PURIFICATION AND CHARACTERIZATION OF MAJOR GELATIN-CLEAVAGE ACTIVITIES IN THE APICALLY LOCATED EXTRACELLULAR MATRIX OF THE SEA URCHIN EMBRYO

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PURIFICATION AND CHARACTERIZATION OF MAJOR GELATIN-CLEAVAGE ACTIVITIES IN THE APICALLY LOCATED EXTRACELLULAR MATRIX OF THE SEA URCHIN EMBRYO

by

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ABSTRACT

The hyaline layer is an apically located extracellular matrix that surrounds the sea urchin embryo from the time of fertilization until metamorphosis occurs. Gelatin-cleavage activities were absent from freshly prepared hyaline layers but a dynamic pattern of activities appeared when layers were incubated at 15°C or 37°C in UV-irradiated and millipore-filtered sea water. Three major gelatin-cleavage activities at 55-, 41-, and 32 kDa were induced. The 55 kDa species was identified as the major activity at 37°C, while the 41- and 32 kDa activities were the minor species. In contrast, both the 41- and 32 kDa activities were the major species at 15°C, the ambient temperature for *Strongylocentrotus purpuratus*. The 55 kDa and other higher molecular weight species appeared only after 92 hours of incubation at 15°C. Interestingly, the gelatin-cleavage activities were absent when the hyaline layers were incubated at 37°C in millipore-filtered sea water containing 10 mM benzamidine hydrochloride, a reversible serine protease inhibitor. The minimum ionic requirements for the 15°C induction of the three activities were determined: both the 55- and 41 kDa species required 300 mM NaCl, while the 32 kDa activity required both 3 mM $CaCl_2$ and 200 mM NaCl. However, none of these activities required $MgCl_2$ for their induction.

The 55-, 41- and 32 kDa gelatinase activities could be dissociated from the hyaline layers when incubated in 50 mM Tris-HCl, pH 8.0 containing 5 mM EDTA for 24 hours at 37°C. All three species were purified using gel filtration

chromatography. In both qualitative and quantitative assays, the 55 kDa gelatinase activity was inhibited by 1,10 phenanthroline (a Zn^{2+} - specific chelator), ethylenediamine tetraacetic acid or ethylenebis (oxyethylenenitrilo) acetic acid. In the presence of 2 mM 1, 10-phenanthroline, the 55 kDa species was inhibited 93.1%. Variations in the percent inhibition of the 55 kDa activity, in the range of 33-100% were observed in the presence of 5 mM ethylenebis (oxyethylenenitrilo) acetic acid. The quantitative gelatinase assay demonstrated that 10 mM $CaCl_2$ stimulated 58.1% of the EGTA-inhibited (33%) 55 kDa species, which resulted in complete recovery of the activity. On the other hand, only 28.2% activation of the EGTA-inhibited (33%) 55 kDa was noticed in the presence of 50 mM, which recovered upto 85% of the total activity. The calcium reactivation of EGTA-inhibited (100%) 55 kDa occurred with an apparent dissociation constant of 1.2 mM. Since the hyaline layer is in direct contact with sea water, which contains 10 mM $CaCl_2$, 50 mM $MgCl_2$ and 500 mM NaCl, the effects of $MgCl_2$ and NaCl were also studied. Our results show that 85% of the total activity of the 33% EGTA-inhibited 55 kDa was reconstituted in the presence of 50 mM $MgCl_2$. The 55 kDa gelatin-cleavage activity was inhibited 57.6% in the presence of 500 mM NaCl. The NaCl-dependent inhibition suggests a possible mechanism for regulating the 55 kDa gelatinase activity.

Developmental substrate gel analysis was performed using embryos of various developmental stages. The 55 kDa species comigrated with a gelatin-cleavage activity present in 45 hours-old embryos and persisted until 93 hours

after fertilization. This result confirms the presence of the 55 kDa species in the sea urchin embryo. We conclude that the sea urchin ECM, the hyaline layer has several gelatin-cleavage activities including a 55 kDa species, which is a Zn^{2+}- and Ca^{2+} dependent, gelatin-specific endopeptidases that belongs to the matrix metalloproteinase class of extracellular matrix remodeling enzymes. Our results suggest that the 55 kDa is secreted as an inactive precursor, which is activated by one or more serine proteinase activity following incubation in MFSW at $15^{\circ}C$ or $37^{\circ}C$. Our data indicate that the 55 kDa species might be the precursor of the 41 kDa gelatinase activity.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

Chapter 2. Materials and Methods

Chapter 3. Results

Chapter 4. Discussion

LIST OF FIGURES

xiii

LIST OF TABLES

LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloproteinase domain
AEBSF	[4-(2-aminoethyl)benzenesulfonylfluoride]
bFGF	Basic fibroblast growth factor
BL	Basal lamina
BMP-1	Bone morphogenetic protein-1
BP10	Blastula protease 10
BPB	Bromophenol blue
CAMs	Cell adhesion molecules
CBB	Coomassie brilliant Blue R-250
cDNA	Complementary deoxyriboneucleic acid
CS	Chondroitin sulfate
DPP	Decapentapleigic
DS	Dermatan sulfate
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylenebis(oxyethylenenitrilo)tetraacetic acid
EMMPRIN	Extracellular matrix metalloproteinase inducer
FACIT	Fibril-associated collagens with interrupted helices
FGFR	Fibroblast growth factor receptor
FN-III	Fibronectin type-III
GAG	Glycosoaminoglycan
GFR	Growth factor receptor
HA	Hyaluronic acid
HE-EGF	Heparin-binding endothelial growth factor
HGF	Hepatocyte growth factor
HL	Hyaline layer
HPF	Hours post fertilization
HS	Heparan sulfate
ICAM	Intracellular cell adhesion molecule
Ig-CAMs	Immunoglobulin superfamily cell adhesion molecules
IL-1	Interleukin-1
KGF	Keratinocyte growth factor
KS	Keratan sulfate
MFSW	Millipore-filtered sea water
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MT-MMP	Membrane type-matrix metalloproteinase
N-CAM	Neural cell adhesion molecules

NGF	Nerve growth factor
PA	Plasminogen activator
PAI	Plasminogen activator inhibitor0
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
pgp-1	Phagocyte glycoprotein-1
PMC	Primary mesenchyme cell
RFU	Relative fluorescence unit
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SERPINS	Serine proteinase inhibitors
Sp	*Strongylocentrotus purpuratus*
SVMP	Snake venom metalloproteinase
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor β
TGF-βR	Transforming growth factor-β receptor
TIMP	Tissue inhibitors of metalloproteinases
tld	Tolloid
TPA	Tissue-type plasminogen activator
UPA	Urokinase-type plasminogen activator
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VEB	Very early blastula
VEGF	Vascular endothelial growth factor
VEGF-R2	Vascular endothelial growth factor-Receptor 2
PECAM-1	Platelet endothelial-cell adhesion molecule

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 The Extracellular Matrix

The extracellular spaces surrounding tissues and organs are filled by complex and intricate networks of macromolecules that are assembled with precision. This network of molecules called the extracellular matrix (ECM) consists of numerous proteins and polysaccharides. The ECM was once thought to be a passive structure that merely provided structural support for the cells and tissues. However, studies in the past few years have established the ECM as a dynamic entity that regulates cell behavior such as adhesion, migration, growth, differentiation, invasion, and apoptosis (Streuli, 1999; Werb, 1997). Cell behavior is controlled by both direct cell-ECM interactions through receptor-mediated signaling and the indirect interaction of ECM components with growth factors (Howe *et al.*, 1998; Adams and Watt, 1993). Cells deposit the ECM molecules at very early stages of development. Hence, the composition of the ECM changes continuously during development. The ECM-degrading endopeptidases, the matrix metalloproteinases and serine proteinases, are the two major classes of proteins involved in tissue remodeling (Stamenkovic, 2003).

I will review the ECM components and cell surface molecules, the biological functions of the ECM as well as the proteinases that are responsible for ECM remodeling. I will also discuss the sea urchin as a model system for studying developmental processes, concluding with a discussion of the sea urchin ECM and thesis focus.

1.1.1 Collagens

Collagens are the most abundant structural molecules of the ECM. They are the essential structural components of all connective tissues, such as cartilage, bone, tendons, ligaments and skin. This large protein family has at least 19 distinct members, which are encoded by at least 34 genes (Ayad *et al.*, 1998). Collagens are predominantly synthesized by fibroblasts, but epithelial cells also synthesize these proteins. Collagenous molecules contain 3 alpha polypeptide chains that wrap into a characteristic triple helical structure with a coiled-coil conformation. Each polypeptide forms a left-handed helix interwined to form the right-handed superhelix (Van der Rest and Garrone, 1991). The aminoacid sequence is enriched in a tripeptide repeat Gly-X-Y, where X and Y are proline and hydroxproline, respectively. These amino acids are responsible for stabilizing the triple helical conformation. Collagens are synthesized as precursor proteins called procollagens. Procollagens contain extensions not only at the amino terminal but also at the carboxy terminal end (Hoffmann *et al.*, 1976). Two procollagen processing metalloproteinases, procollagen N-proteinase and procollagen C-proteinase have been identified (Prockop *et al.*, 1998). Type I and type II procollagens in native conformation are the preferred substrates of N-proteinase. However, the C-proteinase specifically digests both native and denatured forms of type I, II and III procollagens. Proline residues are hydroxylated post-translationally by prolyl 4-hydroxylase and prolyl 3-hydroxylase. Lysine residues are also hydroxylated by lysyl hydroxylase, and can

3

undergo glycosylation (Hay, 1991). Extracellularly, lysyl oxidase, converts lysine and hydroxylysine, in the N- and C- terminal regions, to their corresponding aldehyde forms, which then crosslinks collagen chains intermolecularly and intramolecularly (Hay, 1991).

Collagens have been classified into five groups based on the supramolecular forms they assume within the extracellular matrices namely fibrillar, fibril associated, network-forming filamentous, short chain and long chain collagens (Van der Rest and Garrone, 1991; Olsen, 1995). The collagen molecules involved in fibril formation are referred to as the fibrillar collagens. The fibril-forming collagens include types I, II, III, V, and IX. The collagen fibrils in most connective tissues are heterotypic and contain more than one type of collagen. Fibrillar collagens are prominent components of ECMs, in fact types I, II and III constitute 80-90% of collagen found within ECMs (Ayad *et al.*, 1998). A group of small collagens with interrupted helical domains are called FACIT collagens (fibril-associated collagens with interrupted helices). Collagen types IX, XII, XIV, XVI, and XIX are FACIT collagens (Van der Rest and Garrone, 1991). These collagens have globular domains that extend away from the fibril surface and may facilitate interactions with other matrix components. Type IV collagen is a highly specialized network forming filamentous collagen found only in basement membranes (Kefalides, 1973). Three type IV collagen chains form a triple helix with large globular domains at the C-termini and non-collagenous domain at the N-termini. The triple helical regions from several molecules then

associate laterally in a manner similar to fibril formation to form a network. Type VIII and X are known as short-chain collagens since their single triple helical domain is only half the size of the fibrilar-forming collagens (Van der Rest and Garrone, 1991). Type VII, a long-chain collagen with a longer central helical domain than the fibrillar forming collagens (Olsen, 1995).

1.1.2 Elastin

Elastin is a highly insoluble and a chemically inert protein. This protein is the major component of ECM elastic fibers, and is responsible for its characteristic property of elastic recoil (Partridge, 1962). The elastic fibers are composed of two major proteins: elastin and fibrillin. Elastin has a mass of 70 kDa and possesses an unusual chemical composition rich in lysine, proline and hydrophobic amino acids (Hay, 1991; Ayad *et al.*, 1998). One-third of the amino acids in elastin are lysine, which is distributed randomly throughout the molecule. Mature elastin is formed from tropoelastin, its soluble precursor. The 72 kDa biosynthetic precursor, tropoelastin, is secreted into the extracellular space where it becomes highly cross-linked by covalent bonds into a rubber-like network through the activity of the copper-requiring enzyme lysyl oxidase (Rosenbloom *et al.*, 1993). These covalent bonds link four elastin molecules into either desmosine or isodesmosine crosslinks that are unique to elastin (Raju and Anwar, 1987; Gunja-Smith *et al.*, 1989). The desmosine linkages are formed by up to four oxidized lysine side groups. The purpose of the inelastic collagen fibrils

5

interwoven with the elastic fibers is to limit the extent of stretching and prevents the elastic fibers from tearing.

Considerable sequence heterogeneity exists between the various known tropoelastins and some important genetic variations occur from one species to the other (Boyd *et al.*, 1991). The human elastin gene possesses an additional exon, 26A, that encodes a peculiar tropoelastin. This uncommon precursor is very hydrophilic, which is unusual for a tropoelastin and it contains a histidine residue, which is absent from the other known tropoelastin sequences (Debelle *et al.*, 1998).

1.1.3 Proteoglycans

Proteoglycans function in tissue organization, cell growth, and cell maturation. The ECM proteoglycans are divided into three groups: the basement membrane proteoglycans that includes agrin and perlecan, the hyalectans such as aggrecan and neurocan, and leucine-rich proteoglycans that includes decorin (Zagris, 2001). Proteoglycan molecules have a core protein and one or more covalently attached glycosaminoglycan (GAG) chains. GAGs are located primarily on the surface of cells or in the extracellular matrix. The glycosaminoglycan chains contain different repeating disaccharide structures, which makes this class of ECM components quite diverse. The versatility of proteoglycans and their capacities for multiple interactions with other extracellular matrix molecules give them the ability to perform multifunctional roles (Ruoslahti,

1989). There are four classes of GAG side chains namely hyaluronic acid (HA), chondroitin sulfate (CS) / dermatan sulfate (DS), keratan sulfate (KS) and heparan sulfate (HS) / heparin (Lodish *et al.*, 1999). Hyaluronic acid (HA) is unique among the GAGs because it neither contains any sulfate nor is found covalently attached to proteins. It forms non-covalently linked complexes with proteoglycans in the ECM. Chondroitin sulfate is the most abundant GAG in the ECM. The GAG side chains bind to various extracellular matrix molecules, cell-cell adhesion molecules and growth factors (Ruoslahti, 1989). This binding is determined by the degree of sulfation of the glycosaminoglycan. One of the most important extracellular proteoglycans is aggregan. As its name implies, aggregan forms very large aggregates that give cartilage its resistance to deformation. Decorin is a 90-140 kDa proteoglycan present in the extracellular matrix, so called because it decorates collagen fibers. (Giudetti *et al.*, 2002). Proteoglycans resist compression, possess binding sites for certain signaling molecules, and some proteoglycans remain attached to the cell membrane and interact with cytoskeleton components.

1.1.4 Glycoproteins

A variety of glycoproteins are present in the extracellular matrix having the ability to interact with the cell surface as well as ECM components. The glycoproteins such as laminin, fibronectin, tenascin, and entactin contain multiple domains and interact with other ECM molecules. Laminins are found in both

basal laminae and embryonic tissues and it was the first glycoprotein observed in the ECM of mouse embryos (Leivo *et al.*, 1980). It is a large hetero-trimeric molecule with three large polypeptide chains with binding sites for heparin, collagen type IV and a cell surface-receptor. Laminin forms large aggregates binding to each other (Martin and Timpl, 1987; Martin *et al.*, 1988).

Fibronectin is an important glycoprotein synthesized by fibroblasts that is essential for cell migration during gastrulation. Fibronectin is a large homodimer that binds collagen and entactin (Ruoslahti and Obrink, 1996). Tenascin is a large glycoprotein made of six polypeptide chains. It plays a regulatory role during tissue reorganization, and embryonic development. Tenascin has fibronectin type-III (FN-III) domains that can bind various ECM components and cell surface receptors (Jones and Jones, 2000).

1.2 Cell Surface Molecules

The molecules present on the cell surface play a crucial role in exchanging vital information between the cell and its surrounding environment (Rosales *et al.*, 1995). Cell interactions are responsible for the normal development and functioning of tissues in an organism. The cell-surface molecules consist of three classes of macromolecules namely, the cell adhesion molecules (CAMs) that mediate cell-cell interactions, the cell surface ECM receptors that includes both integrin and non-integrin receptors, and the growth factor receptors (Gumbiner, 1996; Aplin *et al.*, 1998).

1.2.1 Cell Adhesion Molecules

The members of CAM are grouped into three categories, which include cadherins, selectins and immunoglobulin-cell adhesion molecules (Ig-CAM) superfamily (Cunningham, 1995). The cadherins are a large family of transmembrane glycoproteins that are essential for cell-cell adhesion in a calcium-dependant manner (Takeichi, 1990; Gumbiner, 1996). The cadherins are characterized by the presence of the following structural domains: an extracellular domain consisting of one to five cadherin structural domains, a transmembrane, and a cytoplasmic domain. In addition, they also contain DXNDN, LDRE, and DXD conserved sequences (Kreis and Vale, 1999). Members of this subfamily include N-, P-, R-, B-, and E-cadherins. A homologue of vertebrate cadherins was identified in *Drosophila* and was required for early embryonic cell adhesion (Oda *et al.*, 1994). Studies have shown that the cadherins can exist as either homodimers or heterodimers (Takeichi, 1990; Ahrens et al., 2002). Dimerization of cadherins can take place between similar cadherin types or between different cadherin molecules. Heterophilic cis interactions were identified between N- and R-cadherins as well as E- and P-cadherins (Shan *et al.*, 2000; Klingelhofer *et al.*, 2000). Protocadherins, a less studied member of the cadherin family is believed to be essential for neural development in chicks (Capehart and Kern, 2003). The catenins are cadherin-associated polypeptides that have been implicated in cell adhesion and the

9

Wnt/wingless signaling pathway in both *Drosophila* and *Xenopus* embryos (Gumbiner, 1995).

The Ig-CAM family members possess one or more Ig-like domains with two cysteine residues separated by 55 to 75 aminoacids (Kreis and Vale, 1999). The Ig-CAMs are made up of a large N-terminal extracellular domain, a transmembrane segment and a cytoplasmic tail (Aplin *et al.*, 1998). Furthermore, they also contain fibronectin type III-like repeats. The prototype member, neural-cell adhesion molecules (N-CAM) is not only essential for cell-cell adhesion, but is also believed to be important for development. Gicerin is another member of this family, which has been implicated in chick development and in tumor growth, invasion, and metastasis (Tsukamoto *et al.*, 2003; Tsuchiya *et al.*, 2003). Studies have suggested that the *CAM* genes might be controlled by the *Hox* gene products (Cunningham, 1995). The Ig-CAM family also includes vascular cell adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule (ICAM), and platelet endothelial-cell adhesion molecule (PECAM-1) that plays an important rolecated in cell-cell adhesion (Kreis and Vale, 1999).

Selectins are a small group of cell-surface glycoproteins with a lectin-like N-terminal domain, followed by an epidermal growth factor (EGF)-type domain, a transmembrane, and a cytoplasmic domain. There are only three known members of this family E-, P-, and L-selectin names after their localization in endothelial cell, platelets and leucocytes respectively (Ley *et al.*, 2003). The

selectins are involved in interactions of leucocytes with endothelium, and in inflammatory responses (Aplin, 1998).

1.2.2 ECM and Growth Factor Receptors

As previously mentioned, cells lying adjacent to each other communicate through cell-cell interaction, while cells that are located farther apart have to exchange information with the help of the small molecules present in the pericellular environment. Hence, certain molecules in the ECM function as receptors for the rest of the ECM components (Darnell *et al.*, 1990). These cell surface ECM receptors are divided into two groups, integrin and non – integrin receptors (Adams and Watt, 1993).

Integrins are a large family of heterodimeric transmembrane glycoproteins that are made of two subunits α and β. In humans, there are at least 18 α and 8 β subunits, which associate in different combinations to form 24 distinct functional integrins (Hynes, 2002; Van der Flier and Sonnenberg, 2001). Both the α and β subunits contain an extracellular domain, a transmembrane domain and a cytoplasmic tail. The extracellular domains of the α and β subunits contain calmodulin-like divalent cation-binding sites (Loftus *et al.*, 1990). The integrins recognize a wide range of ligands including ECM components such as fibronectin, laminin, vitronectin, entactin, tenascin and several types of collagen (Hemler, 1990). The RGD (Arg-Gly-Asp) tripeptide is the recognition sequence for many integrins that bind ECM (Ruoslahti and Pierschbacher, 1987). The $\alpha2\beta$

1 integrin binds collagens I to V, while $\alpha 1 \beta 1$ binds collagens I to IV. The ligand specificity of some integrins depends on cell type in which they are present. The $\alpha 2 \beta 1$ integrin binds collagens in platelets, but in endothelial cells, it acts as a receptor for both collagens and laminins (Hynes, 1992). The $\alpha 5 \beta 1$ integrin binds fibronectin, $\alpha 3 \beta 1$ and $\alpha 6 \beta 1$ bind laminins, while $\alpha v \beta 3$ and $\alpha v \beta 5$ serve as vitronectin receptors (Hay, 1991).

The family of non-integrin receptors is comprised of cell surface glycoproteins and proteoglycans. For example, CD44 is a transmembrane glycoprotein that functions as a non-integrin receptor. The members of this group are implicated in inflammation and in leukocyte signal transduction (Kries and Vale, 1999). CD44 is also known as phagocyte glycoprotein-1 (pgp-1), which binds collagen types I and VI, fibronectin, and osteopontin (Cichy and Pure, 2003). Syndecans are transmembrane proteoglycans that bind collagen, fibronectin and bFGF (Adams and Watt, 1993). Glypicans are heparan sulfate proteoglycans that serve as laminin receptors. These glypicans are attached to the cell surface by glycosyl phosphatidylinositol (Song and Filmus, 2002).

