

CHARACTERIZATION AND IMMUNOLocalIZATION
OF A 41 kDa COLLAGENASE/GELATINASE ACTIVITY
IN THE SEA URCHIN EMBRYO AND ITS EFFECT(S)
ON DEVELOPMENT

CENTRE FOR NEWFOUNDLAND STUDIES

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41 kDa COLLAGENASE/GELATINASE ACTIVITY IN THE SEA
URCHIN EMBRYO AND ITS EFFECT(S) ON DEVELOPMENT**

by

Janice Ella Mayne

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Dedicated to my wonderful parents,

Ella L. Mayne

and

the late Vincent H. Mayne

ABSTRACT

Using gelatin substrate gel zymography, several activities were detected in sea urchin eggs and in various stage embryos which ranged in apparent molecular masses from 27.5 k to 86.9 k. We have purified and characterized the prominent 41 kDa collagenase/gelatinase activity from the sea urchin egg. The native molecular mass was 160 kDa, suggesting a tetrameric structure. Upon dissociation of calcium, enzyme activity was lost and this inhibition was found to be reversible. Reconstitution of activity occurred with an apparent dissociation constant of 3.7 mM. Up to 200 mols of calcium bound per mol of the 41 kDa subunit. Two classes of calcium binding sites were detected with dissociation constants of 0.5 and 5 mM. Amino acid analysis of the 41 kDa collagenase/gelatinase revealed that it contained twenty-four mol percent carboxy and carboxyamide residues which can function as weak calcium-binding sites. In the presence of calcium no secondary or quaternary structural changes were detected in the collagenase/gelatinase. The 41 kDa species had collagen-cleavage activity which appeared to be specific for echinoderm collagen, and a less specific gelatin-cleavage activity toward both invertebrate and vertebrate gelatin. The kinetic parameters, $K_m V_{max}$ and the ratio k_{cat}/K_m determined for the collagenase and gelatinase activities using peristome collagen and gelatin, were similar to those reported in the

literature for vertebrate collagenase and gelatinase activities. Immunolocalization was carried out to determine the subcellular localization of the 41 kDa collagenase/gelatinase in the unfertilized egg and in various stage embryos. The 41 kDa collagenase/gelatinase was found to be stored in several compartments in the egg: cortical granules (located along the periphery of the egg), and yolk granules and yolk granule-like structures (dispersed throughout the cytoplasm). Following fertilization, the 41 kDa species was detected in both the apical and basal extracellular matrices, the hyaline layer and basal lamina, respectively. In addition, the cytoplasmic storage compartment(s) retained a portion of their label into the gastrula stage. We followed the development of embryos in the presence of the 41 kDa collagenase/gelatinase and in the presence of the anti-41 kDa collagenase/gelatinase antiserum. In both studies embryos developed normally to the mesenchyme blastula stage, while subsequent development was retarded. The process of gastrulation was delayed and embryos failed to develop into plutei with well developed arms.

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LIST OF ABBREVIATIONS

ABS	absorbance
ADAM	a disintegrin and metalloproteinase domain
AEBSF	[4-(2-aminoethyl)benzenesulfonylfluoride]
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BPB	bromophenol blue
BP10	blastula protease 10
BSA	bovine serum albumin
CAMs	cell adhesion molecules
CBB	Coomassie Brilliant Blue R-250
CD	circular dichroism
CMFSW	calcium, magnesium-free sea water
CS	chondroitin sulfate
CUB	complementary subcomponent C1r/C1s
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DPP	decapentapleigic
DS	dermatan sulfate
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FGFR	fibroblast growth factor receptor
GAG	glycosaminoglycan
GFR	growth factor receptor
HA	hyaluronic acid
HFC	human fibroblast collagenase
HL	hyaline layer
HPF	hours post fertilization
HS	heparan sulfate
Ig-CAMs	immunoglobulin superfamily cell adhesion molecules
IL-1	interleukin-1
KS	keratan sulfate
LEF-1	lymphoid enhancer-binding protein-1

MAGP	microfibril-associated glycoprotein
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MT-MMP	membrane type-matrix metalloproteinase
PA	plasminogen activator
PCMB	4-chloromercuribenzoic acid
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKC α	protein kinase C α
PMSF	phenylmethanesulfonyl fluoride
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
<i>Sp</i>	<i>Strongylocentrotus purpuratus</i>
SVMPs	snake venom metalloproteinases
TACE	tumor necrosis factor- α converting enzyme
TCA	trichloroacetic acid
TGF- β	transforming growth factor β
TGF- β -1R	transforming growth factor β -1-cell receptor
TIMP	tissue inhibitors of metalloproteinases
TNF- α	tumor necrosis factor- α
TPA	tissue-type plasminogen activator
UPA	urokinase-type plasminogen activator
UV	ultraviolet
VEGF	vascular endothelial growth factor

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CHAPTER 1: INTRODUCTION

One component of the pericellular environment is the extracellular matrix (ECM), a dynamic structure, both in terms of composition and function. The various types of cells and tissues in an adult organism each have characteristic ECM compositions. As well, for any particular cell type, the ECM is subject to remodelling; new components are synthesized and deposited into the ECM by the cell, and components within the ECM are degraded by extracellular proteinases (Adams and Watt, 1993; Lin and Bissell, 1993). The composition of the ECM is modified throughout embryonic development, and is an important factor which directs, in part, proper development (Adams and Watt, 1993; Lin and Bissell, 1993; Streuli, 1999). The dynamic nature of the ECM is also evident during such biological processes as wound healing, pregnancy and the growth of bones, each of which necessitate precise and rapid ECM remodelling (Wozney *et al.*, 1988; Werb, 1997; Lund *et al.* 1999). The role of the ECM in providing protection and structure for cells has been recognized and accepted for some time. Research over the past several years, however, has increasingly focused attention toward the instructive roles of the ECM. These include directing the processes of cell adhesion and migration, and playing a role in regulating cell growth and differentiation (Thiery and Boyer, 1992; Adams and Watt, 1993 and Streuli, 1999).

