation. Indeed proteolysis of both soluble and insoluble crystallins occurs in cataracts. Since E64 is a charge molecule it may be acting to inhibit autolysis of both the membrane-associated calpain and the cytosolic calpain which would then precipitate in the presence of calcium (6).


LENS
Cytosol
Calpain
Translocation
Calpastatin
4
Calcium
1
Calcium
50 μM
Autolysis
SH
ACTIVE
E64
6
2
EGTA
Autolysis
SH
SH
ACTIVE?
3
cBz
5
Membrane