Several cell surface receptors possess high affinity for growth factors present in the ECM. They are platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and transforming growth factor-β receptor (TGF-βR). Recent studies have indicated that both cell surface receptors and growth factor receptors interact with each other during biological processes such as cell adhesion, migration, and proliferation. (Eliceiri, 2001).

Two receptor tyrosine kinases (RTKs), PDGF-Rβ (platelet-derived growth factor receptor beta), and VEGF-R2 (vascular endothelial growth factor receptor 2) interact with the αvβ3 integrin. PDGF-Rβ associates with the extracellular domain of the β3 integrin subunit, while VEGF-R2 requires both αv and b3 subunits to associate. However, no interaction of PDGF-Rβ and VEGF-R2 was observed with β1 integrins (Borges et al., 2000).

1.3 Biological Functions of ECM

Cell interactions with extracellular matrices are vital for cellular properties such as cell adhesion, migration, growth and differentiation, which will be discussed in this section. Interactions between the cell and the ECM components are mediated through a family of cell-surface receptors called integrins. The ECM components bind integrins on the cell surface; this prompts the integrins to transmit signals inside the cell.

1.3.1 Cell Adhesion and Migration

Cells exchange vital information through cell-ECM interactions. Experiments performed by Boucaut *et al.*, (1984) showed that injecting R-G-D containing synthetic peptides, prevents amphibian gastrulation and neural crest cell migration in avian embryos. This demonstrates a crucial role for fibronectin during development. Tenascin (TN), an ECM glycoprotein has been implicated in cell migration as well as adhesion to some basement membrane proteins.

Inhibition of cranial neural crest cell migration was reported on injecting tenascin antibodies in chick embryos (Bronner-Fraser, 1988). Versican, a large ECM glycoprotein, enhanced the adhesion and proliferation of bovine disc cells *in vivo*. Studies have shown that the globular selectin-like domain of versican was responsible for enhancing the adhesion by 4 to 6 fold (Yang et al., 2003; Wight, 2002).

During morphogenesis, cells move relatively long distances. For example, the mesenchyme cells dissociate themselves from the vegetal plate and ingress during sea urchin gastrulation (Zagris, 2001). Implicated in this process is thrombospondin-1, a calcium-binding protein in the ECM, which contains both adhesive and counter-adhesive activities (Crossin, 1996). In calf pulmonary artery, thrombospondin-1 stimulates smooth muscle cell migration and proliferation (Yabkowitx et al., 1993; Majack, 1998). Similarly, laminin, another key component of the ECM has been implicated in promoting epithelial cell adhesion and Schwannoma cell migration (Trelstad, 1984).

1.3.2 Cell Growth and Differentiation

Laminin promotes epithelial cell growth, while inhibiting the growth of fibroblasts. In addition to this, laminin influences the growth of Schwann cells and B16 melanoma. However, cells cultured in fibronectin-free serum show increased growth in the presence of laminin. The above result suggests a competition in substrate binding (Trelstad, 1984). As mentioned earlier, thrombospondin-1

promotes the growth of smooth muscle cells, but in contrast, it was found to inhibit endothelial cell proliferation (Taraboletti et al., 1990). This behavior illustrates the anti-angiogenic property of thrombospondin-1.

Cell surface heparan sulfate proteoglycans have been implicated in many cellular events. Cell growth and differentiation are controlled by numerous growth factors and cytokines such as HGF (hepatocyte growth factor), HE-EGF (heparin-binding endothelial growth factor), and VEGF (vascular endothelial growth factors) (Tumova et al., 2000).

1.4 Extracellular Matrix Degrading Proteinases

As discussed earlier, the ECM is both a complex and dynamic structure, whose composition changes continuously throughout embryonic development. Cells deposit ECM remodeling endopeptidases into the surrounding matrix. These enzymes include matrix metalloproteinases, serine proteinases, ADAMs, and astacins.

1.4.1 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a major group of enzymes that regulate cell-matrix composition (Massova *et al.*, 1998). The MMPs are zinc-dependant endopeptidases. The metzincin superfamily of metalloproteinases is made of diverse group of zinc endopeptidases that includes the astacins, the reprolysins, the serralysins, and the matrix metalloproteinases. A novel type of

MMP was identified in *Volvox carteri*, which prefers copper for its enzymatic activity rather than zinc (Heitzer and Hallmann, 2002). At present, 25 vertebrate MMP's and 22 human homologues have been identified (Nagase and Woessner, 1999). In addition, several invertebrate MMP's have been identified. Most of the MMP family members are organized into three well-conserved structural domains: an amino terminal propeptide, a catalytic domain and a carboxy-terminal hemopexin-like domain. Most MMPs are secreted in latent precursor, zymogen form and are activated in the extracellular matrix (Harper *et al.*, 1971). It is the amino-terminal prodomain, which confers latency. A highly conserved sequence (...PRCGXPD...) and the cysteine within this propeptide sequence interact with the catalytic zinc to prevent its activity (Massova *et al.*, 1998). All MMPs, with the exception of MMP-23 contain this sequence (Nagase and Woessner, 1999). MMP-11 (stromelysin-3), MMP-27 (epilysin), and the MT-MMPs contain the RXK/RR furin-like enzyme recognition motif between their propeptide and catalytic domains. This allows them to be activated by intracellular subtilinsin-type serine proteinases before they reach the cell surface (Pei and Weiss, 1995).

Structural analysis has shown that the catalytic domain contains two zinc ions and at least one calcium ion (Massova *et al.*, 1998). One of these zinc ions is located at the active site and is involved in catalysis. The other plays a structural role. Calcium ions are present in the catalytic domain approximately 12 Å away from the catalytic zinc. The catalytic domain contains a highly conserved

sequence,   [VAIT]-[AG]-[ATV]-**H**-E-F-[FLIV]-G-**H**-[ALMSV]-[LIM]-G-[LM]-X-**H**-[SITV]-XXXXX-[LAFIV]-**M**, with three conserved histidines (boldface) that chelate the catalytic zinc ion (Massova *et al.*, 1998). The conserved methionine residue (boldface) is part of a beta-turn, also known as methionine-turn, which increases the hydrophobicity in this area to enhance the zinc binding ability of histidines (Bode *et al.*, 1996).

The catalytic domain of the gelatinases MMP-2 and MMP-9 contains three repeats of an additional domain, known as the fibronectic-type II-like domain, which is thought to facilitate substrate binding (Nagase and Woessner, 1999). The C-terminal domain of the MMPs has been implicated in determining the distinct substrate specificities of the various MMPs (Murphy *et al.*, 1992; Sanchez-Lopez *et al.*, 1993). This domain contains sequences similar to the heme binding protein, hemopexin, and the ECM component, Vitronectin (Nagase and Woessner, 1999). All MMPs, with the exception of MMP-7 (Matrilysin), MMP-26 (Matrilysin –2), and MMP-23 contain this domain. MMP-7 and MMP-26 merely lack these extra domains, whereas MMP-23 has unique cysteine-rich, proline-rich, and IL-I type II receptor-like domains instead of a hemopexin domain (Gururanjan *et al.*, 1998, Park *et al.*, 2000).

Membrane-type matrix metalloproteinases (MT-MMPs) are the largest subgroup of MMPs. The members of this subfamily contain an additional structural domain called the transmembrane domain. Unlike the other members of the MMP family, MT-MMPs are not activated in the extracellular matrix but are

processed into the active form intracellularly by proprotein convertase-dependant and -independent pathways (Yana *et al.*, 2000; Rozanov *et al.*, 2001)

The transmembrane domain helps the MT-MMPs to anchor themselves to the cell surface by a type I transmembrane sequence and a cytoplasmic tail (MT1, MT2, MT3, and MT5-MMP) or glycosylphosphatidylinositol linkage (MT4- and MT6-MMP) (Overall, 2001). MT-MMPs are responsible for the cellular activation of zymogens by proteolysis. For instance, MT1-MMP forms a ternary complex with Pro-MMP-2 in the presence of TIMP-2 and activates Pro-MMP-2 by the removal of the propeptide domain (Toth *et al.*, 2000).

The C-terminal domain is important for binding of inhibitor molecules, known as tissue inhibitors of matrix metalloproteinases (TIMPs), to the latent form of gelatinases (Massova *et al.*, 1998). Studies on the mechanism of activation of the latent form of MMP-2 (pro-MMP-2) have been proposed in which the catalytic domain of MT1-MMP binds to the N-terminal portion of TIMP-2, leaving the negatively charged C-terminal region of TIMP-2 which binds the hemopexin-like domain of pro-MMP-2 (Toth *et al.*, 2000). This complex effectively concentrates pro-MMP-2 at the cell surface, where it is activated by the TIMP-free active MT1-MMP molecule (Morrison *et al.*, 2001). Murphy et al. (1992) suggested that the specificity for collagen degradation of MMPs might be facilitated by the hemopexin-like domain. This domain may also be responsible for the difference in substrate specificities for different MMPs (Matrisian, 1992; Nagase and Woessner, 1999).

As mentioned in Appendix C, MMPs were initially classified into four subfamilies based on their specificity for different components of the ECM as substrate, including gelatinases, collagenases, stromelysins and elastases. The members of these subfamilies are also identified as MMP-X, where X is a number assigned to each member (Matrisian, 1992). For example, collagenase-1, gelatinase A, matrilysin, and MT1-MMP are known as MMP-1, 2, 7, and 14, respectively (Nagase and Woessner, 1999). There are only two members in the subfamily of gelatinases, MMP-2 and MMP-9, which prefer gelatin (denatured collagen) as their substrate (Matrisian, 1992). Gelatinases can also degrade collagen I, IV, V, VII, X, and elastin, fibronectin and proteoglycans. The subfamily of collagenases includes four members, namely interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8), collagenase-3 (MMP-13) and collagenase-4 (MMP-18); these cleave collagen molecules at a specific site within the triple-helical region. The other preferred substrates of collagenases are gelatin and proteoglycans (Chintala *et al.*, 1999). The stromelysin subclass of MMPs includes MMP-3, MMP-10, and MMP-11 and has wide substrate specificity for collagen, elastin, fibronectin, laminin and proteoglycans (Chintala *et al.*, 1999). Elastase has only one member (MMP-12) that can cleave elastin, but also has overlapping substrate specificity.

1.4.2 Serine Proteases

Serine proteases also utilize components of the ECM as their substrate, but to a lesser extent. Members of the serine proteases possess one serine residue at their active site. Like MMPs, serine proteases are also secreted as inactive zymogens. Activation occurs by the removal of the N-terminal propeptide sequence by proteolytic cleavage. These endopeptidases include trypsin, chymotrypsin, neutrophil elastase, cathepsin G, plasminogen activator (PA)/plasmin system, etc. The inactive plasminogen is cleaved by the tissue-type and urokinase-type plasminogen activators (tPA and uPA) to form the active plasmin. The activated plasmin reportedly plays some role in the pro-MMP activation instead of the ECM degradation itself (Hay, 1991).

Serine proteases have two distinct families: the chymotrypsin and subtilisin families. Mammalian serine proteases are grouped under the chymotrypsin family, whereas bacterial enzymes such as subtilisin belong to the subtilisin family. Plasminogen activators are regulated by plasminogen activator inhibitors (PAI-1 and PAI-2) and protease nexin-1, while plasmin can be regulated by α_2-antiplasmin, α_1-antitrypsin and protease nexin-2 (Nostrand *et al.*, 1990; Kwaan, 1992). A membrane-associated type II transmembrane serine protease, hepsin, has been isolated and cloned from preimplantation embryos (Vu *et al.*, 1997). Hepsin was predominantly distributed in the liver and kidney. This proteinase was first observed in the mouse at the two-cell stage, but was

present in large amounts only in the early blastula stage. This serine proteinase gene was overexpressed in ovarian carcinoma (Tanimoto *et al.*, 1997).

1.4.3 ADAMs:

The ADAMs (a̲ d̲isintegrin a̲nd m̲etalloproteases) are members of the metzincin superfamily of metalloproteases. ADAMs are also referred to as cellular disintegrins and MDCs (metalloprotease/disintegrin/cysteine). They are cell surface proteins that are membrane bound and contain both disintegrin and metalloprotease domains (Weskamp and Blobel, 1994; Wolfsberg *et al.*, 1995). The domain structure of ADAMs consists of a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic tail. The domain organization of ADAMs is very similar to that of snake venom metalloproteases (SVMPs). There are at least 15 full-length members in the ADAMs family (Wolfsberg and White, 1996). Studies show that the cysteine-rich domain of ADAM13 cooperates intramolecularly with the ADAM13 metalloprotease domain to regulate its function *in vivo*. These results indicate that a downstream extracellular adhesive domain plays a significant role in regulating ADAM protease (White, 2003; Tomczuk *et al.*, 2003). ADAM2 or ADAM3 null mice are viable and healthy with normal development, but male mice are sterile (Cho *et al.*, 1998; Nishimura *et al.*, 2001).

1.4.4 Astacins

The astacin family of metalloproteases was named after the first digestive enzyme astacin, isolated from the freshwater crayfish *Astacus astacus* (Stocker *et al.*, 1993; Bond and Beynon, 1995). Astacins are characterized by the presence of a signal domain, a putative propeptide, a catalytic domain, and an EGF-like domain (Stocker and Bode, 1995). The astacin family also possesses the unique consensus sequence HEXXHXXGFXHEXXRXDRD containing the zinc binding motif in the active center, and another conserved region, SxMHY, called Met-turn. Since, astacins, serralysins, snake venom and matrix metalloproteases possess similar zinc-binding environments they are grouped under a family called Metzincins (Bode *et al.*, 1993; Stocker *et al.*, 1995). Astacin, the smallest member of this family is synthesized as a zymogen (Geier *et al.*, 1997). The x-ray crystal structure of astacin has been recently solved to a resolution of 0.18 nm (Bode *et al.*, 1992). Studies have showed that in crayfish, astacin is secreted in its pro-form and catalyzes its own activation on its way to the stomach (Mohrlen *et al.*, 2001; Yiallouros *et al.*, 2002).

The members of this family are found in many species ranging from sea urchin to humans. They include meprins A and B, membrane-bound metalloproteinases found in the mouse kidney brush borders. The activation mechanism of meprin and other astacins was determined to be different from that of the trypsin family of enzymes (Johnson and Bond, 1997). Studies have shown that the constituents of the basement membrane, laminin 1 and 5 are degraded

22

effectively by recombinant meprin alpha hydrolyzes (Kohler *et al.*, 2000). This family comprises a morphogenetically active protein called human bone morphogenetic protein 1 (BMP-1), which plays a significant role in the embryonic development of amphibians, echinoderms, and insects (Wozney *et al.*, 1988). Two very early blastula (VEB) stage genes are expressed in abundance after 12 to 15 hours of fetilization in the embryo of *Strongylocentrotus purpuratus*. One of those genes encodes SpAN, a metalloproteinase that is similar to the BMP-1 and tolloid, which are required for the dorsal-ventral patterning of the *Drosophila* embryo (Reynolds *et al.*, 1992; Lepage *et al.*, 1992). Astacin-like enzymes were also found to play important roles in egg envelope disruption in *Xenopus laevis* and in degrading of eggshell in Quail (Katagiri *et al.*, 1997; Elaroussi and DeLuca, 1994).

1.5 Physiological Roles of the ECM Remodeling Enzymes

As discussed earlier, the functional role of the ECM is regulated by its own composition. The ECM composition changes continuously due to selective degradation of the ECM components and cell surface proteins by specific matrix degrading enzymes. Direct and indirect interactions of ECM components with growth factors, and cell surface receptors control the remodeling of ECM. MMPs play a significant role in the biological processes, such as cell adhesion, cell migration, cell proliferation, and cell differentiation during embryonic development (Adams and Watt, 1993; Sastry and Horwitz, 1996). The roles of MMPs have

been implicated in wound healing, angiogenesis, bone remodeling, inflammatory and immune responses (Seiki and Yana, 2003; Huachuan, 2003). Wental *et al.* (2003) have showed that MMP-9 could play a role in wound repair, cell migration and neutrophilic inflammation.

The fuctional roles of ADAMs 1 to 6 have been implicated in fertilization and/or spermatogenesis (Barker *et al.*, 1994; Perry *et al.*, 1994). Both ADAM-1 and -2 fully express a disintegrin domain and participate in sperm-egg binding and fusion (Blobel *et al.*, 1992). ADAM-12 has a role in myoblast fusion, hence overexpression of ADAM-12 without pro and metalloprotease domains leads to increased myoblast fusion (Yagami-Hiromasa *et al.*, 1995).

The astacins are found in bacteria, invertebrates and vertebrates serve a variety of physiological functions, such as digestion, hatching, peptide processing, morphogenesis, and pattern formation. The *Drosophila* gene *NAS-39* has a striking resemblance to human BMP-1 and the tolloids. The tolloid gene (*tld*) is responsible for dorsal-ventral patterning of the zygote. Some *tld* alleles possess antagonistic behavior toward decapentaplegic (*dpp*), a second zygotic dorsal-ventral patterning gene whose product is most closely related to the TGF-beta superfamily members (Childs and O'Connors, 1994). Members of the tolloid/bone morphogenetic protein (BMP)-1 family play significant functional roles in the differentiation of cell fates. BMP-1 plays a role in cartilage and bone formation in mammals. Hishida *et al.* (1996) have identified a gene, *hch-1*, which

encodes a protein that is related to tolloid and the BMP family. This gene is required for normal neuroblast migration and normal hatching in *C. elegans*.

In addition to degradation of extracellular matrix components, serine proteases serve important biological functions that include digestion of food, activation of blood coagulation, the anticoagulant pathway, activation of growth and angiogenic factors, and fibrinolysis (Tryggvason *et al.*, 1987; Duffy, 1992). Most of the clotting factors are secreted as inactive precursors. Blood coagulation requires generation of small quantities of thrombin, a serine protease. Coagulation is regulated by the anticoagulant pathway, which includes protein C and serine protease inhibitors in plasma. The fibrinolytic mechanism that comprises the activation of plasminogen into plasmin is caused by plasminogen activators, a member of the serine proteases group. This prevents fibrin over-accumulation and promotes wound healing (Walsh and Ahmad, 2002).

1.6 Regulation of ECM Degrading Enzymes

Since uncontrolled remodeling of ECM by MMP leads to pathological conditions such as: cancer, metastasis, arthritis, nephritis and cardiovascular diseases, MMPs should be very tightly regulated. MMPs are stringently regulated at the gene and protein levels (Sternlicht and Werb, 2001). Most MMPs exhibit transcriptional regulation except for MMP-2, which is expressed and regulated through an enzyme activation mechanism (Strongin *et al.*, 1995). MMP expression is controlled by numerous cytokines and growth factors such as,

interleukins, interferons, EGF, KGF, NGF, VEGF, PDGF, TNF-α, TGF-β, and the extracellular matrix metalloproteinase inducer, EMMPRIN (Fini *et al.*, 1998). For example, TGF-β controls the transcription of MMP1 and MMP3 genes, and upregulates the MMP13 expression (Uria *et al.*, 1998). The expression of MMPs can also be regulated by phorbol ester, ECM proteins, cell stress, and changes in cell shape (Kheradmand *et al.*, 1998). The MMPs are controlled at the post-transcriptional level. For example, the active form of MMP-16 (MT3-MMP) is produced by alternative mRNA splicing, not membrane shedding (Matsumoto *et al.*, 1997).

MMPs are also modulated by their naturally occurring inhibitors called tissue inhibitors of metalloproteinases (TIMPs). As outlined in Appendix D, four TIMPs have been identified and studies show that these inhibitors have broad substrate specificity (Matrisian, 1992). TIMPs are expressed by a variety of cell types and are present in most tissues and body fluids. TIMP inhibits the active MMPs largely by binding to the catalytic (N-terminal) domains of the enzymes. Inhibition of active MMPs by TIMP-1 and TIMP-2 is greatly reduced by removal of the C-terminal domain of the inhibitor (Murphy *et al.*, 1994). TIMPs form high-affinity, noncovalent, and irreversible complexes with active MMPs with a 1:1 stoichiometric ratio. The role of TIMPs in the inhibition of cell invasion and metastasis is widely recognized. TIMP-1 and TIMP-2 possess anti-angiogenic activity through which they inhibit both endothelial cell proliferation and cell migration. MT1-MMP activates the inactive precursor, pro-MMP-2 in the cell

surface into active MMP-2 in the presence of TIMP-2. A complex of active MT1-MMP and TIMP-2 binds pro-MMP-2 forming a ternary complex, which permits pro-MMP-2 activation by a TIMP-2-free neighbouring MT1-MMP. Active MT1-MMP binds the amino-terminal domain of TIMP-2 whereas the carboxyl-terminal domain of the TIMP-2 molecule interacts with the hemopexin C domain of pro-MMP-2. Activation of the tethered pro-MMP-2 is executed by a second active MT1-MMP (Atkinson *et al.*, 1995; Sato *et al.*, 1996; Bernardo and Fridman, 2003).

The regulation of ADAMs is less well understood, although there is some evidence that the ADAMs are modulated at both the gene and protein levels. The expression of ADAM12 was up-regulated by transforming growth factor beta (TGF-beta) in human activated hepatic stellate cells, but no upregulation of ADAM9 was identified (Le Pabic *et al.*, 2003).