The cell and its ECM are coupled through receptors on the cell surface.

These receptors along with several other classes of cell surface molecules, collectively constitute the second component of the pericellular environment. In addition to ECM receptors, several members of the family of ECM degrading proteinases are tethered to the cell surface (Werb, 1997). Other extracellular membrane proteins function as cell surface adhesive proteins which interact directly with neighbouring cells (Gilbert, 1991; Huttenlocher *et al.*, 1995). A fourth class of cell surface molecules are receptors for growth factors, and other cytokines (Thiery and Boyer, 1992; Lodish *et al.*, 1999). A commonality between the cell surface receptors and adhesive molecules is that they connect various components of the ECM with the interior of the cell. Cell surface receptors gather information from ECM components, neighbouring cells and growth factors, then transmit this information through their membrane domains to their cytoplasmic domains. Cytoskeletal and cytosolic proteins respond to this information and transmit signals to the nucleus, which can influence patterns of gene transcription (Juliano and Haskill, 1993; Clark and Brugge, 1995). Therefore, the cell utilizes both cell surface and ECM molecules to gather information and instructions from the extracellular environment. The cell can then integrate these signals and respond appropriately. This process of information gathering, relay and response is central to cell growth and differentiation, and continues throughout the life of the cell.

In the following sections, I will review the classes of ECM components and cell surface molecules. I will discuss in some detail the biological roles of the ECM, the mechanisms through which the ECM affects cell behaviour, and the degradation of ECM components by extracellular proteinases. Since the ECM affects important cellular processes, both during embryonic development and in the adult organism, the effect of unregulated degradation resulting in pathological consequences, such as arthritis and tumour cell metastasis, will be discussed. Following this general introduction, I will examine the utility of the sea urchin as an model to study the ECM and its roles during development, and then briefly discuss the focus of the research contained in this thesis.

1.1 The Extracellular Matrix

As mentioned above, one component of the environment of the cell is the ECM: a network of macromolecules, the composition of which is peculiar for particular tissue types and at various stages of development (Adams and Watt, 1993). For instance, collagen is the major component of cartilage, whereas both collagen and laminin comprise significant portions of the basal lamina (Hay, 1991; Lodish *et al.*, 1999). During embryonic development, the cell tailors the composition of the ECM to its requirements. For example, the ECMs of migrating cells contain large amounts of hyaluronic acid (HA), a

glycosoaminoglycan (GAG) component of ECMs (Hay, 1991; Lodish *et al.*, 1999). As these cells finish migrating and begin to establish connections with other cells, the composition of their ECM changes; the HA content decreases, while other ECM components increase (Hay, 1991; Lodish *et al.*, 1999).

Research has identified the primary sequence of over one hundred distinct ECM components, which are divided into four classes: collagens, elastins, proteoglycans and glycoproteins (Engel, 1991; Hay, 1991). The repertoire of ECM components and their properties generates structural and functional diversity between ECMs. As previously stated, cartilage is comprised mainly of collagen. The particular arrangement of collagen molecules into fibrils (discussed later) enables cartilage to resist tensile stress (van der Rest and Garrone, 1991). Conversely, tendons must be able to withstand compressive forces. The ECM of the tendon is rich in proteoglycans. These ECM molecules resist compressive forces because they have large amounts of water associated with their GAG side chains (Hay, 1991).

Diversity of ECM composition is accomplished through specific expression of genes encoding ECM components (Hay, 1991; Adams and Watt, 1993). A second level of diversity is established through alternative splicing of transcripts encoding some ECM components. This results in several gene products so that a particular ECM component can exist in several isoforms (Hay, 1991; Ayad *et al.*, 1998). An example is that of the dimeric glycoprotein,

fibronectin which, although encoded by a single gene, has an RNA transcript that is spliced in different ways to generate at least twenty isoforms of the polypeptide chain. These isoforms dimerise through disulfide bonding at their C-terminal ends (Ayad *et al.*, 1998). Post-translational modifications also increase the diversity of ECM components (Adams and Watt, 1993). The combination of ECM components, and their isoforms, as well as post-translational modifications generates diversity in ECM composition and function.

This section will review the structural components of the ECM. Functional domains that contribute to the versatility of the ECM components will be highlighted as well as their biological functions. Mechanistic descriptions of the underlying processes will be described in later sections.

1.1.1 Collagen

Collagens, historically regarded as structural components of the ECM, are the most abundant molecules of ECMs. This family consists of at least nineteen different subtypes (Ayad *et al.*, 1998). Collagen molecules have characteristic amino acid compositions, that is, they contain 33% glycine, 10% proline, 10% hydroxyproline and contain lysine and hydroxylysine residues. Also characteristic of collagen molecules is their triple helical structure. Known as collagenous domains, these triple helices are composed of three

left-handed helical polypeptides intertwined to form the right-handed triple helix, which is stabilized by interchain disulfide bonds (Hay, 1991; van der Rest and Garrone, 1991). Glycine is found as every third amino acid along the collagen polypeptide because the small side chain of this amino acid residue allows it to occupy the interior of the triple helical structure. In addition to collagenous domains, two short non-collagenous domains are found at the N- and C-termini of collagen molecules where the polypeptides do not form triple helical structures.

The hydroxylation of both proline and lysine residues occurs post-translationally. The thermal stability of collagen increases as the number of proline plus hydroxyproline (total pyrrolidine) increases (Josse and Harrington, 1964). Lysine can undergo several conversions. Lysines are converted to hydroxylysine by lysyl hydroxylase, and can undergo glycosylation (Hay, 1991). Lysine and hydroxylysine, undergo further modifications to form covalent cross-links between the three polypeptides within a collagen, and between collagen molecules. Extracellularly, lysyl oxidase, converts lysine and hydroxylysine, in the N- and C-terminal regions, to their corresponding aldehyde forms which promotes intermolecular and intramolecular cross-links (Hay, 1991).

Collagen molecules contain a variety of domains which allow them to interact with other ECM components, growth factors, and cell surface

receptors. Collagen type I can interact with decorin, an ECM proteoglycan, and fibronectin, a glycoprotein (Ayad *et al.*, 1998). Collagens interact with members of the integrin family of cell surface receptors (Hay, 1991). Interactions between collagen and other ECM components create the scaffold-like structural organization of the ECM.

Collagens can be subdivided into several classes depending on the supramolecular forms they assume within extracellular matrices: fibrillar, fibril associated, network-forming filamentous, short chain and long chain collagens (van der Rest and Garrone, 1991; Olsen, 1995). The fibrillar collagens, types I, II, III, V and XI form striated fibrils in which the collagen molecules are staggered about one-quarter their length, with a space between the N-terminal of one collagen and the C-terminal of the next (Hay, 1991; Olsen, 1995). They can be composed of only one type of collagen molecule, a homotypic fibril, or more than one, a heterotypic fibril. 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).

Type VIII and X are known as short-chain collagens since their single triple helical domain is only about half the size of the fibrillar-forming collagens (van der Rest and Garrone, 1991; Olsen, 1995). Type X is found as pericellular mats of filamentous material in hypertrophic chondrocytes. Alternatively, type VII, a long chain collagen found solely within anchoring

fibrils, has a longer central triple helical domain than the fibrillar forming collagens (van der Rest and Garrone, 1991; Olsen, 1995).

Fibril-associated collagens interact with the surface of striated collagen fibrils and include types IX, XII, XIV, XVI and XIX (van der Rest and Garrone, 1991). These molecules contain several interrupted collagenous domains. The collagenous domains can associate with fibrillar collagens, while non-collagenous domains act as hinge regions so that other collagenous domains project out into the ECM, providing a region for potential interaction with other matrix components.

Network-forming, filamentous collagens such as type IV are the only collagen type found in basement membranes (van der Rest and Garrone, 1991; Olsen, 1995). These collagens do not form fibrils; instead, the molecules have a non-collagenous domain at their C-terminus which interacts with the C-terminus of other network forming collagens. Their N-terminal ends can also associate together in twos and fours through disulfide bonds, and the triple helical regions from several molecules can associate laterally, thus producing a network.

The beaded filament collagen, type VI, is composed of three different chains, each of which has a central collagenous domain and two large non-collagenous domains at either end of the molecule. These molecules associate into dimers arranged antiparallel to one another, then tetramers

arrange laterally (van der Rest and Garrone, 1991; Olsen, 1995). These tetramers form beaded filaments by associating into linear arrays.

The collagen family characterizes the utility of the cell in creating diversity, not only between classes of ECM components, but within a family. The families of genes encoding collagen polypeptides and their alternative splicing, post-translational modifications and different supramolecular structures in the ECM create a versatile family of ECM components, whose structures reflect the physical requirements of that particular matrix. The variety of roles these collagens play in the functioning of various cell types and tissues reflect the necessity of these different forms.

1.1.2 Elastin

Elastin is a chemically inert, extremely hydrophobic protein which is very stable. Physiological turnover of elastin is slow with the half-life approaching that of the animal, so under normal conditions very little processing of elastin occurs (Hay, 1991). It is the major component of ECM elastic fibers, and is the component responsible for the property of elastic recoil in these fibers. Elastin, like collagen, has a highly characteristic amino acid composition (Hay, 1991; Ayad *et al.*, 1998). It is rich in hydrophobic amino acids, for example, valine comprises 17%, but it is low in both acidic and basic amino acids. One-third of the amino acids are glycine, one-ninth are proline,

and it contains small amounts of hydroxyproline (Hay, 1991; Ayad *et al.*, 1998). It also contains the lysine derivatives allysine aldol and dehydrolysinooreleucine which covalently cross-link the protein monomers. Post-translational modifications of tropoelastin molecules occur extracellularly, and it is within the ECM that it is cross-linked and the elastin fiber assembled (Ayad *et al.*, 1998).

Elastic fibers have two distinct components; a core of polymeric insoluble elastin and peripheral microfibrils. The microfibrillar component consists of a microfibril-associated glycoprotein (MAGP), fibrillin, a 34 kDa glycoprotein with amine oxidase activity, lysyl oxidase and several other glycoproteins. The MAGP forms intermolecular disulfide bonds, while fibrillin (a glycoprotein component of both elastin and non-elastin microfibrils) can interact with growth factors through epidermal growth factor (EGF)-like repeats, and contains an RGD sequence which interacts with cell surface receptors known as integrins (Ayad *et al.*, 1998).

Elastin fibers occur in three morphologically distinct forms which reflect the physical and functional needs of tissues: elastic ligaments found in the lung and skin are ropelike structures of varying lengths, elastic fibers of the major arteries form concentric sheets or lamellae, and a three dimensional array of fibers is found in elastic cartilage (Ayad *et al.*, 1998).

1.1.3 Proteoglycans

Proteoglycans consist of one or more glucosaminoglycan (GAG) side chain(s) covalently attached to a core protein through an oligosaccharide linkage protein (Ayad *et al.*, 1998; Lodish *et al.*, 1999). There are several core proteins, as well as different types and lengths of GAG side chains, which makes this class of ECM component quite diverse. This diversity is reflected not only in the macromolecular structures formed, but in the functions contributed to these ECM components during cell adhesion, migration, and proliferation (Wight *et al.*, 1995; Lodish *et al.*, 1999). Proteoglycans are components of the ECM proper and are found at the cell surface (Esko, 1991; Lodish *et al.*, 1999).

There are four classes of GAG side chains each of which consist of disaccharide repeats of two different sugars: hyaluronic acid (HA), chondroitin sulfate (CS)/dermatan sulfate (DS), keratan sulfate (KS) and heparan sulfate (HS)/heparin (Lodish *et al.*, 1999). Hyaluronic acid, a large polyanionic molecule, is different from other GAGs in that it does not associate with a protein core, instead it exists as a free carbohydrate which can self-associate and form large networks. The other GAG side chains are built on oligosaccharide linkage structures which attach to the core protein (Hay, 1991).