Serine proteinases are regulated by serine proteinase inhibitors (serpins). The serpins are a superfamily of proteins that inhibit serine proteases by an irreversible, suicide substrate mechanism (Potempa *et al.*, 1994). Human serpins are involved in a variety of biological functions, such as inflammation and complement activation, blood pressure regulation, hormone transport, angiogenesis, apoptosis, fibrinolysis, coagulation and sperm development (Silverman *et al.*, 2001).

1.7 The Sea Urchin

The sea urchin is one of the most widely used models to study developmental processes. We utilize the sea urchin species *Strongylocentrotus purpuratus (Sp),* commonly known as the purple sea urchin to investigate the role of ECM components during development. This species is green as a juvenile and purple as an adult. Purple sea urchins are found on the pacific coastline from Alaska to Cedros Island, Mexico. (Olhausen and Russo, 1981). *S. purpuratus* feeds primarily on algae. Primary predators of this species include sea stars and sea otters. The purple sea urchin reaches sexual maturity at the age of two years.

1.7.1 The Sea Urchin as a Model System

The sea urchin is an ideal model to study developmental processes for the following reasons: a large number of gametes can be obtained easily, the ECM components of the sea urchin are very similar to that of the vertebrate system and the number of cells in the embryo is very small compared to other complex organisms. Only 1,500 cells are present in the pluteus, the larval stage of the *S. purpuratus* embryo (Angrerer and Davidson, 1984). The ability to produce and culture transgenic embryos makes the sea urchin an attractive choice (Flytzanis *et al.*, 1983; Cameron and Davidson, 1991). The sea urchin is not without its limitations as a model, however. It is a seasonal spawning animal, hence, the availability of its gametes is reduced to only four months in a year,

from January to late April. Therefore, gametes collected during the spawning season have to be stored for remainder of the year. *Strongylocentrotus purpuratus (Sp)* is the most widely used of the several sea urchin species in the world and is the subject of an on going genome sequencing project, making it the preferred model for our research.

1.7.2 The Extracellular Matrix of the Sea Urchin Embryo

The sea urchin embryo is characterized by the presence of two ECMs: the hyaline layer (HL) on the apical surface and the basal lamina on the basal surface of the ectoderm cells. Fertilization results in the first major secretary event of development with exocytosis of the cortical granule contents. Shortly after fertilization the apically located ECM, the hyaline layer is formed in the embryo. Simultaneously, exocytosis of the matrix proteins occurs from the cortical granules, the site responsible for the synthesis and storage of ECM components (Alliegro and McClay, 1998). The hyaline layer that covers the pluteus larva is evidently lost at metamorphosis during involution of the general epidermis (Cameron and Holland, 1985). Similarities between the sea urchin and vertebrate ECM components have been detected by immunofluorescence assay using polyclonal antibodies prepared against vertebrate ECM components, such as collagen types I, III, and IV; adhesive noncollagenous glycoproteins, such as laminin and fibronectin, and heparan sulfate proteoglycan (Wessel *et al.*, 1984; Spiegel *et al.*, 1989).

The roles of sea urchin ECMs, both hyaline layer and basal lamina (BL), in embryonic development have been well established. The importance of properly constituted ECM for spiculogenesis, cell migration and gastrulation was demonstrated clearly in recent studies. For instance, the PDGF-like growth factor and a TGF-alpha-like growth factor are required for gastrulation and spiculogenesis in the *Lytechinus* embryo (Govindarajan *et al.*, 1995). The significance of the crosslinked-collagen deposition requirement for normal gastrulation was evident when collagen disruption resulted in the arrest of development before gastrulation (Wessel *et al.*, 1987). Developmental delays were also detected on deprivation of exogenous sulfate in the developing sea urchin embryo. In addition to this, sulfate deprivation resulted in disruption of primary mesenchyme cell (PMC) migration in *Strongylocentrotus purpuratus* (Gibson and Burke, 1987; Lane *et al.*, 1993). An antibody against the new ECM component, ECM 18 from *Lytechinus variegatus* resulted in the inhibition of PMC organization and endoderm morphogenesis during gastrulation (Berg *et al.*, 1996).

1.7.3 Extracellular Remodeling in the Sea Urchin Embryo

Matrix metalloproteinases and, to a lesser extent, serine proteinases degrade components of the ECM. Two predominant matrix metalloproteinases with relative molecular weights of 57- and 50 k species were identified in the early developmental stages of *Lytechinus pictus* and *Strongylocentrotus*

purpuratus (Vafa and Nishioka, 1995). BP10 (Blastula Protease 10) found in the early blastula stage was a translation product of an isolated cDNA clone. BP10 is an astacin-like proteinase that was homologous to the tolloid from *Drosophila* (Lepage *et al.*, 1992). Another homologue of BP10, SpAN was identified in *Strongylocentrotus purpuratus* (Reynolds *et al.*, 1992).

The sea urchin hatching enzyme, envelysin, a matrix metalloproteinase cleaves the fertilization envelope specifically during hatching of the blastula stage embryo (Normura *et al.*, 1997). In addition, many research groups have identified collagen and gelatin cleaving endopeptidases present in the sea urchin embryo (Karakiulakis *et al.*, 1993; Quigley *et al.*, 1993). Our lab has previously reported a 41 kDa collagen/gelatin cleaving, serine proteinase activity expressed in the sea urchin embryo (Mayne and Robinson, 2002). Research from our lab has also identified numerous storage-induced gelatin-cleavage activities from the apically located ECM, the hyaline layer (Robinson *et al.*, 2003).

1.8 Thesis Focus

Although the components of the hyaline layer have been investigated for decades, the roles of only a few ECM components are understood. A major component of the hyaline layer, hyalin has been extensively studied (Robinson, 1988; Robinson *et al.*, 1988; Robinson, 1989; Robinson and Brennan, 1991; Robinson *et al.*, 1992; Rimsay and Robinson, 2003). Two other protein components of the sea urchin extracellular matrix, HLC-175 and HLC-32 were

31

also identified (Robinson, 1995; Brennan and Robinson, 1994; Mayne and Robinson, 1998). HLC-175 was found localized to the hyaline layer, while HLC-32 was present in both the hyaline layer and basal lamina.

We are interested in identifying and characterizing the components of the apically located ECM, the hyaline layer, of the sea urchin embryo and its effect on developmental processes. Studies have showed that a properly constituted ECM is essential for normal development. Since the components of ECM are the substrates for endopeptidases that are localized in the ECM, we focused our research on the endopeptidases that utilize gelatin as their substrate. Hence, we employed gelatin substrate zymography, a qualitative assay to analyze the gelatin-cleavage activities present in the hyaline layer. The different patterns of gelatin-cleavage activities induced at 15°C or 37°C were identified. The optimal induction and displacement conditions for the gelatinases were determined. The three major induced gelatinases at 55-, 41- and 32 kDa were identified and purified, while we focused our research mainly on characterizing the 55 kDa species. Quantitative analysis was performed using a fluorescence gelatinase assay to determine the effects of various protease inhibitors and metal-ion chelators on the 55 kDa species. I determined its substrate specificity and confirmed its presence in the sea urchin embryo.

CHAPTER 2: MATERIALS AND METHODS

2.1 Growth of embryos

Strongylocentrotus purpuratus were purchased from Seacology, Vancouver, Canada. Gametes were obtained by intracoelomic injection of 0.5 M KCl (Fisher Scientific, New Jersey, USA). Eggs were washed three times in ice-cold millipore-filtered sea water (0.45-μm-pore-size filter and UV irradiated) and fertilized with a 100-fold numerical excess of sperm. Embryos were cultured with constant aeration, at 12°C, in cylindrical chambers containing paddles rotating at 40 rpm. Embryos were harvested at the times indicated after fertilization (HPF), and stored at −70°C.

2.2 Isolation of intact hyaline layers

To facilitate the isolation of hyaline layers, unfertilized eggs were dejellied by titration with HCl to pH 5.5 and left for 2 min on ice. The pH was then returned to 8.0 with the addition of 1 M Tris-HCl (pH 8.0). After extensive washing in ice-cold MFSW (0.45-μm-pore-size filter and UV irradiated), the eggs were resuspended in MFSW to give a final concentration of 10% (v/v) and their vitelline layer was disrupted with 10 mM dithiotheritol (Epel *et al.* 1970). After extensive washing in ice-cold MFSW, the eggs were fertilized with a 100-fold numerical excess of sperm. Hyaline layers were isolated as follows. One-h-old embryos were washed several times in ice-cold MFSW and resuspended to give a final concentration of 10% (v/v) in MFSW containing 10 mM benzamidine hydrochloride as a protease inhibitor. The embryo suspension was homogenized

34

with 100 strokes in a hand-held Dounce homogenizer (type A) at 0°C. The homogenate was passed by gravity filtration through a 28-μm-pore-size Nitex mesh (B, D and H Thompson and Co., Mount Royal, Quebec), the retarded layers were resuspended in ice-cold MFSW, and the filtration step was repeated twice more. The isolated layers were harvested by centrifugation and the pellets were stored at −70°C. Each preparation was examined by phase-contrast microscopy and showed no evidence of contaminating cytoplasmic debris.

2.3 Substrate gel zymography

Substrate gel zymography was performed essentially as described previously (Heussen and Dowdle 1980). Gels containing sodium dodecyl sulfate were prepared by copolymerizing acrylamide and gelatin or a test substrate at a final concentration of 0.1% (w/v). Samples of embryos, or the purified gelatinase were dispersed for 30 min at room temperature in Laemmli solubilizing solution from which both DTT and bromophenol blue had been omitted (Laemmli, 1970). Electrophoresis was performed at a constant current of 10 mA and at 4°C for 4 h. After electrophoresis the gels were incubated for 60 min at room temperature in 50 mM Tris-HCl, pH 8.0, containing 2.5% (v/v) Triton X-100 followed by 16 h incubation at room temperature in 50 mM Tris-HCl, pH 8.0, containing 10 mM $CaCl_2$. The gels were stained with Coomassie Brilliant Blue R-250. Clear bands of proteolytic activity were visualized against a blue background of stained intact substrate.

2.4 Gel filtration chromatography

Aliquots of intact hyaline layers were resuspended in MFSW and incubated at 37°C. After 24 h incubation, the layers were harvested by centrifugation, and the pellet was resuspended in 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA and incubated for 24 h at 37°C. Following incubation, the suspension was separated into pellet and supernatant fractions. The supernatant containing the displaced gelatin-cleavage activities was applied to a 1 x 28 cm column packed with the gel exclusion resin, Agarose 1.5 m with fractionation range from 10,000 to 1,500,000 (Bio-Rad, Richmond, California). The 55- and 32 kDa species were purified using this column, which was equilibrated in 50 mM Tris-HCl, pH 8.0, and 10 mM benzamidine hydrochloride. However, the 41 kDa gelatinase was purified using the same column equilibrated in 50 mM Tris-HCl, pH 8.0. Chromatography was performed at a flow rate of 1.8 mL/h at 4°C and 0.3 mL fractions were collected.

2.5 Quantitative fluorescence gelatinase assay

The quantitative assay was performed using fluorescein-conjugated gelatin (Molecular Probes Inc., Oregon, USA). The fluorescence of the fluorescein-labeled gelatin is quenched when it is intact. The intact gelatin releases fluorescent peptides following gelatinase digestion. Cleavage activity was monitored by following the release of fluorescent peptides from the

substrate. Reactions contained 10μg of fluorescein-labelled gelatin and aliquots (25 μl) of the purified gelatinase. The fluorescence was measured using a fluorescence microplate reader set to an absorption maximum of 490 nm and emission maximum of 520 nm. The rate of change in relative fluorescence units (RFU/sec) was determined using *SoftmaxPro* software (Molecular Devices, Sunnyvale, California). The increase in fluorescence was assayed at 2 min intervals for 60 min at 37°C.

2.6 Determination of protein concentration

Samples were precipitated with an equal volume of 20% (w/v) TCA at 0°C for 20 min. Samples were centrifuged in a microcentrifuge (Eppendorf, Hamburg, Germany) at 16,000 X g for 10 min and the supernatants discarded. Protein concentrations were determined by the method of (Lowry *et al.* 1951) using bovine serum albumin (Sigma, Ontario, Canada) as a standard. Absorbances were read at 750 nm in a Spectronic 601 spectrophotometer (Milton Roy, Rochester, New York).

CHAPTER 3: RESULTS

3.1 Induction of the gelatin-cleavage activities in isolated layers following incubation in MFSW at 15°C.

Layers were isolated from the 1-h-old sea urchin embryos as described in the Materials and Methods section and stored at −70°C. Gelatin substrate gel zymography was employed to visualize the gelatin-cleavage activities in the isolated layers while freshly prepared layers were devoid of gelatin-cleavage activities. We determined that several activities could be induced upon incubation of layers in MFSW at 15°C (Fig. 1). Layers were incubated at 15°C, the ambient temperature for *Strongylocentrotus purpuratus*, for 24 h (lane 1), 36 h (lane 2), 48 h (lane 3), 60 h (lane 4), 72 h (lane 5), 84 h (lane 6) and 96 h (lane 7) and were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin as the substrate.

We identified three major gelatin-cleavage activities at 55-, 41-, and 32 kDa after incubation in MFSW for 96 h at 15°C. The 32kDa gelatinase activity appeared after 24 h of incubation, while the 41kDa activity appeared between 24 and 36 h of incubation, and both these activities were still detectable after 96 h. The 55 kDa is another major gelatin-cleavage activity that appeared between 84 and 96 h of incubation. In addition to this, two other activities of apparent molecular weight 66 k and 94 k were also induced. The 94 kDa activity appeared as a major species after 36 h (lane 2), but it turned into a minor activity after 48 h of incubation (lane 3), which could not be explained. In order to clearly visualize the pattern of gelatin-cleavage activities after 96 h of incubation (lane 7), a lower

Figure 1. Gelatin substrate gel zymography of hyaline layers incubated at 15°C in millipore-filtered seawater.

Aliquots (15 µg) of isolated layers were incubated at 15°C in millipore-filtered seawater for 24 h (lane 1), 36 h (lane 2), 48 h (lane 3), 60 h (lane 4), 72 h (lane 5), 84 h (lane 6), 96 h (lane 7) and were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% gelatin. The gel was stained with Coomassie Brilliant Blue. Lane 8 is a lower exposure of lane 7. The molecular weights of gelatin-cleavage activities were estimated using molecular mass markers (Sigma Chem Co.); Myosin - 205,000; β-Galactosidase - 116,000; Phosphorylase b - 97,400; Bovine albumin - 66,000; Egg albumin - 45,000; Carbonic anhydrase - 29,000.
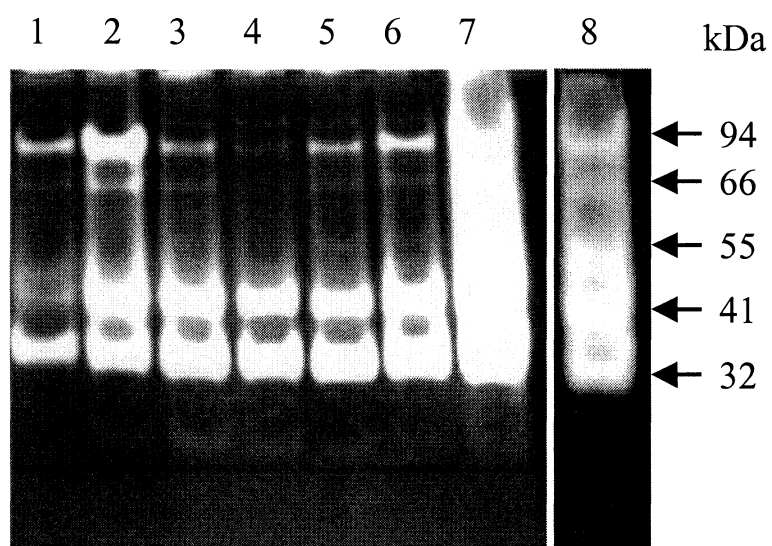
exposure of the same lane is shown (Lane 8). The data obtained from this experiment suggest that the gelatin-cleavage activities were present as one or more precursor(s) in the freshly prepared layers and these species were activated on incubation in MFSW at 15°C.

3.2 Gelatin substrate gel analysis of gelatin-cleavage activities induced in layers after incubation in MFSW at 37°C.

The layers stored at –70°C were incubated at 37°C to study the induction profile of gelatin-cleavage activities and to compare it with the pattern seen at 15°C. Isolated layers were incubated in MFSW at 37°C for 24 h (lane 1), 48 h (lane 2), 72 h (lane 3), 96 h (lane 4), 120 h (lane 5), 144 h (lane 6) and were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin (Fig. 2). The three major gelatin-cleavage activities at 55-, 41- and 32 kDa induced at 15°C were also present at 37°C, but the temporal sequence of induction varied at these two different temperatures. The 55 kDa species appeared after 24 h of incubation and remained as the major activity. In contrast, the 55 kDa activity appeared only after 96 h of incubation at 15°C (Fig. 1, lane 7). At 37°C, the amount of the 32 kDa gelatin-cleavage activity present after 144 h of incubation (lane 6) was relatively low compared to the amount of this gelatinase present after 24 h of incubation (lane 1). This interesting observation suggests a possible turn-over of the 32 kDa activity between 120 and 144 h of incubation at 37°C in MFSW. Two other activities of apparent molecular weight 66 k and 94 k

Figure 2. The pattern of gelatin-cleavage activities induced when isolated hyaline layers were incubated in MFSW at 37°C.

Aliquots (15 μg) of isolated hyaline layers were resuspended in millipore-filtered seawater and incubated at 37°C for 24 h (lane 1), 48 h (lane 2), 72 h (lane 3), 96 h (lane 4), 120 h (lane 5), 144 h (lane 6). Lane 7 contained molecular mass markers (Sigma Chem Co.); Myosin, Rabbit Muscle 205,000; β-Galactosidase, *E.coli* 116,000; Phosphorylase b, Rabbit Muscle - 97,400; Albumin, Bovine - 66,000; Albumin, Egg - 45,000; Carbonic Anhydrase, Bovine Erythrocytes 29,000.
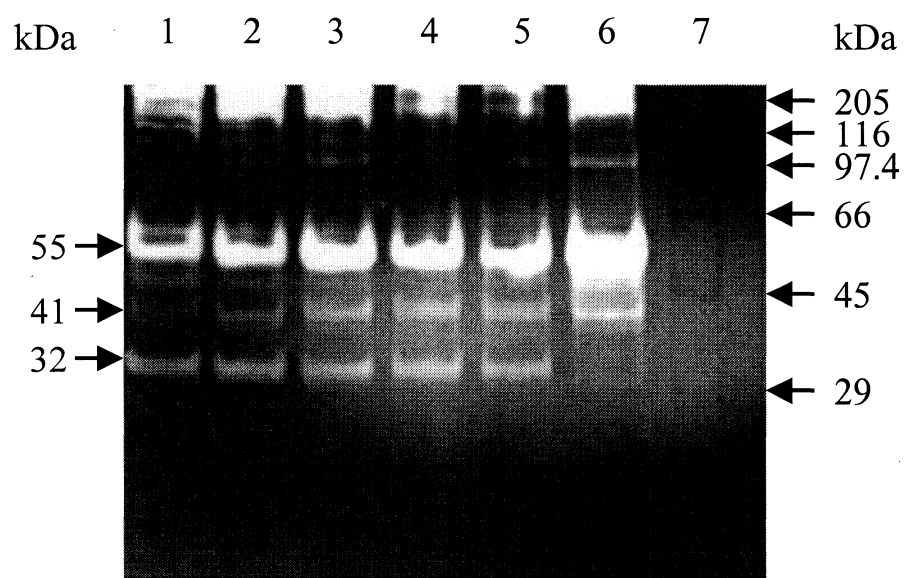
induced at 15°C were also present at 37°C. In addition to this, a very high molecular weight species around 200 kDa was observed following induction at 37°C. This activity was absent at 15°C.

The above data collectively suggest that the incubation of layers in MFSW at 15°C or 37°C can induce gelatin-cleavage activities, which are present as one or more inactive precursors in the freshly prepared layers. This experiment also demonstrates that induction of the 55 kDa activity at 37°C occurs within 24 h. However, 96 h of incubation is required for the induction of the 55 kDa gelatinase at 15°C suggesting that the induction at 37°C is at least 4 times faster than at 15°C. Using the above data we have established that both the 15°C and 37°C inductions produced the same pattern of gelatin-cleavage activities except for differences in the order of appearance of the activities. Layers were induced at 37°C in some experiments because the 37°C induction is faster than that at 15°C.

3.3 Induction of the gelatin-cleavage activities in isolated layers following incubation in the presence of benzamidine hydrochloride, a reversible serine protease inhibitor at 37°C.

Aliquots (15 μg) of isolated layers stored at –70°C were incubated at 37°C in MFSW containing 10 mM benzamidine hydrochloride, a reversible serine protease inhibitor (Sigma Chem. Co.) for 24 h (lane 1), 48 h (lane 2), 72 h (lane 3), 96 h (lane 4), 120 h (lane 5) and 144 h (lane 6). These samples were

Figure 3. The effects of benzamidine hydrochloride on the induction of gelatin-cleavage activities in hyaline layers at 37°C.

Equal amounts (15 μg) of isolated hyaline layers were incubated at 37°C in millipore-filtered seawater containing 10 mM benzamidine hydrochloride for 24 h (lane 1), 48 h (lane 2), 72 h (lane 3), 96 h (lane 4), 120 h (lane 5), 144 h (lane 6) and were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin.
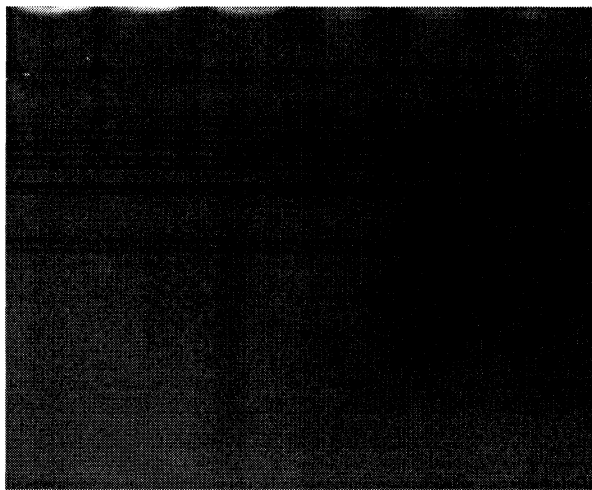
fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin (Fig. 3). Layers incubated in MFSW containing 10 mM benzamidine hydrochloride had no gelatin-cleavage activities. However, layers incubated in the absence of benzamidine hydrochloride had all three major gelatin-cleavage activities in addition to the 66-, 94- and 200 kDa activities (Fig. 2). These data suggest that serine protease activity is essential for the activation of the gelatin-cleavage activities, which are present as one or more inactive precursors in layers.

3.4 Determination of the minimum time required for induction of the 55- and 41 kDa gelatin-cleavage activities at 37°C.

Freshly prepared layers stored at –70°C were incubated in MFSW at 37°C to determine the minimum time required for induction of the three major gelatin-cleavage activities (Fig. 4). The layers (15 μg) were incubated for 2.5 h (lane 1), 5 h (lane 2), 7.5 h (lane 3), 10 h (lane 4), 12.5 h (lane 5) and 15 h (lane 6) in MFSW at 37°C and were fractionated in a gelatin substrate gel.

Gelatin-cleavage activities appeared between 7.5 and 10 h of incubation. The 55- and 41 kDa gelatinases were the two major activities present after 10 h of incubation. In addition, the 94- and 200 kDa gelatin-cleavage activities were also present after 10 h of incubation. Trace amounts of the 32 kDa species appeared after 15 h of incubation.

Figure 4. Estimation of the minimum time required for the induction of the 55- and 41 kDa gelatin-cleavage activities at 37°C.

Aliquots (15 µg) of isolated hyaline layers were incubated at 37°C in millipore-filtered seawater for 2.5 h (lane 1), 5 h (lane 2), 7.5 h (lane 3), 10 h (lane 4), 12.5 h (lane 5), 15 h (lane 6). The apparent molecular weights of gelatin-cleavage activities are shown. Lane 7 contained molecular mass markers (Sigma Chem Co.); Myosin, Rabbit Muscle 205,000; β-Galactosidase, *E.coli* 116,000; Phosphorylase b, Rabbit Muscle - 97,400; Albumin, Bovine - 66,000; Albumin, Egg - 45,000; Carbonic Anhydrase, Bovine Erythrocytes 29,000.
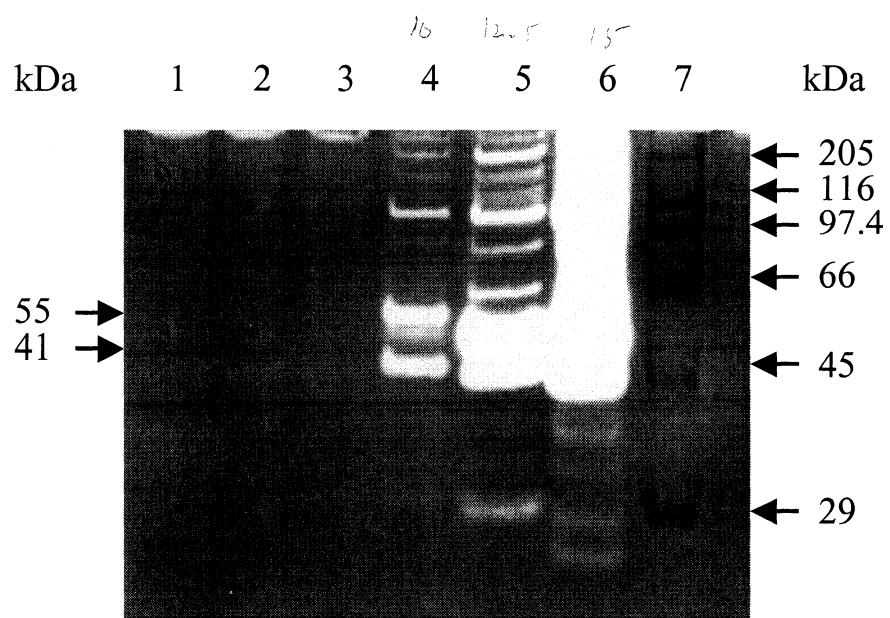
The data from this experiment show that the 55- and 41 kDa gelatin-cleavage activities require between 7.5 and 10 h of incubation at 37°C in MFSW for their induction and the 32 kDa did not appear as a major gelatin-cleavage activity following up to 15 h incubation.

3.5 Estimation of the minimum time required for the induction of the 32 kDa gelatin-cleavage activity at 37°C.

Isolated layers were incubated at 37°C in MFSW to determine the minimum time required for the induction of the 32 kDa gelatin-cleavage activity. The layers (15 μg) were incubated for 18 h (lane 1), 24 h (lane 2), or 48 h (lane 3) in MFSW at 37°C and were fractionated in a gelatin substrate gel (Fig. 5). The molecular mass markers are shown in lane 4. The three major gelatin-cleavage activities were induced after 18 h of incubation. The 32 kDa gelatinase was present from 18 to 48 h of incubation, signifying that at least 18 h of incubation at 37°C in MFSW is essential for the induction of the 32 kDa gelatinase. In addition to this, the 41, 55, 66, 94 and ~200 kDa gelatinase activities were also induced.

Collectively, the results obtained from the last two experiments indicate that the 55- and 41 kDa species were induced after 10 h of incubation while induction of the 32 kDa activity required between 15 and 18 h of incubation in MFSW at 37°C. Based on these results, all our inductions at 37°C were performed for 24 h in MFSW.

Figure 5. Determination of the minimum time required for the induction of the 32 kDa gelatin-cleavage activity at 37°C.

Equal amounts (15 μg) of isolated hyaline layers were incubated at 37°C in millipore-filtered seawater for 18 h (lane 1), 24 h (lane 2), 48 h (lane 3) and molecular weight markers (lane 4). These samples were fractionated in a gelatin substrate gel and stained in Coomassie Brilliant Blue.
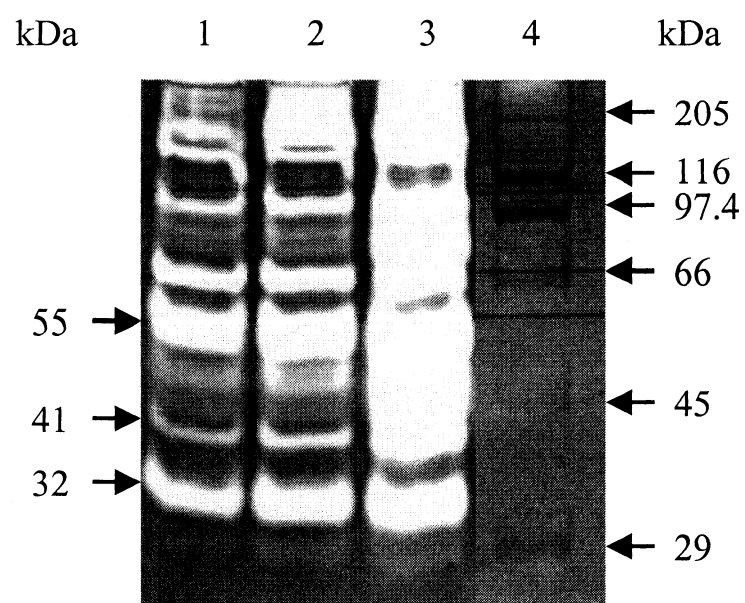
3.6 Determination of the minimum time required for the induction of the three major gelatin-cleavage activities at 15°C.

Aliquots (15 μg) of freshly prepared layers were incubated in MFSW for 2.5 h (lane 1), 5 h (lane 2), 7.5 h (lane 3), 10 h (lane 4), 12.5 h (lane 5) and 15 h (lane 6) were fractionated in a gelatin substrate gel (Fig. 6). Gelatin-cleavage activities were absent in all the six lanes. This result shows that the gelatin-cleavage activities were not induced in the first 15 h of incubation in MFSW at 15°C.

As shown in Fig.1, the 32 kDa gelatin-cleavage activity is induced after 24 h of incubation in MFSW at 15°C (Fig. 1). Collectively, these results suggest that the 32 kDa gelatin-cleavage activity requires between 15 and 24 h of incubation at 15°C, while the 41 kDa species requires between 24 and 36 h for its induction. However, the 55 kDa was induced between 92 and 96 h of incubation in MFSW at 15°C (data not shown). Henceforth, all inductions at 15°C were performed in MFSW for 96 h.

3.7 Estimation of the minimum ionic requirements for the induction of the three major gelatinase activities at 15°C.

Seawater contains high concentrations of salts, namely 10 mM $CaCl_2$, 50 mM $MgCl_2$, 500 mM NaCl, and other salts in trace amounts. Hence, we wished to examine the requirement of these salts for the induction of the 55-, 41- and 32 kDa species.

Figure 6. Estimation of the minimum time required for the induction of the major gelatin-cleavage activities at 15°C.

Equal amounts (15 μg) of isolated hyaline layers stored at −70°C were incubated in millipore-filtered seawater at 15°C for 2.5 h (lane 1), 5 h (lane 2), 7.5 h (lane 3), 10 h (lane 4), 12.5 h (lane 5), 15 h (lane 6) and molecular weight markers (lane 7) and were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin as the substrate. The gel was stained with Coomassie Brilliant Blue.
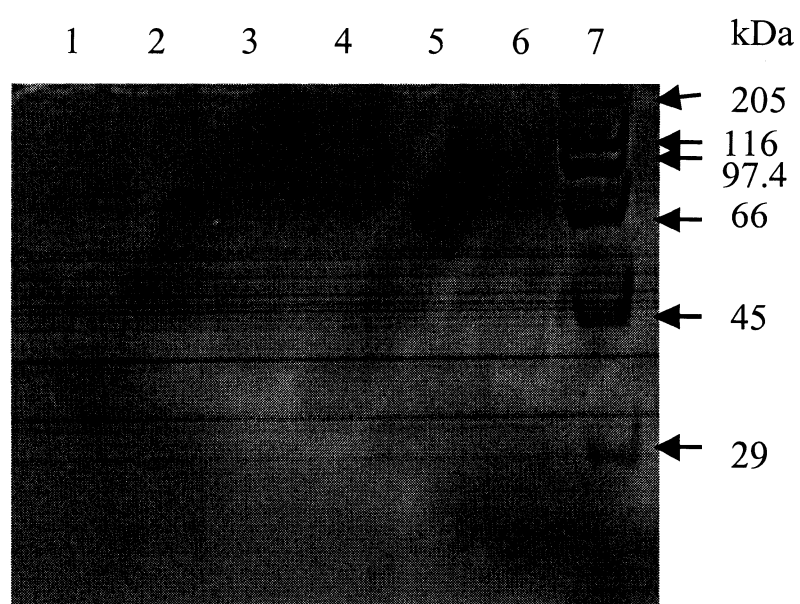
Aliquots (15 µg) of isolated layers, stored at −70°C, were incubated at 15°C for 96 h in a 50 mM Tris-HCl, pH 8.0, buffer containing the following combination of salts: no salt (lane 1), 10 mM $CaCl_2$ (lane 2), 50 mM $MgCl_2$ (lane 3), 500 mM NaCl (lane 4), 10 mM $CaCl_2$ and 50 mM $MgCl_2$ (lane 5), 10 mM $CaCl_2$ and 500 mM NaCl (lane 6), 50 mM $MgCl_2$ and 500 mM NaCl (lane 7), millipore-filtered seawater (lane 8) along with molecular weight markers (Sigma Co.) in lane 9 (Fig. 7). The first three lanes were devoid of any activities, suggesting that buffer alone (lane 1) or buffer containing 10 mM $CaCl_2$ (lane 2) or buffer containing 50 mM $MgCl_2$ (lane 3) are not sufficient to induce the major gelatin-cleavage activities. In contrast, buffer containing 500 mM NaCl induced the 55- and 41 kDa major gelatin-cleavage activities (lane 4). Both the 55- and 41 kDa gelatinases were overlapped in this figure. A similar pattern of induced gelatin-cleavage activities appeared in buffer containing 50 mM $MgCl_2$ and 500 mM NaCl (Lane 7). A comparison of lanes 4 and 7 indicate that 50 mM $MgCl_2$ is not required for the induction of the 55- and 41 kDa, while 500 mM NaCl is essential for their induction at 15°C. The layers incubated in MFSW for 96 h (lane 8) served as a positive control for this experiment. Interestingly, identical patterns of induced gelatinases were present in buffer containing 10 mM $CaCl_2$ and 500 mM NaCl (lane 6), and in MFSW, which contains 10 mM $CaCl_2$, 50 mM $MgCl_2$ and 500 mM NaCl (lane 8). In both lanes 6 and 8 the three major gelatin-cleavage activities were induced.

Figure 7. Determination of the minimum ionic requirements for the induction of the three gelatin-cleavage activities at 15°C.

Aliquots (15 µg) of isolated hyaline layers were incubated at 15°C in 50mM Tris-HCl pH 8.0 (lane 1), 50mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$ (lane 2), 50mM Tris-HCl pH 8.0 containing 50 mM $MgCl_2$ (lane 3), 50mM Tris-HCl pH 8.0 containing 500 mM NaCl (lane 4), 50mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$ and 50 mM $MgCl_2$ (lane 5), 50mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$ and 500 mM NaCl (lane 6) 50mM Tris-HCl pH 8.0 containing 50 mM $MgCl_2$ and 500 mM NaCl (lane 7), millipore-filtered seawater (lane 8) and molecular weight markers (lane 9). These samples were fractionated in a gelatin substrate gel and stained with Coomassie Brilliant Blue.
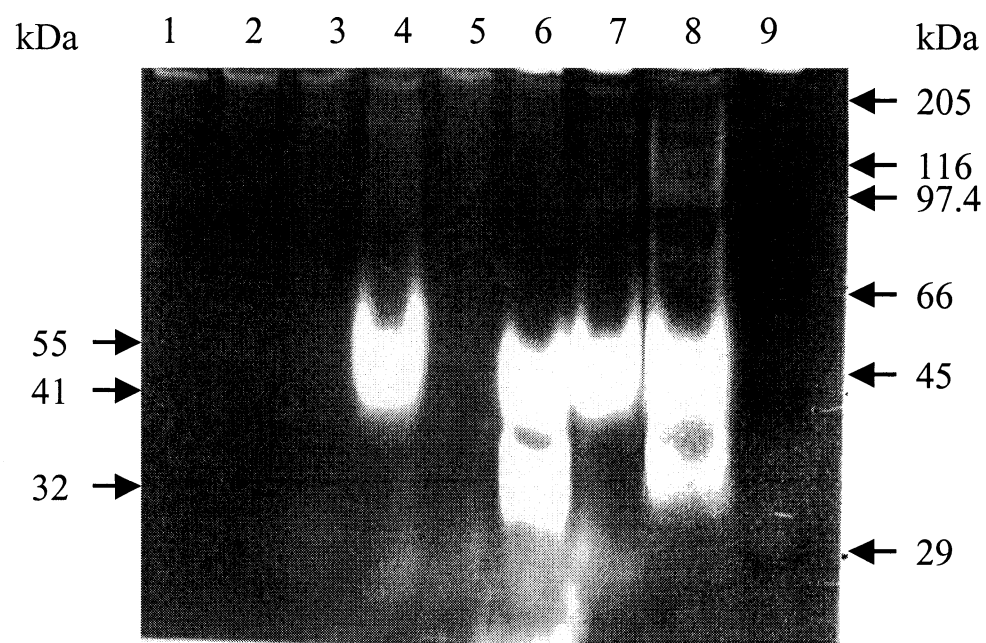
Collectively, the above results show that both the 55- and 41 kDa required only 500 mM NaCl for their induction, while the 32 kDa required both 10 mM CaCl$_2$ and 500 mM NaCl.

3.8 Determination of the minimum sodium chloride concentration required for the induction of the 55- and 41 kDa species at 15°C.

Aliquots (15 μg) of isolated layers, stored at −70°C, were incubated at 15°C (Fig. 8) for 96 h in 50 mM Tris-HCl, pH 8.0, containing: 100 mM NaCl (lane 1), 200 mM NaCl (lane 2), 300 mM NaCl (lane 3), 400 mM NaCl (lane 4), 500 mM NaCl (lane 5), and millipore-filtered seawater (lane 6).

In Fig. 8, gelatin-cleavage activities were absent in the first two lanes, but lanes 3, 4 and 5 had both the 55- and 41 kDa activities. This result confirms that only sodium chloride is required for the induction of the two major gelatin-cleavage activities at 55- and 41 kDa in isolated layers. These data also suggest that 300 mM NaCl is the minimum concentration required for inducing both the 55- and 41 kDa activities at 15°C. The layers incubated in millipore-filtered seawater for 96 h (lane 6) was the positive control for this experiment.

3.9 Determination of the minimum sodium chloride concentration required for the induction of the 32 kDa gelatinase at 15°C.

Isolated layers stored at −70°C were incubated at 15°C for 96 h to induce the three major gelatin-cleavage activities at 55-, 41- and 32 kDa. Equal amounts

Figure 8. Estimation of the minimum NaCl concentration required for induction of both the 55- and 41 kDa gelatin-cleavage activities at 15°C.

Equal amounts (15 μg) of freshly prepared hyaline layers, stored at –70°C, were incubated at 15°C in 50mM Tris-HCl pH 8.0 containing 100 mM NaCl (lane 1), 50mM Tris-HCl pH 8.0 containing 200 mM NaCl (lane 2), 50mM Tris-HCl pH 8.0 containing 300 mM NaCl (lane 3), 50mM Tris-HCl pH 8.0 containing 400 mM NaCl (lane 4), 50mM Tris-HCl pH 8.0 containing 500 mM NaCl (lane 5), and millipore-filtered seawater (lane 6). The above samples were fractionated in a gelatin substrate gel and stained with Coomassie Brilliant Blue.
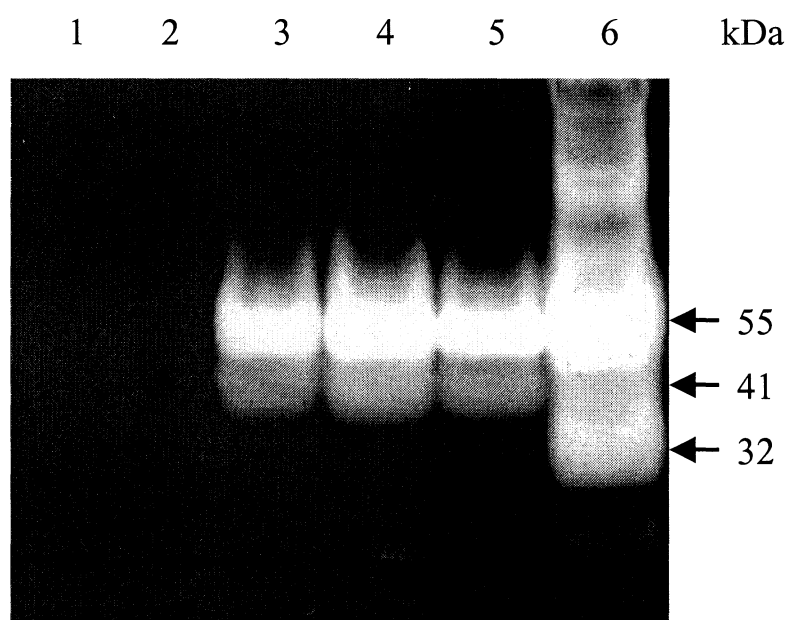
(15 μg) of layers incubated in 50 mM Tris-HCl, pH 8.0, containing: 10 mM CaCl$_2$ and 100 mM NaCl (lane 1), 10 mM CaCl$_2$ and 200 mM NaCl (lane 2), 10 mM CaCl$_2$ and 300 mM NaCl (lane 3), 10 mM CaCl$_2$ and 400 mM NaCl (lane 4), 10 mM CaCl$_2$ and 500 mM NaCl (lane 5), and millipore-filtered seawater (lane 6) were fractionated in a gelatin substrate gel (Fig. 9).

Gelatin-cleavage activities were absent in the first lane, but a wide range of gelatinase activities appeared in lanes 2 to 6. The three major gelatin-cleavage activities at 55-, 41- and 32 kDa were present in lanes 2, 3, 4, 5 and 6. Layers incubated in MFSW for 96 h (lane 6) was the positive control for this experiment. This result confirms that both sodium chloride and calcium chloride are required for the induction of the 32 kDa species at 15°C. These data also clearly demonstrate that only 200 mM NaCl is required in addition to 10 mM CaCl$_2$ for the induction of 32 kDa gelatin-cleavage activity. Interestingly, both the 55- and 41 kDa gelatinase required a minimum of 300 mM NaCl for their induction (Fig. 8, lane 3). However, both the 55- and 41 kDa gelatinase were induced at 200 mM NaCl (Fig. 9, lane 2), which is lower than the minimum NaCl requirement. This could be due to the presence of 10 mM CaCl$_2$, which could accelerate the induction of the 55- and 41 kDa gelatinase activities. Hence, we suggest that both the 55- and 41 kDa requires only NaCl for their induction. However, the 32 kDa gelatinase requires a minimum of 200 mM NaCl in addition to CaCl$_2$.