The diversity within the proteoglycan family is contributed not only by the

GAG side chains, but also in the type of core protein to which these side chains are attached. The primary amino acid sequence of more than a dozen core proteins are known. As is the case with other ECM components, cells of various types or at different stages of development express particular proteoglycans (Esko, 1991). For instance, aggrecan is a CS proteoglycan which forms large aggregates in the ECMs and is abundant in cartilage, brevican is a CS proteoglycan found in brain tissue, and perlecan is a basement membrane HS proteoglycan (Ayad *et al.*, 1998).

The functions played by various proteoglycans throughout development can be attributed both to their GAG side chain and to the protein core which contains several domains. Aggrecan contains binding sites for HA, lectin repeats, which may interact with carbohydrate ligands of other ECM components, and EGF-like repeats (Ayad *et al.*, 1998). Perlecan can interact with other basement membrane components such as collagen type IV and laminin, and can serve as an attachment substrate for cells (Ayad *et al.*, 1998).

1.1.4 Glycoproteins

These ECM proteins contain carbohydrate moieties covalently attached through O- or N-linkages. An important aspect of the large number of glycoproteins present in the ECM is their ability to interact not only with the cell surface, but also with other matrix molecules in the ECM (Ayad *et al.*,

1998). Glycoproteins are a versatile family of ECM components which carry out a variety of functions in the ECM. Several types of glycoproteins carry out adhesion roles in tissues, while others are associated with anti-adhesive properties, and as mentioned in a previous section, a number of glycoproteins are associated with elastin fibrogenesis.

Fibronectin, a dimeric glycoprotein composed of three repeating domains, has long been characterized as an adhesive glycoprotein (Schwarzbauer, 1991). Its structural domains can bind other ECM components such as heparin and collagen, as well as, cell surface receptors (Schwarzbauer, 1991; Moyano, 1997). The large heterotrimeric glycoproteins known as laminins are major constituents of the basal lamina. Laminins also contain biologically important domains which include a cell binding domain, a heparin binding domain, and an entactin binding domain (Ashkenas *et al.*, 1996). Through binding entactin, laminin can interact with collagen type IV. Therefore laminin can serve to connect the cell to the ECM through its interaction with both ECM components and cell surface receptors.

Contrasting with the adhesion-promoting glycoproteins are the anti-adhesive glycoproteins such as tenascin, found transiently in the ECM during embryonic development, and at sites of tissue remodelling in adult organism such as during wound repair (Hay, 1991). Addition of several cell types to tenascin-coated substrates resulted in loss of attachment (Sage and Bornstein,

1991). The adhesive and non-adhesive glycoproteins exert contrasting affects on cells, wherein varying the relative amounts of these ECM components varies the adhesion properties of a particular cell for its ECM. Thus, glycoproteins play important roles in the assembly of ECMs because of their numerous interactions with a variety of other ECM components and cell surface receptors. Consequently, these molecules are important in processes involving cell adhesion, cell migration and cell growth.

1.2 Cell Surface Molecules

As mentioned earlier, several classes of molecules located at the cell surface constitute the second component of the pericellular environment: cell adhesion molecules (CAMs) mediate cell - cell interactions, integrins and cell surface proteoglycans mediate cell - matrix and cell - cell interactions, while other cell surface molecules act as receptors for soluble proteins found within the ECM, such as growth factors (Aplin *et al.*, 1998; Liu *et al.*, 1998; Lodish *et al.*, 1999). Collectively, these molecules can interact with cytoskeletal components and/or cytoplasmic proteins through their cytoplasmic domains to induce or effect signal transduction pathways. They act as messengers, shuttling information from the exterior of the cell to the interior, and vice versa, so that the cell can communicate with and respond to its extracellular environment. This section will review three classes of cell surface molecules;

the CAMs, ECM receptors, and growth factor receptors. Cell surface ECM proteinases, although a part of the cell surface environment, will be discussed later in the context of ECM remodelling.

1.2.1 Cell Adhesion Molecules

Cell adhesion molecules are involved in both homophilic and heterophilic adhesion between cells. These molecules can be divided into three classes: cadherins, immunoglobulin superfamily CAMs (Ig-CAMs), and selectins (Aplin *et al.*, 1998; Lodish *et al.*, 1999). Cadherins contain extracellular 'cadherin' domains of 110 amino acid repeats with a calcium-binding site between each repeat, and mediate calcium-dependent, homophilic cell - cell adhesion (Aplin *et al.*, 1998). The cadherin family consists of more than forty members which can be divided into several subfamilies: classical cadherins, desmosome-associated cadherins, and protocadherins (Gumbiner, 1996; Aplin *et al.*, 1998; Yap, 1998). The highly conserved cytoplasmic domains of classical cadherins interact with a family of periplasmic proteins known as catenins to mediate binding to the actin cytoskeleton and strengthen cell - cell adhesions, while additional research has shown that these interactions can also affect signal transduction pathways (Aplin *et al.*, 1998; Yap, 1998; Vleminckx and Kemler, 1999). Unlike the classical cadherins, the cytoplasmic domains of protocadherins do not

associate with intracellular adhesion proteins. However, they can mediate weak, calcium-dependent cell - cell adhesion. The desmosome-associated cadherins, desmoglein and desmocollin, interact with desmoplakin and γ -catenin which are attached to keratin filaments, intracellularly (Gumbiner, 1996; Aplin *et al.*, 1998).

The extracellular domains of the second family of CAMs, the Ig-CAMs contain cell binding domains that are held in place by disulfide bonds which resemble those of antibody molecules (Hortsch and Goodman, 1991; Gilbert, 1991; Lodish *et al.*, 1999). In addition, some Ig-CAMs contain copies of a fibronectin type III domain, thrombospondin-1 domain or an EGF-like domain, following their Ig domain (Aplin *et al.*, 1998; Hutter *et al.*, 2000). Cell-cell adhesion is mediated by sialylation of the extracellular domains; the more heavily sialylated, the weaker the interaction between these molecules because of repulsion of negative charges. In addition to their extracellular domains, Ig-CAMs have a single transmembrane domain and a cytoplasmic tail (Aplin *et al.*, 1998). They are important in developing and adult organisms, functioning in cell adhesion and migration, immune system processes and in signal transduction events.