Figure 9. Estimation of the minimum NaCl concentration required for induction of the 32 kDa gelatin-cleavage activities at 15°C.

Aliquots (15 μg) of isolated hyaline layers were incubated at 15°C for 96 h in 50mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$ and 100 mM NaCl (lane 1), 50mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$ and 200 mM NaCl (lane 2), 50mM Tris-HCl pH 8.0, 10 mM $CaCl_2$ and 300 mM NaCl (lane 3), 50mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$ and 400 mM NaCl (lane 4), 50mM Tris-HCl pH 8.0 containing 10 mM $CaCl_2$ and 500 mM NaCl (lane 5), and millipore-filtered seawater (lane 6) and were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin.
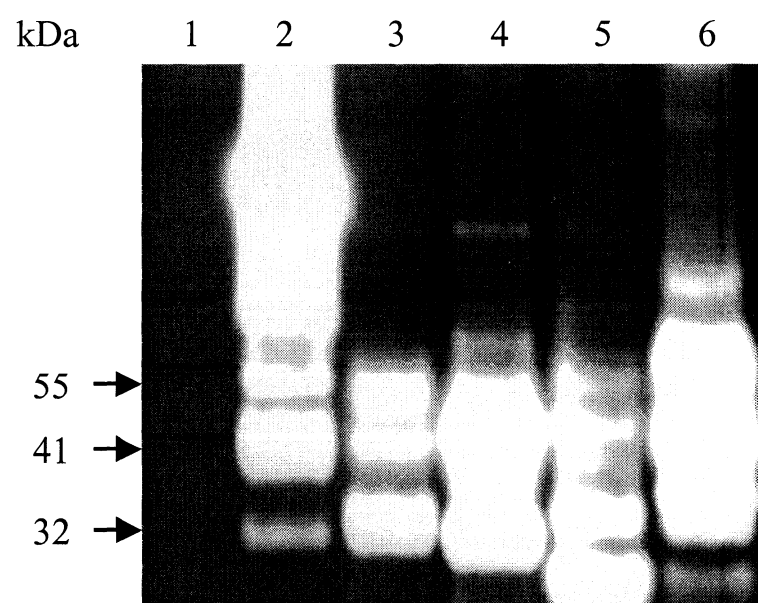
3.10 Estimation of the minimum calcium chloride concentration required for the induction of the 32 kDa species at 15°C.

Equal amounts (15 μg) of layers were incubated for 96 h at 15°C in 50 mM Tris-HCl, pH 8.0 and 500 mM NaCl containing: 1 mM $CaCl_2$ (lane 1), 3 mM $CaCl_2$ (lane 2), 5 mM $CaCl_2$ (lane 3), 7 mM $CaCl_2$ (lane 4), 10 mM $CaCl_2$ (lane 5), and millipore-filtered seawater in lane 6 (Fig. 10). Aliquots of these samples were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin as the substrate.

As expected the 55- and 41 kDa gelatinase activities were present in all of the six lanes. Except for lane 1, the 32 kDa was present in all the other five lanes (lanes 2, 3, 4, 5 and 6). This demonstrates that in addition to 200 mM NaCl, the 32 kDa also requires a minimum of 3 mM $CaCl_2$ for induction at 15°C.

3.11 Dissociation of the induced gelatin-cleavage activities from isolated layers.

We first incubated the isolated layers in MFSW for 24 h at 37°C to induce the gelatin-cleavage activities. After induction, the suspension was separated into pellet and supernatant fractions. The pellet fraction, containing layers were retained, while the supernatant was discarded. Aliquots (15 μg) of the layers (pellet) were incubated in 50 mM Tris-HCl, pH 8.0, (Fig. 11, lanes 1 and 2) or 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA (Fig. 11, lanes 3 and 4) for 24 h at 37°C. The pellet and supernatant fractions were collected, and were analyzed in

Figure 10. Identification of the minimum $CaCl_2$ concentration required for induction of the 32 kDa gelatin-cleavage activity at 15°C.

Equal amounts (15 µg) of isolated hyaline layers, stored at –70°C, were incubated at 15°C for 96 h in 50mM Tris-HCl pH 8.0, 500 mM NaCl and 1 mM $CaCl_2$ (lane 1), 50mM Tris-HCl pH 8.0, 500 mM NaCl and 3 mM $CaCl_2$ (lane 2), 50mM Tris-HCl pH 8.0, 500 mM NaCl and 5 mM $CaCl_2$ (lane 3), 50mM Tris-HCl pH 8.0, 500 mM NaCl and 7 mM $CaCl_2$ (lane 4), 50mM Tris-HCl pH 8.0, 500 mM NaCl and 10 mM $CaCl_2$ (lane 5) and millipore-filtered seawater (lane 6) and were fractionated in a gelatin substrate gel.
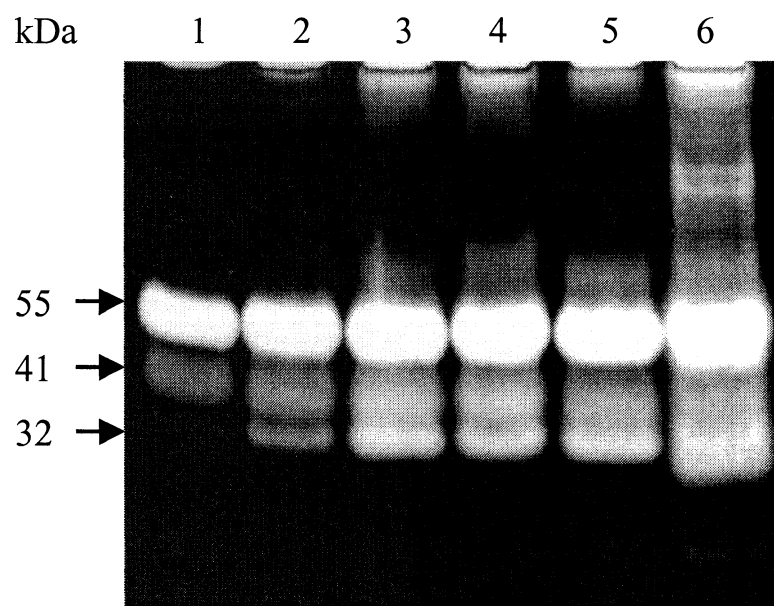
gelatin substrate gel. The patterns of gelatinase activities for pellets (Fig. 11, lanes 1 and 3) and supernatants (Fig. 11, lanes 2 and 4) were fractionated in a gelatin substrate gel.

The purpose of this experiment was to identify the optimal conditions that would facilitate the dissociation of the major gelatinase activities from the induced layers, which would then enable us to attempt to purify these activities. This experiment would also provide us with additional information about the metal ions required to maintain the association of these activities with the layers. The induced activities were displaced more efficiently by 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA than 50 mM Tris-HCl, pH 8.0, alone. The above conclusion was reached by comparing the supernatants from 50 mM Tris-HCl, pH 8.0, (Fig. 11, lane 2) and 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA (Fig. 11, lane 4). Both the 55- and 32 kDa activities were displaced in the presence of EDTA. In contrast, in the absence of EDTA only the 32 kDa was fully displaced (Fig. 11, lane 2). These data indicate that Tris-EDTA is more efficient than Tris alone in displacing the induced gelatinase activities. This also suggests that the 55 kDa requires a divalent metal ion to remain bound to the layers, whereas the 32 kDa dissociates readily dissociates from the hyaline layers in 50 mM Tris-HCl, pH 8.0, alone.

Figure 11. Displacement of the gelatin-cleavage activities from the layers in the presence and absence of EDTA.

Isolated hyaline layers were incubated at 37°C in millipore-filtered seawater for 24 h to induce the gelatin-cleavage activities. Lanes 1 and 2 show the pellet and supernatant fractions respectively, isolated from hyaline layers incubated for 24 h at 37°C in 50 mM Tris-HCl pH 8.0 and lanes 4 and 5 show the pellet and supernatant fractions respectively, isolated from hyaline layers incubated for 24 h at 37°C in 50 mM Tris-HCl pH 8.0 containing 5 mM EDTA. Lane 5 contained molecular weight markers (Sigma Chem Co.); Myosin, Rabbit Muscle 205,000; β-Galactosidase, E.coli 116,000; Phosphorylase b, Rabbit Muscle - 97,400; Albumin, Bovine - 66,000; Albumin, Egg - 45,000; Carbonic Anhydrase, Bovine Erythrocytes 29,000.
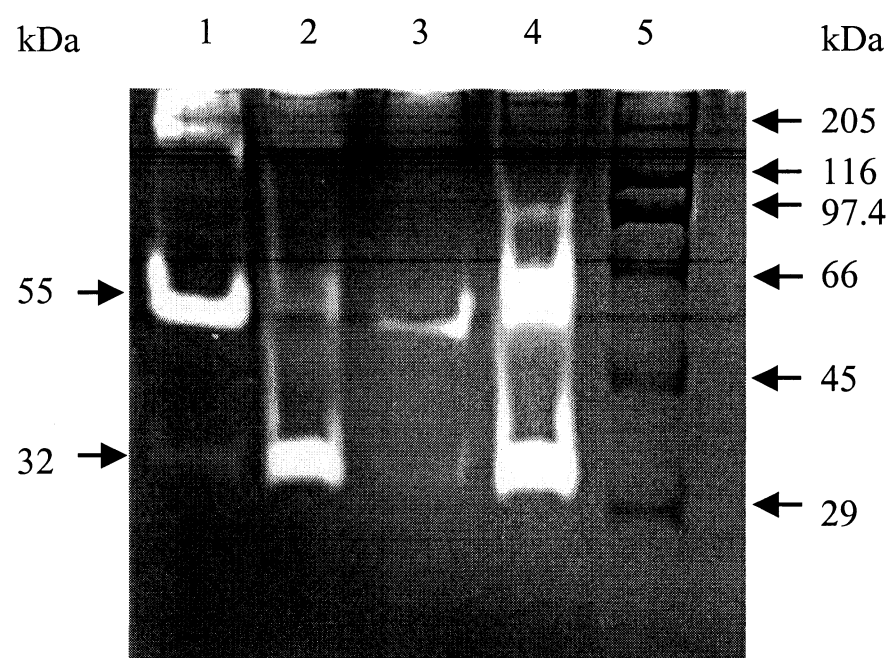
3.12 Gelatin substrate gel analysis of the fractions eluted from a 1.5 M-Agarose gel exclusion column.

Isolated layers were incubated for 24 h at 37°C in MFSW to induce the gelatin-cleavage activities followed by incubation in 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA for 24 h at 37°C to dissociate those activities from the layers. The supernatant containing the displaced gelatin-cleavage activities was loaded in a 1.5 M-Agarose gel filtration column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 10 mM benzamidine hydrochloride. The eluant from this column was collected and analyzed in a gelatin substrate gel.

Equal amounts (5 μg) of fraction No. 15, 18 and 21 were fractionated in lanes 1, 2, and 3 respectively (Fig. 12). Lane 1 shows the enriched 55 kDa gelatinase activity in elution No. 15 and lane 4 shows the highly enriched 32 kDa gelatin-cleavage activity in elution No. 21.

3.13 Purification of the 41 kDa gelatin-cleavage activity using a 1.5 M-Agarose gel exclusion column.

The previous experiment (Fig. 12) was repeated, but with benzamidine hydrochloride omitted from the equilibration buffer. The supernatant containing the displaced 55- and 32 kDa gelatinase activities was loaded onto the column and the fractions eluted were analyzed in a gelatin substrate gel (Fig. 13).

Equal amounts (5 μg) of the fractions No. 12, 15, 18 and 21 were analyzed in lanes 1, 2, 3 and 4 respectively. Interestingly, the eluted fractions

Figure 12. Purification of the 55- and 32 kDa activities using a 1.5 M-Agarose gel filtration chromatography column.

Isolated hyaline layers were incubated at 37°C in millipore-filtered seawater for 24 h, followed by 24 h incubation at 37°C in 50 mM Tris-HCl pH 8.0 containing 5 mM EDTA to displace the major gelatin-cleavage activities. The displaced gelatin-cleavage activities were loaded on a 1.5 M Agarose gel exclusion column equilibrated in 50 mM Tris-HCl pH 8.0 containing 10 mM benzamidine hydrochloride (BioRad.) and 5 μg of elutions No.15 (lane 1), No.18 (lane 2), No.21 (lane 3) were analyzed in a gelatin substrate gel. Lane 4 contained molecular weight markers (Sigma Chem Co.) 205, 116, 97.4, 66, 45, 29 kDa.
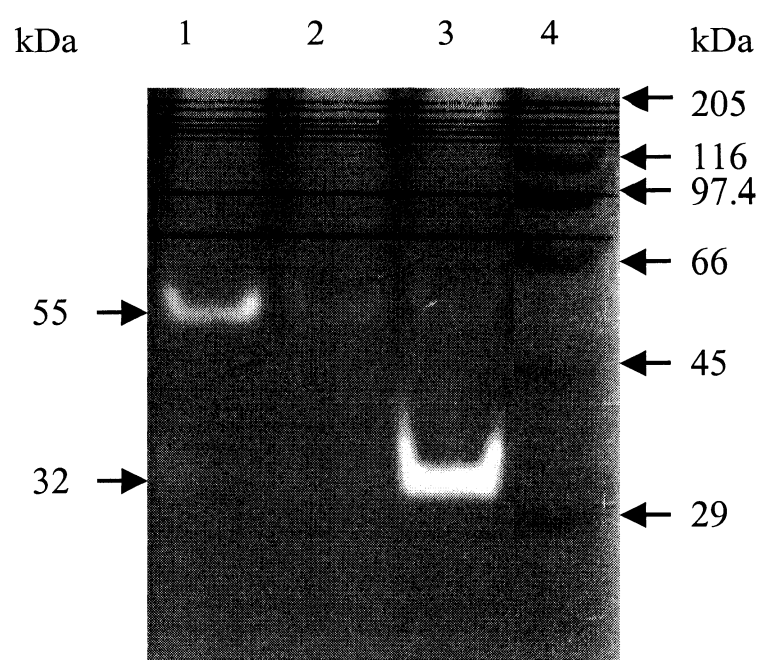
had neither the 55 kDa nor the 32 kDa gelatinase activities. Surprisingly, only 41 kDa activity was present in the eluant suggesting that both the 55- and 32 kDa gelatinase were processed and the 41 kDa could be a product of the 55 kDa processing. The 32 kDa gelatinase might be processed into very small peptides that cannot be visualized in this gel. These data strongly suggest a precursor-product relationship between the 55- and 41 kDa gelatinase activities.

3.14 The effects of various protease inhibitors on the 55 kDa gelatin-cleavage activity.

Aliquots (15 μL) of the purified 55 kDa activity were fractionated in a gelatin substrate gel (Fig.14). After electrophoresis, the gel was cut into strips, which were processed for 1 h in 50 mM Tris-HCl, pH 8.0, and 2.5 %(v/v) Triton X-100 containing: no inhibitor (lane 1), 2 mM 1, 10 phenanthroline (lane 2), 10 mM benzamidine hydrochloride (lane 3), 0.6 mM AEBSF (lane 4). This was followed by 16 h of processing in 50 mM Tris-HCl, pH 8.0, containing: no inhibitor (lane 1), 2 mM 1, 10 phenanthroline (lane 2), 10 mM benzamidine hydrochloride (lane 3), 0.6 mM AEBSF (lane 4). Lane 5 contained molecular weight markers (Sigma Chem. Co.).

Lane 2 shows the total inhibition of the 55 kDa gelatinase by 2 mM 1, 10-phenanthroline, a largely specific Zn^{2+} chelator, suggesting that this protease requires Zn^{2+} for its activity. This indicates that the 55 kDa species might be a MMP since many vertebrate MMPs are known to be Zn^{2+} dependant. In the presence of 10 mM benzamidine hydrochloride, the reversible serine protease

75

Figure 13. Isolation of the 41 kDa gelatin-cleavage activity using a 1.5 M-Agarose gel filtration column.

Isolated hyaline layers were incubated at 37°C in millipore-filtered seawater for 24 h to induce the gelatin-cleavage activities. This was followed by 24 h incubation at 37°C in 50 mM Tris-HCl pH 8.0 containing 5 mM EDTA to displace all those major gelatin-cleavage activities. The displaced supernatant was loaded on a 1.5 M-Agarose (Bio-Rad) column equilibrated in 50 mM Tris-HCl pH 8.0 and 5 μg of elutions No.12 (lane 1), No.15 (lane 2), No.18 (lane 3), and No. 21 (lane 4) were analyzed in a gelatin substrate gel.
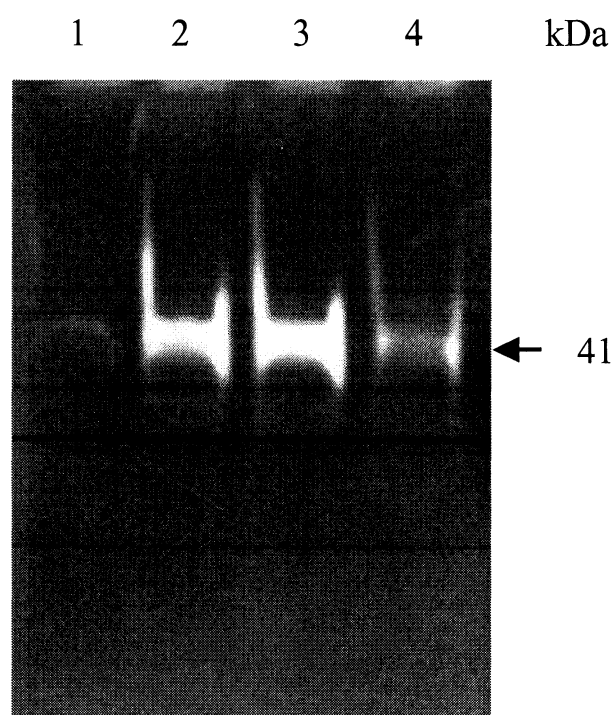
Figure 14. Determination of the effects of various protease inhibitors on the 55 kDa gelatin-cleavage activity.

Aliquots (15 μL) of the purified 55 kDa gelatin-cleavage activity were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin. After electrophoresis, the gel was sliced into pieces and processed overnight in 50 mM Tris-HCl pH 8.0 (lane 1), 50 mM Tris-HCl pH 8.0 and 2 mM 1,10-phenanthroline (lane 2), 50 mM Tris-HCl pH 8.0 and 10 mM benzamidine hydrochloride (lane 3), 50 mM Tris-HCl pH 8.0 and 0.6 mM [4-(2-aminoethyl) benzenesulfonylfluoride] (lane 4).
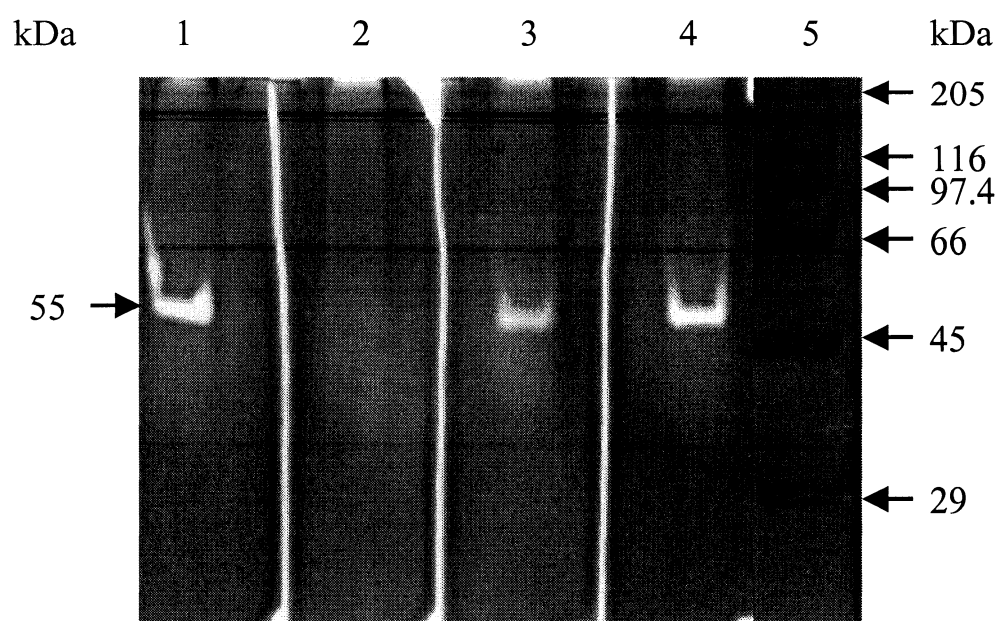
inhibitor, the 55 kDa specie was inhibited to some extent, whereas no inhibition was noticed in the presence of 0.6 mM AEBSF, the irreversible serine protease inhibitor. The above data demonstrate that the 55 kDa gelatinase was totally inhibited by 1, 10-phenanthroline, partial inhibition with benzamidine hydrochloride and no inhibition with AEBSF.

3.15 Quantitative analysis of the inhibition of the 55 kDa gelatin-cleavage activity by 1, 10 phenanthroline, a largely specific Zn^{2+} chelator.

Equal amounts (25 μL) of the purified 55 kDa gelatinase was added to the reaction buffer containing 50 mM Tris-HCl, pH 8.0, in the presence or absence of 2 mM 1,10 phenanthroline. In addition to the above samples, appropriate blanks were also monitored. This reaction mixture was incubated at 37°C for 15 min before adding 10μg of fluorescein-conjugate gelatin. The increase in fluorescence was monitored every 2 min for 1 h at 37°C. The sample containing the 55 kDa gelatinase in the absence of the zinc chelator, 1, 10 phenanthroline is the positive control for this experiment. The rate of change in relative fluorescence unit (RFU/sec) in the presence of 1, 10 phenanthroline (Δ-Δ) was calculated to be 0.251, whereas the rate for the sample containing 1, 10-phenanthroline (◊-◊) was measured as 0.002 (Fig. 15). The percent inhibition of the 55 kDa gelatinase in the presence of 1, 10 phenanthroline was determined to be 99.2% in this experiment. In the first 3 readings, an increase in the fluorescence is observed in the reaction mixture containing both the 55 kDa species and 1, 10-phenanthroline (◊-◊). This initial "bump" is observed

Figure 15. Determination of the effect of phenanthroline on the 55 kDa gelatin-cleavage activity using the fluorescence gelatinase assay.

Aliquots (25 μL) of the purified 55 kDa activity were preincubated in the absence of 1,10-phenanthroline (Δ-Δ) or in the presence 10 mM of 1,10-phenanthroline (◊-◊) for 15 min, after which the fluorescence was read to calculate the enzyme activity. The blanks were also read in the absence (o-o) and presence of 1,10-phenanthroline (□-□). The enzyme activity was measured by the increase in relative fluorescence unit (RFU) per second.
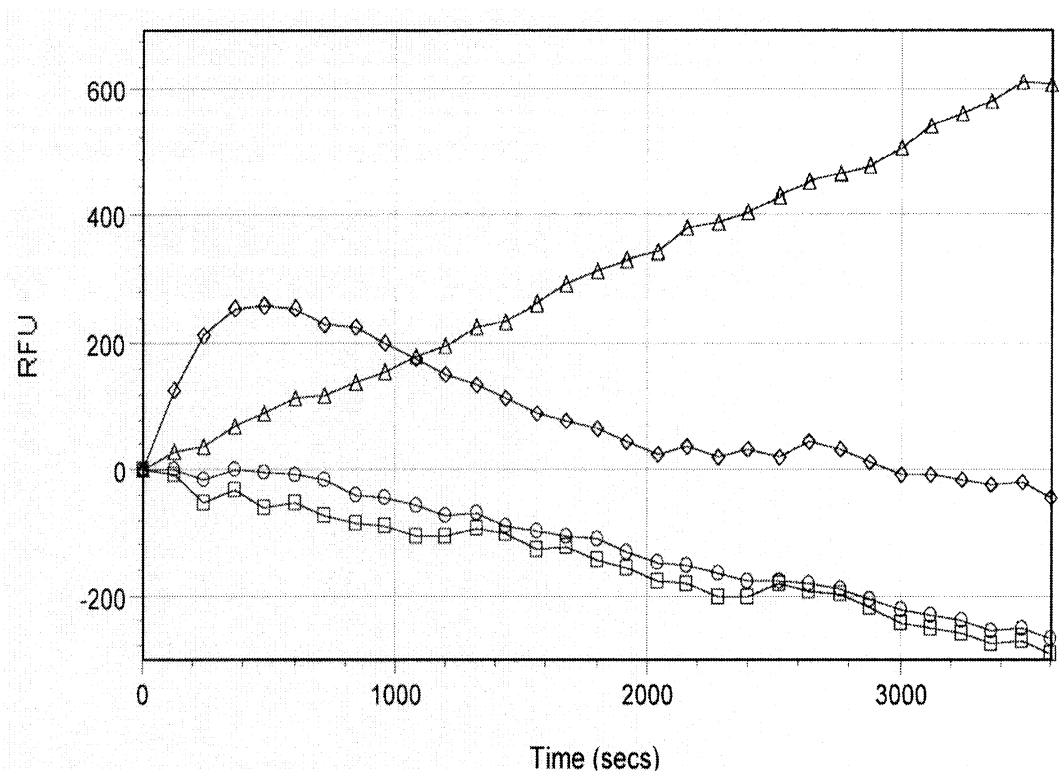
occasionally and could not be explained. Hence, the rate of change in (RFU/sec) was calculated excluding the first three readings. The negative slopes observed in both the blanks (o-o) and (□-□) were corrected by adding those values to their corresponding samples (Δ-Δ) and ((-() respectively. The fluorescence assay is used to quantify gelatinase activity as well as to confirm the results obtained from the substrate gel zymography. The amount of protein used in the fluorescence assay was less than 1 μg.

3.16 The effect of 1, 10 phenanthroline or benzamidine hydrochloride on the 55 kDa gelatinase species using the fluorescence assay.

Aliquots (25 μL) of the purified 55 kDa species were preincubated in the presence of 2 mM 1,10 phenanthroline or 10 mM benzamidine hydrochloride (Table 1). The 55 kDa activity preincubated in the absence of the inhibitors served as a control for this assay, whose gelatin-cleavage activity was normalized to 100%. The largely specific Zn^{2+} chelator, 1, 10 phenanthroline inhibited the 55 kDa activity by 93.1±11.9%, while the reversible serine protease inhibitor, benzamidine hydrochloride inhibited 4.1±1.4% (n=3). These data confirmed that the 55 kDa gelatinase required Zn^{2+} for its enzymatic activity. The above results also suggest that the 55 kDa is not a serine proteinase, since the inhibition observed in the presence of 10 mM bezamidine hydrochloride was negligible. Similar results were obtained from the substrate gel zymography.

Table 1. Quantitative analysis of the effect of the protease inhibitors on the 55 kDa gelatin-cleavage activity.

Aliquots (25 μL) of the purified 55 kDa species were preincubated in the presence of 2 mM 1,10 phenanthroline or 10 mM benzamidine hydrochloride and the fluorescence was monitored. The specific Zn^{2+} chelator, phenanthroline inhibited the 55 kDa activity by 93.1±11.9%, while the reversible serine protease inhibitor, benzamidine hydrochloride inhibited 4.1±1.4% (n=3).

Exp. No	2 mM Phenanthroline	10 mM Benzamidine	Inhibition (%)
1	-	-	0
	[+]	-	93.1±11.9
2	-	-	0
	-	[+]	4.1±1.4

3.17 Determination of the effects of EDTA and EGTA on the major gelatin–cleavage activities.

Gelatin-cleavage activities were induced by incubating layers in MFSW for 24 h at 37°C and those activities were dissociated from the layers by incubating in 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA for 24 h at 37°C. Aliquots (15 µg) of the supernatant containing gelatin-cleavage activities were fractionated in a gelatin substrate gel (Fig. 16). Following electrophoresis, the gel was sliced into strips and these strips were processed for 1 h in 50 mM Tris-HCl, pH 8.0, and 2.5% (v/v) Triton X-100 containing: no chelator (lane 1), 5 mM EDTA (lane 2), or 5 mM EGTA (lane 3). This was followed by 16 h processing in 50 mM Tris-HCl, pH 8.0, containing: no chelators (lane 1), 5 mM EDTA (lane 2), or 5 mM EGTA (lane 3).

In Fig. 16, both 5 mM EDTA and 5 mM EGTA inhibited the 55- and 41 kDa gelatinase activities. The EDTA inhibition suggests a divalent cation requirement for both the 55- and 41 kDa gelatinases since the EDTA is a divalent cation chelator, but the EGTA inhibition of the 55- and 41 kDa indicates that Ca^{2+} might be the divalent cation required for the activity of the 55- and 41 kDa since EGTA is a specific Ca^{2+} chelator.

Figure 16. The effects of EDTA and EGTA on the 55- and 41 kDa gelatin-cleavage activities.

Aliquots (15 µg) of displaced gelatin-cleavage activities were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin. After electrophoresis, the gel was sliced into pieces and processed overnight in 50 mM Tris-HCl pH 8.0 (lane 1), 50 mM Tris-HCl pH 8.0 and 5 mM ethylenediaminetetraacetic acid (lane 2), and 50 mM Tris-HCl pH 8.0 and 5 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (lane 3).
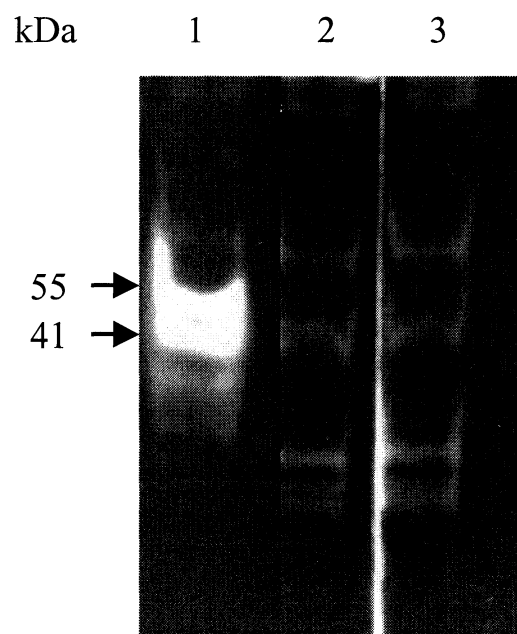
3.18 The divalent cation requirement for the reactivation of the EGTA-inhibited 55- and 41 kDa gelatin-cleavage activities.

We incubated freshly prepared layers, stored at $-70^{\circ}C$, for 24 h in MFSW at $37^{\circ}C$ to induced the gelatin-cleavage activities. The induced layers were incubated in 50 mM Tris-HCl, pH 8.0 containing 5 mM EDTA for 24 h at $37^{\circ}C$ to displace those activities from the layers. Aliquots (15 µg) of the displaced gelatinase activities were fractionated in a gelatin substrate gel (Fig. 17).

After electrophoresis, the gel was cut into strips and the strips were processed for 2 h in 50 mM Tris-HCl, pH 8.0, and 2.5%(v/v) Triton X-100 containing: no chelator (lane 1), 5 mM EGTA (lanes 2, 3, 4, 5 and 6). This was followed by 16 h of processing in 50 mM Tris-HCl, pH 8.0, containing: none (lanes 1 and 2), 100 µM $CaCl_2$ (lane 3), 150 µM $CaCl_2$ (lane 4), 200 µM $CaCl_2$ (lane 5), or 250 µM $CaCl_2$ (lane 6).

This experiment was performed to examine whether $CaCl_2$ could reactivate the EGTA-inhibited 55- and 41 kDa gelatinase activities. The 55- and 41 kDa activities processed in the absence of exogenously added $CaCl_2$ served as a positive control for this experiment (lane 1). Remaining gel strips were processed in the presence of 5 mM EGTA for 2 h instead of the usual 1 h, in order to completely inhibit the 55- and 41 kDa gelatinase activities. After the 2 h processing, the EGTA was discarded and the gel strips were thoroughly washed in deionized water to remove the remaining EGTA present in the gel. The 55- and 41 kDa activities processed in the presence of EGTA and in the absence of

Figure 17. Reactivation of the EGTA inhibited-55 kDa gelatin-cleavage activity by calcium chloride.

Equal amounts (15 μg) of displaced gelatin-cleavage activities were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% gelatin as the substrate. After four h of electrophoresis, the gel was sliced into pieces and processed for two h in 50 mM Tris-HCl pH 8.0 (lane 1), 50 mM Tris-HCl pH 8.0 and 5 mM EGTA (lane 2, 3, 4, 5, 6), which was followed by 16 h of processing in the absence of exogenously added $CaCl_2$ (lanes 1 and 2), 100 μM $CaCl_2$ (lane 3), 150 μM $CaCl_2$ (lane 4), 200 μM $CaCl_2$ (lane 5), 250 μM $CaCl_2$ (lane 6).
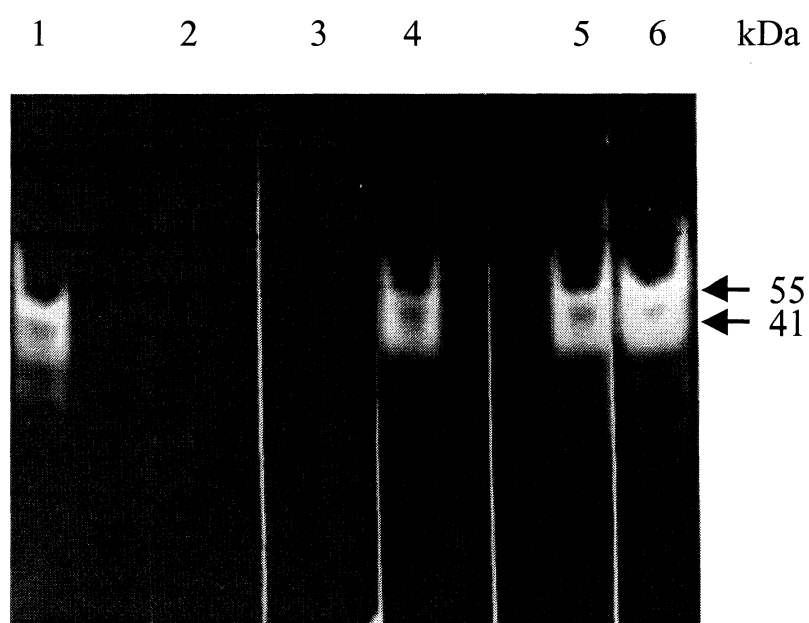
exogenously added $CaCl_2$ served as a negative control for this experiment (lane 2). Reactivation of the EGTA-inhibited 55- and 41 kDa was observed in the gel strips processed in the presence of 150 μM, 200 μM and 250 μM $CaCl_2$ (lanes 4, 5 and 6 respectively) but not in 100μM $CaCl_2$ (lane 3). This result clearly shows that the EGTA-inhibited 55- and 41 kDa gelatin-cleavage activities could be reactivated by a minimum of 150 μM $CaCl_2$.

3.19 Quantitative analysis of calcium concentration dependence of the EGTA-inhibited 55 kDa gelatin-cleavage activity.

Equal amounts (25 μL) of the purified 55 kDa gelatinase were preincubated in 5 mM EGTA at 4°C. After the incubation, the EGTA was removed by dialyzing the samples in 500 mL of 50 mM Tris-HCl, pH 8.0 at room temperature for 1 h. The 55 kDa was completely inhibited by 5 mM EGTA. These aliquots were incubated for 15 min at 37°C with increasing concentrations of calcium chloride (0, 100 μM, 250 μM, 500 μM, 1 mM, 5mM and 10 mM). Finally 10 μg of fluorescein-conjugate gelatin was added and the fluorescence was monitored for 1 h at 37°C at 2 min intervals (Fig. 18). The 55 kDa activity assayed in the presence of 10 mM $CaCl_2$ was normalized to 100% in this experiment. The apparent dissociation constant for calcium reactivation of the EGTA-inhibited 55 kDa was calculated to be 1.2 mM (Fig. 18).

Figure 18. Calcium concentration dependence of reactivation of the EGTA-inhibited 55 kDa gelatinase activity using fluorescence assay.

Aliquots (20 μg) of the purified 55 kDa were incubated in the presence of 5 mM EGTA, which completely inhibited the 55 kDa activity. The EGTA was removed from the reaction mixture by dialysis before the addition of assay buffer containing various concentrations of calcium (0, 100 μM, 250 μM, 500 μM, 1 mM, 5mM and 10 mM). Non-linear regression curve-fitting analysis was performed using *Graphpad Prism*, V. 3.0.
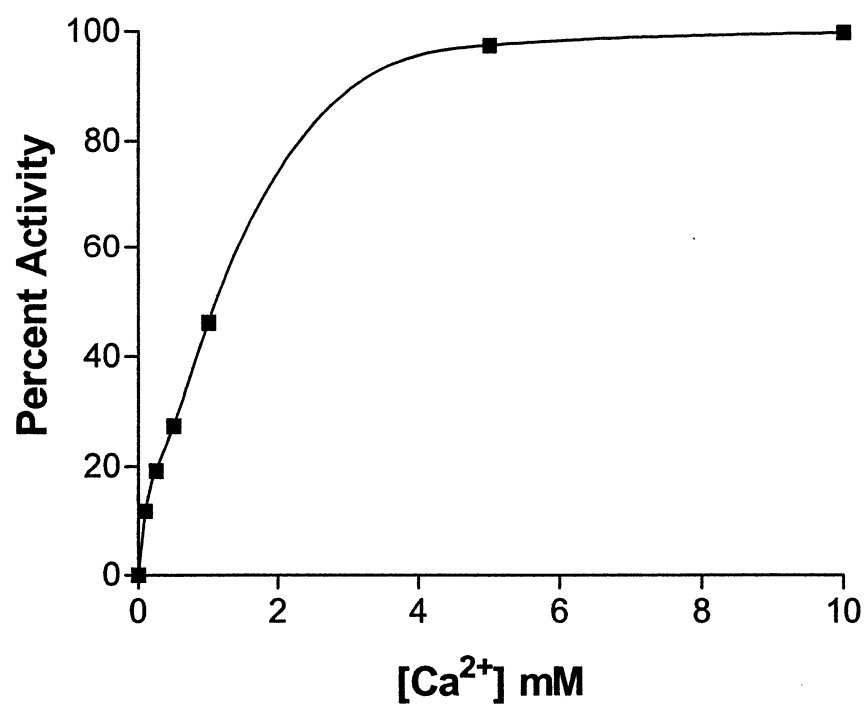
3.20 Identification of the calcium ion requirement of the 32 kDa gelatin-cleavage activity.

Isolated layers were incubated in MFSW for 24 h at 37°C to induce the gelatin-cleavage activities and the activities were dissociated from the layers on incubation in 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA for 24 h at 37°C. Equal amounts (15 μg) of the displaced gelatinases were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin as the substrate (Fig. 19). Following electrophoresis, the gel was sliced into strips and these strips were processed for 2 h in 50 mM Tris-HCl, pH 8.0, and 2.5%(v/v) Triton X-100 containing: no chelator (lane 1), 5 mM EDTA (lane 2), 5 mM EGTA (lanes 3, 4 and 5). This was followed by 16 h processing in 50 mM Tris-HCl, pH 8.0, containing: none (lanes 1, 2 and 3), 1 mM $CaCl_2$ (lane 4) or 1 mM $MgCl_2$ (lane 5).

The purpose of this experiment was to determine the effects of EDTA and EGTA on the 32 kDa gelatinase activity. The 32 kDa activity was totally inhibited by both the EDTA (lane 2) and EGTA (lane 3), which had similar inhibitory effect on the 55- and 41 kDa gelatin-cleavage activities. The EGTA-inhibited 32 kDa activity was reactivated by the addition of 1 mM $CaCl_2$ (lane 4). The addition of 1 mM $MgCl_2$ to the EGTA-inhibited 32 kDa gelatinase resulted in very little reactivation (lane 5). The data obtained from this experiment indicate that both EDTA and EGTA inhibited the three major gelatin-cleavage activities at 55-, 41- and 32 kDa. In addition, gelatin-cleavage activities at 200-, 94-, 66-, and 28 kDa

Figure 19. Reactivation of the EGTA-inhibited 32 kDa gelatinase by CaCl$_2$ or MgCl$_2$.

Aliquots (15 µg) of gelatin-cleavage activities dissociated from the hyaline layers were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin. Following electrophoresis, the gel was sliced into pieces in 50 mM Tris-HCl pH 8.0 (lane 1), 50 mM Tris-HCl pH 8.0 and 5 mM EDTA (lane 2), 50 mM Tris-HCl pH 8.0 and 5 mM EGTA acid (lane 3), 50 mM Tris-HCl pH 8.0, 5 mM EGTA and 1 mM CaCl$_2$ (lane 4) and 50 mM Tris-HCl pH 8.0, 5 mM EGTA and 1 mM MgCl$_2$ (lane 5).
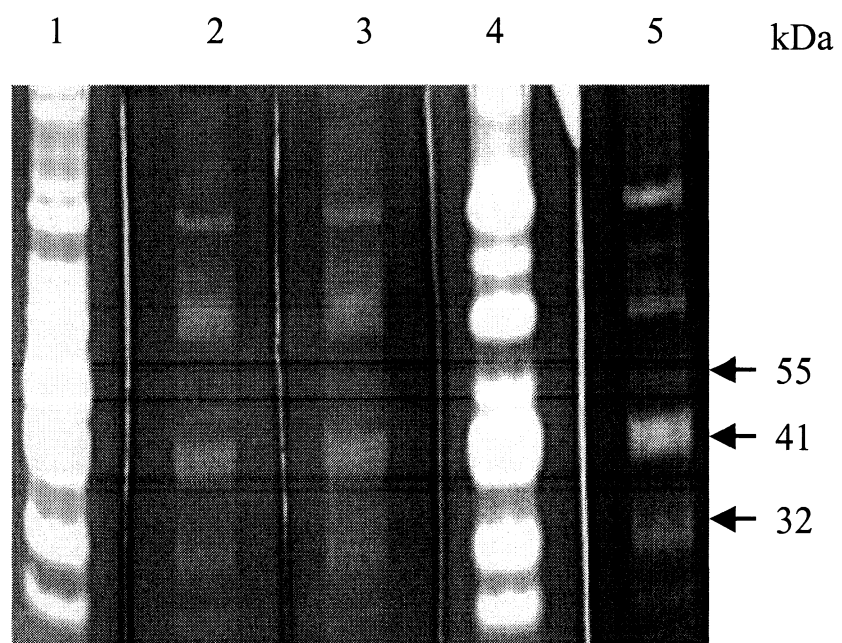
were also inhibited by EDTA and EGTA. Calcium chloride reactivated the EGTA-inhibited gelatinases and only a partial or faint reactivation was observed by $MgCl_2$ reactivation. This clearly shows the Ca^{2+} ion requirement of these species for their enzymatic activity, which is a characteristic of the vertebrate matrix metalloproteinase group of ECM remodeling enzymes.