Selectins mediate calcium-dependent, heterotypic interactions between leukocytes and endothelial cells during processes such as inflammation (Aplin *et al.*, 1998; Lodish *et al.*, 1999). The extracellular domain of selectins

resemble lectins, a family of saccharide binding proteins. The lectin-like domain is followed by an EGF-like domain and complement regulatory protein repeats. Selectins, like cadherins and Ig-CAMS, have a single transmembrane segment and a cytoplasmic tail (Aplin *et al.*, 1998).

1.2.2 Growth Factor Receptors

There are a number of growth factor receptors found at the cell surface: including transforming growth factor β -1-cell receptor (TGF- β -1R), epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR) and fibroblast growth factor receptor (FGFR) (Lodish *et al.*, 1999). The combination of growth factor receptors at the cell surface will vary with cell type and during development. In addition, the cellular response of a particular growth factor - growth factor receptor complex will vary between cell types.

Most of these transmembrane protein receptors are receptor tyrosine kinases (RTKs) (eg. PDGFR, FGFR and EGFR), while others are receptor serine/threonine kinases (eg. TGF- β -R). Binding of ligand(s) in the extracellular domain usually results in dimerization of the receptors, inducing a conformational change in the cytoplasmic domains so that the tyrosine (serine/threonine) kinase activity becomes active (Juliano and

Haskill, 1993; Lodish *et al.*, 1999). The protein kinase activity of each receptor molecule will phosphorylate a tyrosine (serine/threonine) residue in the cytoplasmic domain of the other receptor that makes up the dimer, so that the cytoplasmic domains can now catalyze phosphorylation of tyrosine (serine/threonine) residues on adjacent proteins, initiating signal transduction cascades that result in messages being relayed to the nucleus which affect cell growth and differentiation.

Growth factor receptors bind the soluble growth factors which are transient molecules of the ECM. Growth factors must first pass through the ECM to bind to their cell surface receptors. In addition many growth factors must bind ECM components before interacting with their cell surface receptors, and can be sequestered by ECM components. The role of ECM components in regulating growth factor - growth factor receptor interactions will be discussed in a later section.

1.2.3 Extracellular Matrix Receptors

ECM proteins contain several cell binding domains through which individual ECM components are able to interact directly with the cell. Interactions are mediated by cell surface receptors for ECM components of two major classes: the integrin and non-integrin receptors (Adams and Watt, 1993). The non-integrin receptors include cell surface proteoglycans such as

heparin sulfate (Yayon *et al.*, 1991; Wight *et al.*, 1992; David, 1993). Several heparan sulfate proteoglycans have been identified whose core proteins include a type I membrane spanning domain and a cytoplasmic tail that contains four conserved tyrosine residues. These heparan sulfate proteoglycans include syndecan, fibroglycan, syndecan-3, and ryudocan (Esko, 1991; David, 1993). These transmembrane proteins can interact both with growth factors (such as FGF, VEGF, and TGF- β) and with matrix components (such as fibronectin, vitronectin, thrombospondin, laminin and fibrillar collagens) in the ECM (Esko, 1991; David, 1993). The cell surface proteoglycans, betaglycan and CD44, contain an extracellular domain with sites for binding of GAG chains, a site near the transmembrane domain for potential cleavage of the ectodomain, a single transmembrane segment and a cytoplasmic tail (Esko, 1991; Wight *et al.*, 1992; David, 1993). The cytoplasmic domain of betaglycan differs in that it contains two cysteine-rich regions separated by short sequences and does not contain tyrosine residues. It does contain serine and threonine, and a potential protein kinase C substrate site (Wight *et al.*, 1992; David, 1993). Other heparan sulfate proteoglycans, such as glypican, are covalently linked to the cell surface through glycosyl phosphatidylinositol. Glypican is rich in cysteine and contains a repeating sequence of serine-glycine residues at its N-terminus (David, 1993). Since these core proteins do not span the cell membrane they do not interact

intracellularly (Esko, 1991; David, 1993). Many ECM components contain domains that can bind to the GAG chains of cell surface proteoglycans. Cell surface heparan sulfate proteoglycans can mediate attachment to type V collagen, thrombospondin and fibronectin (Esko, 1991). As well, heparan sulfate proteoglycans play important roles in growth factor - growth factor receptor interactions (discussed in the following section) (David, 1993).

Integrins are a family of heterodimeric, transmembrane glycoproteins consisting of an α - and a β -subunit, and are responsible for the majority of cell interactions with the ECM, although they can mediate cell - cell interactions through membrane receptors on other cells (Damsky and Werb, 1992; Juliano and Haskill, 1993; Aplin *et al.*, 1998; Boudreau and Jones, 1999). Integrin receptors provide the link between ECM components and intracellular cytoskeletal proteins and signalling pathways.

In vertebrates, there are 16 α -subunits and 8 β -subunits which combine to create a diverse family of more than 22 ECM receptors (Clarke and Brugge, 1995; Boudreau and Jones, 1999). Each subunit consists of an extracellular domain, a single transmembrane domain and a cytoplasmic domain. The particular combination of an α -subunit with a β -subunit determines the specificity of the ligand binding site (Damsky and Werb, 1992; Juliano and Haskill, 1993; Aplin *et al.*, 1998; Boudreau and Jones, 1999). In some cases, a

particular integrin heterodimer will bind to a particular ligand; for instance, $\alpha 5 \beta 1$ interacts with fibronectin only (Juliano and Haskill, 1993). More commonly however, a specific integrin heterodimer will interact with several ECM components. An example is the integrin $\alpha 5 \beta 3$, which interacts with vitronectin, fibronectin, collagen, tenascin C, thrombospondin and fibrinogen (Boudreau and Jones, 1999). Although different integrin heterodimers possess overlapping substrate specificity, the actual interaction of ligand to integrin will elicit a different cellular response, depending on the integrin involved, since their intracellular signalling domains can be quite divergent (Aplin *et al.*, 1998; Boudreau and Jones, 1999).