3.21 Reactivation of the EGTA-inhibited 55 kDa species in the presence of $CaCl_2$ or $MgCl_2$.

Aliquots (25 μL) of the purified 55 kDa activity were preincubated in 5 mM EGTA for two weeks at 4°C. Following 24 h incubation, the 55 kDa activity was inhibited only 33% in the presence of 5 mM EGTA. In order to inhibit the 55 kDa activity completely, aliquots of the purified 55 kDa were incubated under the same condition for two weeks. The percent inhibition of the 55 kDa activity in the presence of 5 mM EGTA still remained at 33%. After incubation, the EGTA was removed by dialyzing the samples in 500 mL of 50 mM Tris-HCl, pH 8.0 at room temperature for 1 h. Following dialysis, the samples were incubated in 10 mM $CaCl_2$ or 50 mM $MgCl_2$ for 15 min at 37°C (Table 2). The fluorescence was read after adding 10 μg of fluorescein conjugate gelatin. The EGTA-inhibited 55 kDa was reactivated 58.1±6.95% by 10 mM $CaCl_2$, which completely recovered the initial activity of the 55 kDa (n=2). However, EGTA-inhibited 55 kDa was reactivated 28.18±2.72% in the presence of 50 mM $MgCl_2$, which recovered 85%

98

Table 2. Quantitative analysis of the reactivation of the EGTA-inhibited 55 kDa gelatin-cleavage activity with 10 mM CaCl$_2$ or 50 mM MgCl$_2$.

Aliquots (25 μL) of the EGTA-inhibited 55 kDa activity were incubated in 10 mM CaCl$_2$ or 50 mM MgCl$_2$ for 15 min at 37°C. The fluorescence was read after the addition of 10 μg of fluorescein conjugate gelatin. The EGTA-inhibited 55 kDa was reactivated 58.1±6.95% by 10 mM CaCl$_2$ and 28.18±2.72 by 50 mM MgCl$_2$ (n=2).

Exp. No	10 mM CaCl$_2$	50 mM MgCl$_2$	Activation (%)
1	-	-	0
	[+]	-	58.1 ± 6.95
2	-	-	0
	-	[+]	28.18 ± 2.72

of the total activity of the 55 kDa species (n=2). This result was consistent with the data obtained from substrate gel zymography (Fig. 19).

3.22 Determination of the effect of 500 mM NaCl on the 55 kDa activity using the fluorescence assay.

Aliquots (25 μL) of the purified 55 kDa activity were preincubated in the presence and absence of 500 mM NaCl for two h on ice (Table 3). The gelatin-cleavage activity of the 55 kDa was inhibited $57.64\pm5.8\%$ in the presence of 500 mM NaCl (n=3).

To rule out the possibility that this inhibition could be due to the salting out effect the following procedure was done. After preincubation with 500 mM NaCl for 2 h the reaction mixture was centrifuged for 1 h in an airfuge and the pellet and supernatant were examined for gelatin-cleavage activity. The gelatin-cleavage activity of the 55 kDa species incubated in the absence of 500 mM NaCl was normalized to 100%. The pellet contained $30.33\pm12.3\%$ and the supernatant had $69.67\pm12.3\%$ activity, but both this activity accounted only for 42.36% of the total activity of the control. This confirmed that the 500 mM NaCl inhibition was not due to salting out. This NaCl-dependent inhibition suggests a possible mechanism for regulating the 55 kDa gelatinase activity.

101

Table 3. Quantitative analysis of the effect of the 500 mM NaCl on the purified 55 kDa gelatin-cleavage activity.

Aliquots (25 µL) of the purified 55 kDa activity were preincubated in the presence or absence of 500 mM NaCl for 2 h on ice. The gelatin-cleavage activity of the 55 kDa was inhibited $57.64\pm5.8\%$ in the presence of 500 mM NaCl (n=3). To exclude the possibility that the NaCl-dependent inhibition is not due to salting out effect, the reaction mixture containing the 55 kDa species and 500 mM NaCl was centrifuged for 1 h in an airfuge and the pellet and supernatant were examined for gelatin-cleavage activity. The pellet fractions contained $30.33\pm12.3\%$ gelatin-cleavage activity and the supernatant had $69.67\pm12.3\%$ activity. However, both this activity accounted only for 42.36% of the total activity.

Exp. No	50 mM Tris-HCl pH 8.0	500 mM NaCl	Activity in Pellet (%)	Activity in Supernatant (%)	Inhibition (%)
1	[+]	[-]	-	-	0
	[+]	[+]	30.33±12.3	69.67±12.3	57.64±5.8

3.23 Determination of the effects of the CaCl₂, MgCl₂ and NaCl on the major gelatin-cleavage activities at 55-, 41-, and 32 kDa.

The sea urchin lives in the sea water that contains salts in high concentrations namely: 10 mM $CaCl_2$, 50 mM $MgCl_2$ and 500 mM NaCl. Hence, we decided to determine the effects of these salts on the three gelatin-cleavage activities at 55-, 41-, and 32 kDa.

Freshly prepared layers stored at -70°C were incubated in MFSW for 24 h at 37°C to induce the gelatin-cleavage activities, which was followed by the dissociation of those gelatinase activities from the layers by incubation in 50 mM Tris-HCl, pH 8.0, containing 5 mM EDTA for 24 h at 37°C. Aliquots (15 µg) of the displaced gelatin-cleavage activities were fractionated in a gelatin substrate gel (Fig. 20). After electrophoresis, the gel was sliced into strips and all these strips were processed for 1 h in 50 mM Tris-HCl, pH 8.0, and 2.5%(v/v) Triton X-100. This was followed by 16 h processing in 50 mM Tris-HCl, pH 8.0, containing: none (lanes 1), 10 mM $CaCl_2$ (lane 2), 50 mM $MgCl_2$ (lane 3), 500 mM NaCl (lane 4) or 10 mM $CaCl_2$, 50 mM $MgCl_2$ and 500 mM NaCl (lane 3).

Gelatin-cleavage activities processed in the absence of exogenously added calcium chloride served as a control in this experiment (lane 1). The addition of 10 mM exogenous $CaCl_2$ activated the enzymatic activity of the three gelatin-cleavage activities at 55-, 41- and 32 kDa (lane 2). Both 50 mM $MgCl_2$ (lane 3) and 500 mM NaCl (lane 4) had no effect on the three major activities. However, the effect of all the above mentioned salts together seemed to activate

Figure 20. The effects of 10 mM $CaCl_2$, 50 mM $MgCl_2$ and 500 mM NaCl on the three major gelatin-cleavage activities.

Aliquots (15 μg) of displaced gelatin-cleavage activities were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin. After electrophoresis, the gel was sliced into pieces and processed overnight in 50 mM Tris-HCl pH 8.0 (lane 1), 50 mM Tris-HCl pH 8.0 and 10 mM $CaCl_2$ (lane 2), 50 mM Tris-HCl pH 8.0 and 50 mM $CaCl_2$ (lane 3), 50 mM Tris-HCl pH 8.0 and 500 mM NaCl (lane 4) and 50 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$, 50 mM $MgCl_2$ and 500 mM NaCl (lane 5).

106

the 55-, 41- and 32 kDa gelatinase activities (lane 5). In contrast, the results obtained from the quantitative fluorescence assay (Table 2 and 3) show that 50 mM MgCl$_2$ stimulates the 55 kDa species, while the 500 mM NaCl has an inhibitory effect on the 55 kDa activity. The inhibitory effect of 500 mM NaCl could not be visualized in the substrate zymogen gel, due to overloading of gelatinases (Fig. 20). Since, the fluorescence assay is a more accurate technique, the results obtained from this method are reliable.

3.24 The effects of various protease inhibitors on the 41- and 32 kDa gelatinase activities.

Isolated layers stored at −70°C were incubated in MFSW at 37°C for 24 h to induce the gelatin-cleavage activities. The induction was followed by the dissociation of those gelatinase activities from the layers in by incubating in 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA for 24 h at 37°C. Equal amounts (15 μg) of the dissociated gelatin-cleavage activities were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin (Fig. 21). After electrophoresis, the gel was cut into strips and these strips were processed in 50 mM Tris-HCl, pH 8.0, and 2.5 %(v/v) Triton X-100 containing: no inhibitor (lane 1), 2 mM 1, 10 phenanthroline (lane 2), 10 mM benzamidine hydrochloride (lane 3), 0.6 mM AEBSF (lane 4). This was followed by 16 h of processing in 50 mM Tris-HCl, pH 8.0, containing: none (lane 1), 2 mM 1, 10 phenanthroline (lane 2),

107

Figure 21. Determination of the effects of various protease inhibitors on the 41- and 32 kDa gelatin-cleavage activities.

Aliquots (15 µg) of the induced gelatin-cleavage activities were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin. Following electrophoresis, the gel was sliced into pieces and processed overnight in 50 mM Tris-HCl pH 8.0 (lane 1), 50 mM Tris-HCl pH 8.0 and 2 mM 1,10-phenanthroline (lane 2), 50 mM Tris-HCl pH 8.0 and 10 mM benzamidine hydrochloride (lane 3), 50 mM Tris-HCl pH 8.0 and 0.6 mM [4-(2-aminoethyl) benzenesulfonylfluoride] (lane 4). Lane 5 contained molecular weight markers.

the 55-, 41- and 32 kDa gelatinase activities (lane 5). In contrast, the results obtained from the quantitative fluorescence assay (Table 2 and 3) show that 50 mM MgCl$_2$ stimulates the 55 kDa species, while the 500 mM NaCl has an inhibitory effect on the 55 kDa activity. The inhibitory effect of 500 mM NaCl could not be visualized in the substrate zymogen gel, due to overloading of gelatinases (Fig. 20). Since, the fluorescence assay is a more accurate technique, the results obtained from this method are reliable.

3.24 The effects of various protease inhibitors on the 41- and 32 kDa gelatinase activities.

Isolated layers stored at -70°C were incubated in MFSW at 37°C for 24 h to induce the gelatin-cleavage activities. The induction was followed by the dissociation of those gelatinase activities from the layers in by incubating in 50 mM Tris-HCl, pH 8.0, and 5 mM EDTA for 24 h at 37°C. Equal amounts (15 µg) of the dissociated gelatin-cleavage activities were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin (Fig. 21). After electrophoresis, the gel was cut into strips and these strips were processed in 50 mM Tris-HCl, pH 8.0, and 2.5 %(v/v) Triton X-100 containing: no inhibitor (lane 1), 2 mM 1, 10 phenanthroline (lane 2), 10 mM benzamidine hydrochloride (lane 3), 0.6 mM AEBSF (lane 4). This was followed by 16 h of processing in 50 mM Tris-HCl, pH 8.0, containing: none (lane 1), 2 mM 1, 10 phenanthroline (lane 2),

110

Figure 22. Determination of the substrate specificity of the 55-, 41- and 32 kDa gelatin-cleavage activities.

Equal amounts (15 µg) of the gelatin-cleavage activities displaced from hyaline layers were fractionated in gels containing 0.1% (w/v) gelatin (lane 1), 0.1% (w/v) casein (lane 2), 0.1% (w/v) bovine serum albumin (lane 3) or 0.1% (w/v) bovine hemoglobin (lane 4). All gels were processed in the presence of 10 mM $CaCl_2$.
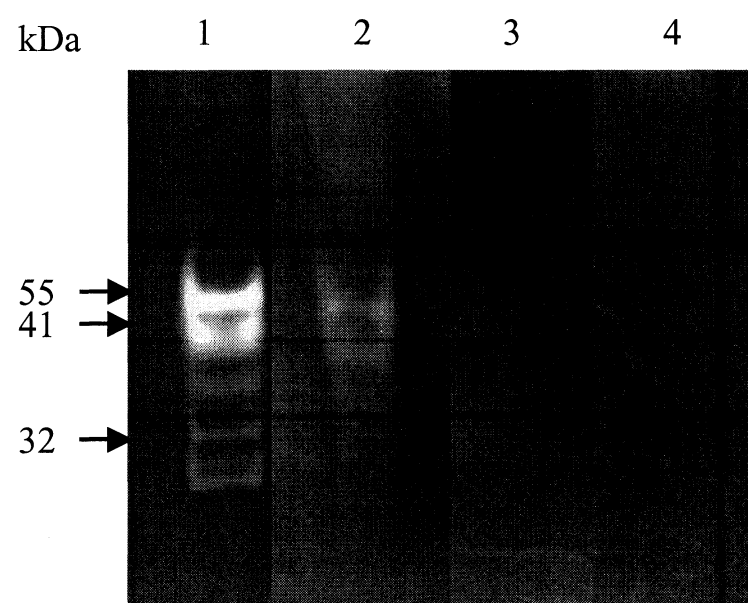
casein (lane 2), 0.1% (w/v) bovine serum albumin (lane 3), or 0.1% (w/v) bovine hemoglobin (lane 4) as their substrates (Fig. 22). The purpose of this experiment was to rule out the possibility that the gelatin-cleavage activities that were visualized in the gelatin substrate gels were not coincidental gelatin-cleavage activities caused by nonspecific endopeptidases.

The data shows the highest cleavage activity in the gelatin substrate gel (lane 1) and faint activities in casein substrate gel (lane 2). No cleavage activity was detected when bovine serum albumin (lane 3) and bovine hemoglobin (lane 4) were used as substrates. This result clearly demonstrates gelatin to be the preferred substrate of the 55-, 41- and 32 kDa gelatin-cleavage activities.

3.26 Comparison of the comigration pattern of the embryos with the 55 kDa gelatin-cleavage activity.

This experiment was performed to prove that the activities induced in the isolated layers were present in the sea urchin embryo. Aliquots (15 μg) of embryos of various stages namely, 22 HPF (lane 1), 45 HPF (lane 2), 65 HPF (lanes 3 and 4), 93 HPF (lanes 5 and 6) and displaced gelatinase activities (lane 7) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin (Fig. 23). After electrophoresis, the gels were processed for an h in 50 mM Tris-HCl, pH 8.0, containing 2.5 %(v/v) Triton X-100 and 16 h in 50 mM Tris-HCl, pH 8.0, and 10 mM $CaCl_2$.

Figure 23. Comparative analysis of the comigration pattern of the gelatin-cleavage activities from various stage embryos with the 55 kDa gelatinase activity.

Aliquots (15 μg) of various stage embryos 22 HPF (lane 1), 45 HPF (lane 2), 69 HPF (lanes 3 and 4), 93 HPF (lanes 5 and 6) were resuspended in equal volume of solubilizing solution (Laemmli, 1970) from which both DTT and bromophenol blue had been omitted. The samples were left at room temperature for 30 min and were fractionated in a gelatin substrate gel along with induced gelatin-cleavage activities (lane 7).
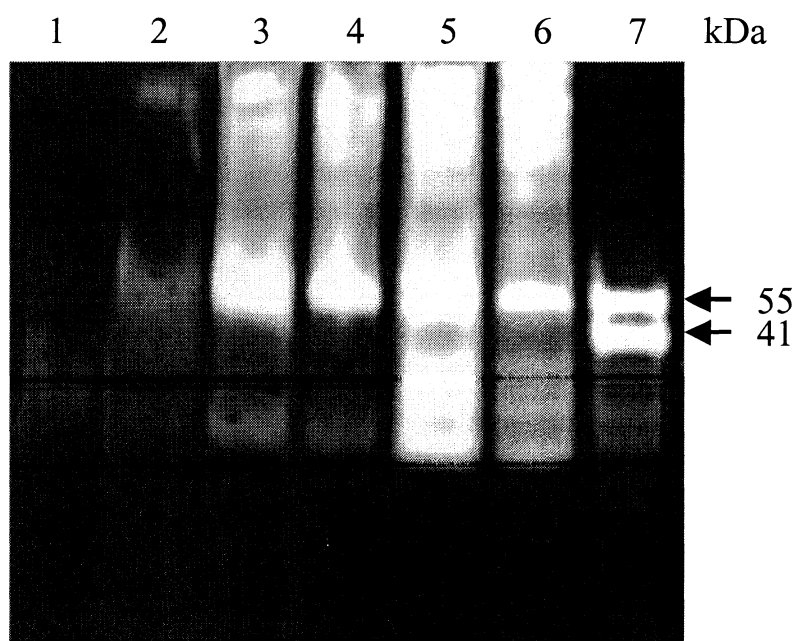
A gelatin-cleavage activity present in the embryo from the early gastrula stage (45 HPF, lane 2) to the pluteus stage embryo (93 HPF, lane 6) was found to comigrate with the 55 kDa gelatin-cleavage activity from the induced layers (lane 7). These data indicate both the gelatin-cleavage activity in the embryo and the 55 kDa gelatinase induced from the isolated layers could be the same proteolytic activity.

3.27 Comparing the properties of the 55 kDa gelatinase with a gelatin-cleavage activity from the 93 HPF embryos.

This experiment was carried out to check whether the gelatin-cleavage activity from 45 to 93 HPF embryos that comigrated with the 55 kDa activity, induced from the isolated layers possessed similar properties. Aliquots (15 µg) of gelatin-cleavage activities containing the 55 kDa species, induced in the layers (lanes 1, 4, 6 and 8) were fractionated along with aliquots of 93 HPF embryos (lanes 2, 5, 7 and 9) in a gelatin substrate gel (Fig 24). Following electrophoresis, the gel was sliced into four pieces and was processed for 2 h in 50 mM Tris-HCl, pH 8.0, and 2.5% Triton X-100 containing: none (lanes 1 and 2), 2 mM 1,10 phenanthroline (lanes 4 and 5), 5 mM EGTA (lanes 6, 7, 8 and 9). Then, the gels were processed overnight in 50 mM Tris-HCl, pH 8.0, containing: none (lanes 1, 2, 6 and 7), 2 mM 1,10 phenanthroline (lanes 4 and 5), 1 mM $CaCl_2$ (lanes 8 and 9).

116

Figure 24. Comparison of the characteristics of the 55 kDa gelatinase with a gelatin-cleavage activity found in the 93 hours post fertilization (HPF) embryos.

Aliquots (15 µg) of gelatin-cleavage activities containing the 55 kDa species, induced from the hyaline layers (lanes 1, 4, 6 and 8) were fractionated along with aliquots of 93 HPF embryos (lanes 2, 5, 7 and 9) in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) containing 0.1% (w/v) gelatin. After electrophoresis, the gel was sliced and processed in the absence of exogenous $CaCl_2$ (lanes 1 and 2), 10 mM 1,10-phenanthroline (lanes 4 and 5), and in 5 mM EGTA(lanes 6, 7, 8 and 9). Lanes 7 and 8 were processed overnight in the presence of 1 mM $CaCl_2$. Lane 3 contained molecular mass markers.
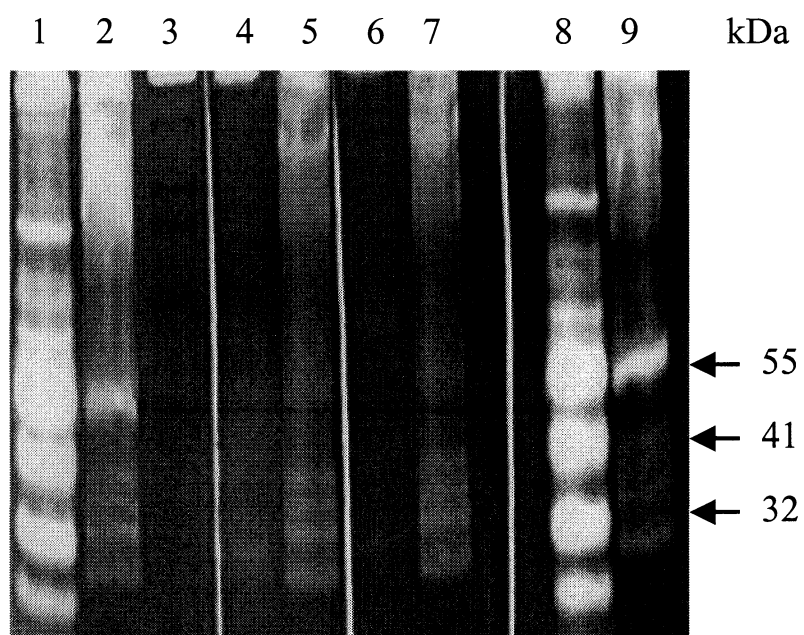
The data obtained from this experiment clearly show that both the 55 kDa species and the gelatinase from the 93 HPF embryos are inhibited by 2 mM 1,10-phenanthroline (lanes 4 and 5) and 5 mM EGTA (lanes 6 and 7). Both the gelatin-cleavage activities were reactivated by 1 mM $CaCl_2$ as shown in lanes 8 and 9. Hence, this experiment confirms that both the activities posses similar migration pattern and properties. This proves that the 55 kDa gelatin-cleavage activity induced from the isolated layers is present during the developmental stages of the sea urchin embryo especially from the early gastrula stage (45 HPF) to the pluteus stage (93 HPF).

CHAPTER 4: DISCUSSION

4.1　Role of the ECM components in sea urchin development

The ECM is a dynamic structure that fills the space between cells enabling cells to exchange information through cell-ECM interactions. These interactions are mediated by specific cell-surface receptors called integrins. The ECM composition changes throughout embryonic development. The components of ECM play a critical role in cell adhesion, migration, proliferation, and differentiation (Hay, 1991).