Integrins recognize specific sequences within ECM components, such as RGD and EILDV. In some cases however, the conformation of the ligand may be important. This appears to be the case for the interaction of integrins with other CAMs, such as Ig-CAMs and cadherins (Aplin *et al.*, 1998). The binding site for integrins includes a series of seven repeats of 60 amino acids (each of which contain a potential binding site for calcium) in the N-terminal portion of the α -chain, an inserted domain (I domain) which contains both a nucleotide binding fold and potential divalent cation coordination site of 200 amino acids in the α -chain, and a second I domain-like region of 250 amino acids found in the N-terminal region of the β -subunit (Aplin *et al.*, 1998).

Upon binding of ligand, an integrin will undergo several structural changes; the relative positions of the subunits and the domains change, as well, conformational changes within domains occur (Aplin *et al.*, 1998).

While both the α and β subunits are essential for binding in the ECM, only the β -subunit appears to play a critical role in the transmembrane connection to the integral cytoskeleton and, unlike the cytoplasmic domain of the α -subunit, the β -domains do show sequence similarities (Clark and Brugge, 1995). The cytoplasmic domain of integrin β -subunits interacts with cytoskeletal proteins such as talin, vinculin, α -actinin, tensin and focal adhesion kinase (FAK), and the cytoplasmic adaptor molecules paxillin, RACK1 and p130^{Cas} (Aplin *et al.*, 1998; Boudreau and Jones, 1999). The α -subunit is thought to interact with the β -subunit, inhibiting certain functions of the β subunit (Aplin *et al.*, 1998; Boudreau and Jones, 1999). Binding of a ligand may cause the cytoplasmic domains to swing apart relieving the inhibition.

1.3 Biological Roles of the Extracellular Matrix

As mentioned earlier, variations in the composition of ECMs, both during development and between tissues in adult organisms, reflect, in part, the

physical demands of that environment. This section will review the role of ECM components in processes such as cell adhesion and migration, as well as the role they play during cell growth and differentiation.

1.3.1 Cell Adhesion and Cell Migration

The integrated effect of multiple interactions between two neighbouring cells influences the decision of cells to adhere. Cell migration, a process dependent upon regulated cellular adhesion is important during embryonic development, wound healing and the immune response. Extracellular matrix components and cell surface adhesion molecules are important in these processes since cells must detach from both their ECM and neighbouring cells to migrate, and because these molecules enable cells to transmit information from the extracellular environment to their cytoplasm, and vice versa.

Evidence that components of the ECM and CAMs affect these biological processes has been collected from both *in vitro* and *in vivo* studies (Adams and Watt, 1993; Lin and Bissell, 1993; Sastry and Horwitz, 1996). In cell culture, a monolayer of epithelial cells will detach from one another when anti-E-cadherin antibody is added, demonstrating the effect of CAMs on both cell migration and cell shape (Lodish *et al.*, 1999). Adhesive mechanisms involving cell-matrix interactions are important in determining the cellular

architecture of mammary epithelial cells (Lin and Bissell, 1993; Ashkenas *et al.*, 1996). In the absence of ECM components, these cells failed to interact with one another in culture. Addition of laminin resulted in cell clustering and, in some cases, formation of alveolar structures (Ashkenas *et al.*, 1996). In other studies, addition of antibodies against $\alpha 3$ or $\beta 1$ integrin subunits prevented morphogenesis of human mammary cells (Ashkenas *et al.*, 1996).

Experiments carried out by Liu *et al.* (1998) illustrate the contrasting effects of cell surface proteoglycans on the processes of cell adhesion and migration. They transfected a cell line which expressed low levels of cell surface heparan sulfate and that invaded type I collagen gels, with cDNA for syndecan-1, -2 and -4 or glypican-1. Expression of syndecans on the surface of these cells promoted binding to type I collagen and prevented invasion into the collagen gel, whereas expression of glypican-1 on the surface of these cells, did not promote binding to type I collagen and the cells remained invasive. They also concluded that adhesion alone could not inhibit invasive behaviour, since cells expressing chimeric cell surface heparan sulfates, constructed from the extracellular domain of glypican and the transmembrane and cytoplasmic domains of syndecan, bound type I collagen but remained invasive (Liu *et al.*, 1998).

Wound repair depends upon regulated cell adhesion and migration, necessitating degradation of ECMs as keratinocytes migrate to the site of

injury, re-establish cell-cell interactions, and differentiate to form epidermis. Some serine proteinases are extracellular matrix proteinases and have been shown to degrade ECM components during wound repair (discussed later). Kainulainen *et al.* (1998) demonstrated a role for syndecans in the balance of the activity of serine proteinases during wound repair. They showed that the ectodomains of these cell surface proteoglycans are cleaved, releasing soluble syndecan ectodomains into the ECM (a process known as shedding) and that these soluble syndecans were responsible for maintaining proteolytic balance during wound repair. The purified syndecan-1 ectodomain bound to the serine proteinases and prevented binding to their inhibitors.

Hynes (1996) reviewed the results of experiments examining the biological effects of knockout mutations of genes encoding CAMs and ECM components, as well as their receptors. Although some null mutations resulted in lethal phenotypes, other resulted in less severe phenotypes or developmental effects than expected from *in vitro* studies. Since many ECM components are part of larger families, and ECM component - integrin receptor interactions overlap, other members of a particular family or receptor may compensate for null mutations. Similarly, the elimination of one cell adhesion system may result in the up-regulation and compensation by another. In addition, some extracellular components may play roles in events such as repair processes, rather than during development.