Wessel and McClay (1987) studied the effect of collagen disruption on sea urchin embryogenesis. *Lytechinus variegatus* embryos cultured in the presence of a lathyritic agent or proline analogs remained in the mesenchyme blastula stage and did not gastrulate. Once the collagen disruption agents were removed, these embryos continued their developmental course to reach the pluteus larval stage. Immunofluorescence studies have shown that the deposition of collagen types IV and I in the ECM are greatly reduced by a lathyritic agent, β-aminopropionitrile (BAPN). The presence of BAPN stops sea urchin gastrulation by inhibiting the intra- and intermolecular collagen crosslinking, but does not affect normal cellular functions such as respiration and protein synthesis. These results collectively suggest that ECM collagen is required for gastrulation and not for developmental stages prior to gastrulation. In addition to collagen disruption, inhibition of sulfated glycosaminoglycan synthesis or laminin expression arrests gastrulation in sea urchin embryos (Karp and Solursh, 1974). Inhibition of

collagen deposition also prevents sea urchin spiculogenesis (Mintz *et al.*, 1981; Blankenship and Benson, 1984).

ECM disruption has a profound influence over gene expression in the sea urchin embryo. The role of the sea urchin ECM in differential gene expression during development was studied u sing cDNA probes that were specific to cell types (Wessel *et al.*, 1989). The mRNA levels of various genes from *Strongylocentrotus purpuratus*, *Lytechinus variegatus*, and *Lytechinus pictus* were compared in embryos cultured both in the presence and absence of BAPN. The BAPN treated embryos had reduced levels of a 1.2 kb mRNA, a homologue of LpS1 from *L. variegatus*, which is a homologue of Spec1 - Spec2 genes in *S. purpuratus*. The Spec genes encode a calcium binding protein that is a member of the calmodulin superfamily (Ramachandran *et al.*, 1993). These Spec proteins are expressed only in aboral ectoderm.

Ramachandran *et al.* (1993) have demonstrated the importance of a growth factor mediated pathway for sea urchin gastrulation and spiculogenesis. The development of BAPN-treated *Lytechinus* embryos was restored by a human recombinant platelet derived growth factor-BB (PDGF-BB) and transforming growth factor- α (TGF-α). The critical role of the growth factors was confirmed using nonspecific antagonists of PDGF, which arrested both gastrulation and LpS1 mRNA accumulation. Similar results were obtained when synthetic peptides, possessing a heparan sulfate proteoglycan binding sequence to PDGF-B, were administered. Antibodies to PDGF-B and TGF-α inhibited *Lytechinus*

embryo gastrulation and spiculogenesis suggesting a requirement for PDGF-like and TGF-α like ligands during early development (Govindarajan et al., 1995).

4.2 Components of the sea urchin ECM, the hyaline layer

The sea urchin embryo is characterized by the presence of two extracellular matrices: the hyaline layer and the basal lamina. Following fertilization, the embryo is surrounded by the hyaline layer, an extraembryonic matrix located on the apical surface of the embryo (Matese et al., 1997). The hyaline layer is assembled on the surface of the zygote by exocytosis from various storage compartments present in the egg. Numerous proteins are exocytosed from five different compartments, namely: cortical granules, basal laminar vesicles, apical vesicles, echinonectin vesicles, and vesicles containing membrane bound cadherin. These proteins are released and assembled in a sequential manner to form hyaline layer (Alliegro and McClay, 1988; Alliegro et al., 1988).

The major component of the hyaline layer is the protein hyalin. Hyalin is a 350 kDa glycoprotein that is capable of polymerization in the presence of calcium (Robinson, 1988; Robinson et al., 1992). Another protein component, HLC-175 was found localized to the hyaline layer using indirect immunofluorescence (Robinson, 1995). A large multidomain protein, Endo16 is present in both the hyaline layer and on the surface of endodermal cells. Endo16 is protected from proteolytic degradation when it is bound to calcium (Soltysik-Espanola et al.,

1994). HLC-32 is a 32 kDa protein present in the cytoplasm of the unfertilized egg and is released to both the hyaline layer and the basal lamina after fertilization (Brennan and Robinson, 1994).

4.3 Induction of gelatin-cleavage activities in isolated hyaline layers

The hyaline layer was isolated from 1-h-old sea urchin embryos as described in the Materials and Methods section. Freshly isolated layers stored at –70°C, were devoid of gelatin-cleavage activities. A dynamic pattern of activities appeared when the layers were incubated in MFSW at 15°C or 37°C. To rule out the possibility t hat these a ctivities d erived from microbial c ontamination o f s ea water, the sea water was both UV-irradiated and millipore-filtered.

Three major gelatin-cleavage activities at 55-, 41- and 32 kDa emerged after incubation in MFSW at both 15°C, the ambient temperature for *Strongylocentrotus purpuratus* and at 37°C. The pattern of gelatin-cleavage activities induced in the layers was similar at both 15°C and 37°C. However, differences w ere o bserved i n t he temporal sequence o f the i nduction. A t 1 5°C induction, the 32 kDa appeared after 24 h (Fig. 1, lane 1), followed by the 41 kDa species between 24 and 36 h of incubation (Fig.1, lane 2); the 55 kDa appeared after 96 h of incubation (Fig.1, lane 7 and 8). In contrast, at 37°C both the 55- and 41 kDa species appeared between 7.5 and 10 h, while the 32 kDa gelatinase emerged as a minor activity after 15 h of incubation (Fig. 4). Based on the above results, the layers were incubated in MFSW for 96 h at 15°C or 24 h at 37°C in

order to induce all three major gelatin-cleavage activities. Robinson *et al.*, (2002) have identified two gelatin-cleavage activities of apparent molecular weight 40- and 50 k that were induced in isolated layers stored at 4°C for 4 days in MFSW containing 10 mM benzamidine hydrochloride.

The induced gelatin-cleavage activities were absent when the layers were incubated in MFSW containing 10 mM benzamidine hydrochloride, a reversible serine proteinase inhibitor (Fig. 3). This result suggests a requirement for serine proteinase activity during the induction process. Studies have shown that vertebrate MMPs are secreted as inactive zymogens, which are proteolytically processed to active forms. Plasmin, a serine proteinase cleaves the N-terminal propeptide domains of proMMP-2, proMMP-9, and proMMP-12 to form active MMPs (Mazzieri *et al.*, 1997; Carmeliet *et al.*, 19997). Thrombin is also a serine proteinase, which has been implicated in progelatinase A (proMMP-2) activation in vascular endothelial cells (Zucker *et al.*, 1998).

We have extended our analysis of the induction process by determining the minimum ionic requirements for the induction of all three major gelatin-cleavage at 15°C. The hyaline layer is in contact with sea water, which contains 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 500 mM NaCl. Hence, the isolated layers were incubated under various ionic conditions at 15°C for 96 h. (Fig. 7). The data obtained from this experiment suggested that both the 55- and 41 kDa species require only NaCl for their induction (Fig. 7, lane 4). However, the 32 kDa species required both $CaCl_2$ and NaCl for its induction (Fig. 7, lane 6). The minimum

sodium chloride concentration required for induction of the 55- and 41 kDa species was determined to be 300 mM NaCl (Fig. 8), while the 32 kDa species required both 3 mM $CaCl_2$ and 200 mM NaCl (Figs. 9 and 10).

4.4   Dissociation of induced gelatin-cleavage activities from isolated hyaline layers

Previous studies from our lab have shown the presence of two structural domains in the isolated hyaline layers. The first domain is mainly composed of hyaline, the major component of the layer, while the second domain is made of several polypeptides (Robinson, 1990; Robinson 1991). The induced gelatin-cleavage activities have to be displaced from the hyaline layers, which would then enable us to attempt to purify these activities. The hyaline layers stored at − 70°C, were incubated in MFSW for 24 h at 37°C to induce gelatinase activities. The pellet containing the induced gelatinases was incubated in 50 m Tris, pH 8.0 both in the presence and absence of 5 mM EDTA for 24 h at 37°C. The pellet and supernatant fractions were separated and analyzed in a gelatin substrate gel (Fig.11). The results showed that the gelatinases were more efficiently displaced from the layers into the supernatant in the presence of EDTA (Fig. 11, lane 4), than in the absence of EDTA (Fig. 11, lane 2). Since EDTA is a divalent metal ion chelator, the above results indicate the requirement of a divalent cation such as Ca^{2+} and/or Mg^{2+} for the gelatinases to remain bound to the intact layers.

Flood *et al.* (2000) have reported similar results on dissociation of gelatin-cleavage activities in the presence of EDTA. Quantitative analysis of the displacement assay has demonstrated that 79% of the total gelatinase activity was displaced from the hyaline layers in the presence of EDTA.

4.5 Purification of the three major gelatin-cleavage activities 55-, 41- and 32 kDa using a gel filtration column

The isolated layers were induced at 37°C for 24 h followed by incubation in 50 mM Tris-HCl, pH 8.0 containing 5 mM EDTA for 24 h at 37°C to dissociate the induced gelatinases from the layers into the supernatant. The displaced 55- and 32 kDa gelatinases were then separated from each other on 1.5 M-Agarose column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 10 mM benzamidine hydrochloride (Fig. 12).

When gel permeation chromatography was performed in the absence of the inhibitor, only the 41 kDa activity eluted from the column. Neither the 55 kDa nor the 32 kDa species are observed (Fig. 13). This suggests that one or more serine proteinases is responsible for processing the 55 kDa into 41 kDa activity, while the 32 kDa was degraded into very small polypeptides that cannot be visualized in a gelatin substrate gel. Collectively, the above data indicate a precursor-product relationship between the 55- and the 41 kDa species. A possibility that one of the higher molecular weight gelatinase activity could be a precursor for the 55 kDa species has yet to be explored.

4.6 Characterization of the three gelatin-cleavage activities

Using both qualitative and quantitative assays we attempted to characterize the three major gelatin-cleavage activities at 55-, 41- and 32 kDa. The effects of various protease inhibitors on these activities were determined using a gelatin substrate gel (Figs. 14 and 21). All three activities were totally inhibited in the presence of 2 mM 1,10-phenanthroline and partially inhibited by 10 mM benzamidine hydrochloride. However, 0.6 mM AEBSF did not have any effect on all three activities. Similar results were obtained from the quantitative assay, which confirmed the Zn^{2+} requirement of these activities (Table 1). The above results indicate that the induced gelatin-cleavage activities possess a characteristic of many vertebrate MMPs, which are Zn^{2+} dependent (Chintala and Rao, 1999; Egeblad and Werb, 2002; Somerville *et al.*, 2003).

Both EDTA and EGTA had an inhibitory effect on all three activities (Figs. 16 and 19). EDTA inhibition suggests a divalent cation requirement, while EGTA inhibition indicates that Ca^{2+} could be the divalent cation required for their enzymatic activity. Gelatin-cleavage activities can be visualized even in the absence of exogenously added $CaCl_2$, which is due to the presence of endogenous Ca^{2+} in the hyaline layers.

Variations in the percent inhibition of the 55 kDa activity, in the range of 33-100% were observed in the presence of 5 mM ethylenebis (oxyethylenenitrilo) acetic acid. The quantitative gelatinase assay demonstrated that 10 mM $CaCl_2$ stimulated 58.1% of the EGTA-inhibited (33%) 55 kDa species, which resulted in

complete recovery of the activity (Table 2). On the other hand, only 28.2% activation of the EGTA-inhibited (33%) 55 kDa species was noticed in the presence of 50 mM $MgCl_2$, which recovered upto 85% of the total activity. The calcium reactivation of EGTA-inhibited (100%) 55 kDa occurred with an apparent dissociation constant of 1.2 mM (Fig. 18). Several vertebrate MMPs were known to possess both Zn^{2+} and Ca^{2+} binding sites in their catalytic domain (Massova *et al.*, 1998; Chintala and Rao, 1999).

Since the hyaline layer is always surrounded by sea water that contains 10 mM $CaCl_2$, 50 mM $MgCl_2$ and 500 mM NaCl, the effects of all three salts were investigated (Fig. 20). Fluorescence gelatinase assays suggest that 10 mM $CaCl_2$ and 50 mM $MgCl_2$ serve as activators of the 55 kDa species, while 500 mM NaCl inhibited 57.6% activity of the 55 kDa gelatinase (Tables 2 and 3). However, the effect of all the above mentioned salts together seemed to activate the 55-, 41- and 32 kDa gelatinase activities (Fig. 20, lane 5). To exclude the possibility that the inhibition may be due to a salting out effect caused by 500 mM NaCl, the reaction mixture containing the purified 55 kDa species and 500 mM NaCl was centrifuged in an airfuge for 1 hour, after which both the pellet and supernatant fractions were assayed for gelatinase activity. The pellet fractions contained 30.3% and the supernatants contained 69.7% of the gelatinase activity. However, the combined gelatinase activity obtained from both the pellet and supernatant only accounted for 42.4% of the total activity present in the reaction mixture. This result confirms that 500 mM NaCl has an inhibitory effect

on the 55 kDa gelatin-cleavage activity. The NaCl-dependent inhibition suggests a possible mechanism for regulating the 55 kDa gelatinase activity. Interestingly, 50 mM $MgCl_2$ and 500 mM NaCl did not have any effect on the 55 kDa in a gelatin substrate gel (Fig. 20). We suggest that the effects of $MgCl_2$ and NaCl cannot be visualized in the gel, due to overloading of the samples in the lanes.

Since the hyaline layer is surrounded by sea water, which contains 10 mM $CaCl_2$, weak interactions are possible between Ca^{2+} and 55 kDa activity. Carafoli (1987) has shown that mammalian ECM has up to 3 mM Ca^{2+} that supports low-affinity binding sites to interact with Ca^{2+}. Mayne and Robinson, (1998) have suggested a possible role of the hyaline layer as a Ca^{2+} reservoir with both Ca^{2+} and Mg^{2+} microenvironments.

Substrate gels containing either gelatin, casein, bovine serum albumin, or bovine hemoglobin as substrates were used to determine whether the three gelatinases induced from the isolated hyaline layers were nonspecific endopeptidases with accidental gelatin-cleavage activity (Fig. 22). Gelatin substrate gel had the highest cleavage activity (lane 1), while the casein gel displayed only low levels of cleavage-activity (lane 2). However, no detectable cleavage-activities were visualized in bovine serum albumin or bovine hemoglobin gels (lanes 3 and 4). The above results collectively suggest that the gelatin-cleavage activities were specific endopeptidases, which prefer gelatin as their substrate. The physiological substrates of vertebrate MMPs include ECM components such as, the growth factor receptors, the cell adhesion molecules

(CAMs), inactive MMP zymogens, other active MMPs and proteinase inhibitors (Egeblad and Werb, 2002). The physiological substrates of 55 kDa activity is yet to be identified.

A dynamic pattern of gelatin-cleavage activities was found during sea urchin embryonic development (Mayne and Robinson, 1996). The comigration pattern of the induced 55 kDa species was compared with embryos from various developmental stages to show that the 55 kDa activity is present in the embryo during its development (Fig. 23). A gelatin-cleavage activity found in the early gastrula stage embryo was found to comigrate with the 55 kDa gelatin-cleavage activity from the induced layers (lane 7). This cleavage-activity persisted until the pluteus stage. The effects of various inhibitors and chelators on the 55 kDa species and the activity that comigrated from 93 HPF embryos were studied (Fig. 24). These results showed that both these activities possessed similar characteristics confirming the presence of 55 kDa species in the sea urchin embryo. Both the 41- and 32 kDa could not be detected in the embryos. We speculate that these activities might be the intermediary products, which get processed further into another activity.

4.7   General Conclusions

As mentioned earlier, the ECM was first believed to be a passive storage compartment. The ECM is now widely recognized as a dynamic entity whose composition changes throughout embryonic development. The ECM degrading

proteinases utilize the ECM components as their substrates. Therefore, the focus of this project was to identify and characterize the gelatin-cleavage activities present in the sea urchin ECM, the hyaline layer.

We have identified different patterns of induced gelatin-cleavage activities in the isolated hyaline layers. Three major inducible gelatin-cleavage activities 55-, 41- and 32 kDa appeared after incubation of isolated hyaline layers in MFSW at 15°C or 37°C. We have purified all three major gelatinase activities, and focused on extensively characterizing the 55 kDa species. The hyaline layers contain several induced gelatin-cleavage activities, which are secreted as one or more inactive precursor. The activation of this precursor is mediated by one or more serine proteinase activity. The induced gelatinases require divalent cations to be bound to the layers. We conclude that the three major induced gelatinases are Zn^{2+}- and Ca^{2+}-dependent, gelatin-specific endopeptidase that belonged to the MMP class of ECM remodeling enzymes. The 55 kDa species was identified in the early gastrula stage embryos and remained until the pluteus stage. We also suggest a possible precursor-product relationship between the 55- and 41 kDa species, mediated through one or more serine proteinases.

4.8 Future Work

Three inducible major gelatin-cleavage activities have been identified in the sea urchin hyaline layer. Elucidating the physiological substrates of these proteases, and their modes of regulation, comprise logical avenues for future

endeavor. Experimentation outlined in the thesis shows that sea urchin MMPs are processed by serine proteinases, but more work is required before it can be firmly concluded that a precursor-product relationship exists between the 55- and 41 kDa species. Another natural extension of the thesis is to explore the regulation of sea urchin MMPs by TIMPs using reverse zymography, as documented in vertebrate systems.

A big step forward in characterizing the roles of MMPs in the sea urchin would be to isolate these enzymes to biochemical homogeneity, thus facilitating antiserum production. Such antisera would be useful for a number of reasons. One, to map, by immunofluorescence, the distribution of antigen during development. Two, to identify antigen storage compartments (using immunogold labeling in conjuction with electron microscopy) and three to probe antigen-dependent cellular processes (by injecting an antibody into cultured embryos). Finally, microsequencing of purified MMPs and identification of the corresponding cDNA will pave the way to using RNA interference (RNAi) to manipulate gene expression.

APPENDICES

Appendix A
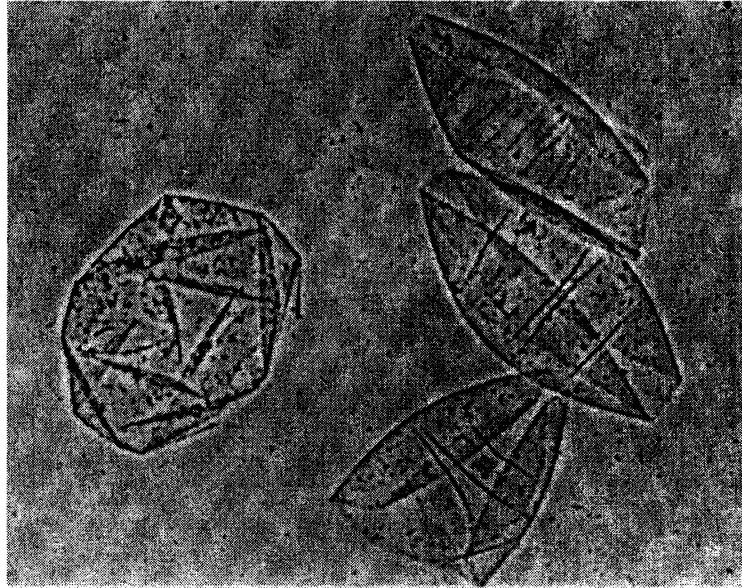
Phase contrast microscopy of isolated hyaline layers (Robinson, 1991).

Appendix B

Treatment	% gelatinase activity in the supernatant fraction	% protein in the pellet fraction
MFSW	49.4 ± 5.6	92.1 ± 3.8
Tris-EDTA	100	46.9 ± 4.7
Tris-EDTA-Ca^{2+}	23.8 ± 4.2	76.0 ± 5.0
Tris-EDTA-Mg^{2+}	55. 5 ± 4.9	72.0 ± 4.4
CMFSW	48. 3 ± 6.9	70.1 ± 6.1
CMFSW-EDTA	71. 2 ± 5.4	72.9 ± 5.3
Tris	43. 3 ± 5.1	64.8 ± 6.1
Tris-NaCl	41. 9 ± 3.4	69.4 ± 6.2
Tris-NaCl-EDTA	80.7 ± 6.8	68.5 ± 5.1

Dissociation of induced gelatin-cleavage activities from hyaline layers (Flood *et al.*, 2000).

Appendix C

MMP Family	Preferred Substrates	Members
Gelatinases	Collagen I, IV, V, VII, X, gelatin, Fibronectin, proteoglycans	MMP-2 and 9
Collagenases	Collagen I, II, II, VII, VIII, X, gelatin, proteoglycans	MMP-1, 8, 13, and 18
Stromelysins	Collagen IV, IX, X, proteoglycans, Elastin, fibronectin	MMP-3, 10, and 11
Elastase	Elastin	MMP-12
MT-MMPs	Activates MMP-2	MMP-14 and 16
	Unknown	MMP-15 and 17

Classification of matrix metalloproteinases (Adapted from Chintala and Rao, 1999)

Appendix D

	TIMP-1	TIMP-2	TIMP-3	TIMP-4
Molecular mass (kDa)	28	21	24-27	22
Glycosylation sites	2	0	1	0
Solubility	Soluble	Soluble	-	Soluble
MT-MMP inhibition	Poor	Good	Good	Good
ADAM inhibition	ADAM 10	None	ADAM 12, 17,19,10	None
Tumourigenesis	Inhibits	Inhibits	Inhibits	Inhibits
Metastasis	Stimulates	-	-	Stimulates

Characteristics of tissue inhibitors of metalloproteinases (TIMPs) (Adapted from Baker et al., 2002)

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