1.3.2 Cell Growth and Differentiation

Cells receive and interpret signals from different components of the ECM to determine whether to grow or differentiate, and must be able to respond to both normal and abnormal changes (for instance during injury) throughout their growth. There is a large body of evidence supporting a role for ECM proteins in regulating cell growth and differentiation (Lin and Bissell, 1993; Sastry and Horwitz, 1996). For instance, components found within the ECM are important during differentiation of keratinocytes (Lin and Bissell, 1993). Keratinocytes in contact with basement membrane did not differentiate, however differentiation occurred when these cells were placed in suspension cultures. The presence of fibronectin (one component of basement membranes) and its integrin receptor ($\alpha 5\beta 1$) prevented keratinocyte differentiation (Lin and Bissell, 1993).

Osteoblast cells are important during bone formation; precursors to these cells migrate and proliferate at the site of bone formation where they synthesize type I collagen and other matrix components (Takeuchi *et al.*, 1996). Following matrix deposition, these cells differentiate so that mineralization occurs forming bone. Transforming growth factor- β is important during bone formation, stimulating production of type I collagen, proteoglycans and fibronectin. However, its action must be suppressed

during the mineralization phase of bone formation and has been shown to inhibit further differentiation of the osteoblasts. Takeuchi *et al.* (1996), employing osteoblast-like MC3T3-E1 cells, demonstrated that the interaction of type I collagen with its integrin receptor, $\alpha 2\beta 1$, reduced the actions of TGF- β through reduction of cell surface receptors competent to bind TGF- β , thus promoting osteoblast differentiation and matrix mineralization.

Majack *et al.* (1986) postulated that the composition of the ECM can, in part, influence cell growth. This group has shown that PDGF stimulated growth of smooth muscle cells, and resulted in synthesis and deposition of thrombospondin into the ECM. Conversely, heparin, a known inhibitor of smooth muscle cell growth, prevented incorporation of thrombospondin into the ECM. In their study, Majack *et al.* (1986), showed that EGF, unlike PDGF, did not promote appreciable smooth muscle cell growth. However, addition of both EGF and thrombospondin to smooth muscle cells, in culture, resulted in cell growth. Presumably, the thrombospondin enriched ECM was required to facilitate cell growth. This concept was supported by the inhibitory effect of heparin on cell growth, since heparin effectively inhibits binding of thrombospondin to ECM components, fibroblasts and glycolipids on the cell membrane. Similarly, antibodies against thrombospondin reduced mitogenesis of smooth muscle cells. They concluded that production of a

thrombospondin-rich ECM by PDGF allowed other factors, such as EGF, to exert their biological effects.

During early development, some ECM components are expressed widely but become restricted to certain cell types as development proceeds, while other components only appear during later development. To illustrate, syndecan-1 is expressed by ectoderm cells at the four cell stage of mouse embryonic development (Tumova *et al.*, 2000). However following gastrulation this cell surface proteoglycan is restricted to mesoderm, and in the mature mouse it is found on epithelial cells (Tumova *et al.*, 2000). In contrast to syndecan-1, syndecans-2- and -4 are not expressed until 10.5 days following fertilization (Tumova *et al.*, 2000). Such a pattern of expression may reflect the roles these components play during various stages of growth or development.

Mutations in ECM proteins and/or receptors have been shown to affect development. For example, in *Drosophila* the maternal gene toll, expressed during embryonic development is responsible for establishment of the dorsal-ventral body axis. Embryos from females that are mutated in the toll gene all adopt a dorsal polarity. Also in *Drosophila*, when Fasciclin I, a cell surface molecule expressed on central and peripheral nervous system axons is mutated, adults have uncoordinated behaviour, suggesting that this gene is involved in nervous system connectivity.

Behrens *et al.* (1996) showed that β -catenin (which interacts with the cytoplasmic domain of cadherins) interacts with a transcription factor, lymphoid enhancer-binding protein-1 (LEF-1), in mammalian cells. The β -catenin - LEF-1 complex translocated to the nucleus where it interacted with DNA. They demonstrated in *Xenopus* embryos that interaction of LEF-1 and β -catenin was necessary to induce formation of a secondary body axis. In *Drosophila*, Armadillo (whose vertebrate equivalent is β -catenin) played a role in both cadherin cell adhesion and in the wnt/wingless signalling pathway, important in establishing segment polarity (Brunner *et al.*, 1997). These observations provide evidence of a relationship between cell adhesion and cell signalling events.

1.4 Mechanisms of Regulation by Extracellular Matrix Molecules

The previous section illustrated the role of the ECM in processes such as embryonic development and wound repair. Earlier sections discussed how components of the ECM interact with one another, with regulatory molecules (such as growth factors) present in the ECM, and with cell surface receptors. The notion of the ECM as a static structure has been dispelled and it is clear that its role in cell growth and differentiation necessitates an equilibrium between ECM synthesis, remodelling and degradation. Three mechanisms

have been proposed through which the ECM molecules regulate cellular properties: (a) indirectly through the interaction of ECM molecules with growth factors, (b) through direct interactions between components of the ECM and cell surface receptors, and (c) through changes to the composition of the ECM (Adams and Watt, 1993).

1.4.1 Interactions Between Growth Factors and the Extracellular Matrix

Interactions between growth factors and ECM components through specific peptide domains can indirectly regulate cell behaviour in two ways; the interaction of growth factor - ECM component can increase the stability of the growth factor or modify the diffusion of the growth factor within the ECM and effectively alter the concentrations of the growth factor at the cell surface (Flaumenhaft and Rifkin, 1991; Sastry and Horwitz, 1996). As well, these interactions confine the activity of the growth factor to the appropriate time and location. Therefore, binding of a growth factor to its cell surface receptor can be altered by its interaction with an ECM component, and, in some cases, a growth factor can only bind to its cell surface receptor after first binding with an ECM molecule.

Cell surface heparan sulfate proteoglycans play important roles in receptor - growth factor interactions. Yayon and colleagues (1991) demonstrated that basic fibroblast growth factor (bFGF) binds to its cell surface receptor on

Chinese hamster ovary cells (mutant in their metabolism of GAGs) after it has first bound to heparin sulfate proteoglycans present at the cell surface or in the ECM. This ternary interaction of heparan sulfate proteoglycan - bFGF - FGFR also provides a pathway for endocytosis of this growth factor (David, 1993). Heparin sulfate proteoglycans are also required for the binding of VEGF (vascular endothelial growth factor) to its corresponding receptors at the cell surface (David, 1993).

The signal transduction pathways initiated through binding of growth factors to their receptors can increase or decrease the transcription of genes, including those that encode ECM molecules. For example, binding of TGF- β -1 to its receptor induces expression of genes encoding ECM proteoglycans such as decorin and biglycan (Flaumenhaft and Rifkin, 1991).

Growth factor receptors undergo ligand-induced dimerization, an event necessary for transmembrane signalling (Spivak-Kroizman, 1994). Growth factors such as PDGF, colony stimulating factor-1, and stem cell factor are dimeric proteins and can induce dimerization of their receptors if each molecule of a dimer binds to a separate receptor molecule. Other growth factors such as human growth hormone and EGF contain two binding sites for their receptors, allowing them to cross link receptors. In other cases, proteoglycans induce oligomerization of growth factors so they can then interact with receptors. DiGabriele *et al.* (1998) demonstrated that, upon

binding to heparin decasaccharides, acidic FGF (aFGF) dimerizes, which promotes tyrosine autophosphorylation and activation of the FGF receptor.

Richard *et al.* (1995) showed that bFGF could mediate cell attachment by linking receptors and heparan sulfate proteoglycans on neighbouring cells. Using a cell line that did not express either FGFR or heparan sulfate proteoglycans, they showed that these cells remained in suspension when co-cultured with Chinese hamster ovary cells, which contain cell surface heparan sulfate proteoglycans. When they transfected the cell line with cDNA encoding the FGFR-1 in the presence of bFGF these cells adhered to the Chinese hamster ovary cells, and binding could be inhibited with addition of free heparan sulfate proteoglycans (Richard *et al.*, 1995). Therefore proteoglycans can function as reservoirs for growth factors and facilitate binding of growth factors to cells, triggering intracellular pathways.

1.4.2 Interactions Between the Cell Surface and the Extracellular Matrix

ECM proteins contain several cell-binding sites so that the ECM molecule is able to interact directly with the cell through the non-integrin and integrin ECM receptors (Adams and Watt, 1993). Upon binding of an ECM molecule to a cell surface receptor, a message is relayed via the transmembrane segments of the receptor to the cytoplasm of the cell. These changes initiate signal transduction processes which transmit signals to the nucleus. In this

manner, the ECM can stimulate transcription of specific genes through its interactions with the cell surface proteins. Interactions between cell surface receptors and the ECM molecules are important for cell adhesion and migration and can be developmentally regulated (Adams and Watt, 1993; Hortsch and Goodman, 1991).

Hyaluronic acid associates with two classes of cell surface proteoglycans: syndecans and glypicans (Horowitz and Simons, 1996). Syndecans can carry both CS and HS chains in their extracellular domains, while their cytoplasmic domains contain four invariant tyrosines and one invariant serine. The cytoplasmic tail of syndecan-4 appears to bind and activate PKC α and the four tyrosines and one serine may act as kinase substrates *in vivo* (Horowitz and Simons, 1996). They showed that the extent of phosphorylation of the cytoplasmic tail was determined by the activity of a novel PKC enzyme and a bFGF-activated phosphatase. Dephosphorylation of the residues may allow for oligomerization of the receptors, and activation of PKC α and downstream signalling events. Subsequently, Horowitz and Simons (1996(b)) showed that both the multimerization and activation of PKC α by syndecan-4 is dependent upon interaction of the cytoplasmic tail of syndecan-4 with phosphatidylinositol 4,5-bisphosphate (PIP₂). They found that phosphorylation of the serine residue in the cytoplasmic tail reduced its

affinity for PIP_2 and prevented multimerization and activation of $\text{PKC}\alpha$. Phosphatidylinositol 4,5-bisphosphate binds to the cytoplasmic tail and must be present for multimerization and activation of $\text{PKC}\alpha$ (Horowitz and Simons, 1996(b)).

The integrins are the major receptors for ECM protein attachment to the cell (Hynes, 1992). The cytoplasmic domain of integrin subunits interacts with the actin cytoskeleton and related proteins, such as vinculin, talin and α -actinin (Boudreau and Jones, 1999). Integrins can transmit signalling events by organizing the cytoskeleton, regulating cell shape and architecture, which can regulate the biosynthetic capabilities of the cell, and affect cell growth and differentiation (Juliano and Haskill, 1993). In some systems the binding of integrin with an ECM component was followed by clustering of integrin receptors and increased tyrosine phosphorylation of proteins, such as the novel tyrosine kinase termed pp125 focal adhesion kinase (pp125^{fak}) (a substrate for the *src* family of tyrosine kinases) which is found at focal adhesive contacts and which may mediate signal transduction (Juliano and Haskill, 1993). Focal adhesions are enriched in cytoskeletal proteins such as actin filaments and vinculin, talin, paxillin and tensin, but they do not form if tyrosine kinase activity is inhibited (Boudreau and Jones, 1999). Although pp125^{fak} undergoes phosphorylation upon integrin - ECM component

interaction and integrin clustering, it does not phosphorylate other substrates. Instead, phosphorylation allows it to interact with adaptor proteins such as paxillin and tensin which can activate downstream signalling mediators such as Src, Ras and Raf (Boudreau and Jones, 1999). Growth factor receptors are also recruited to focal adhesion complexes upon integrin clustering and may be involved in cross-talk between these two types of receptors. In fact, pp125^{fak} can be activated by some growth factors (Boudreau and Jones, 1999). For example, EGFRs bind to actin, which increases EGF-dependent autophosphorylation and activation of downstream substrates. When the actin cytoskeleton is disrupted, it prevents formation of focal adhesions and activation of GFRs, indicating a role for the actin cytoskeleton in coordinating signals from integrins and growth factors (Boudreau and Jones, 1999).

Adhesion of cells to ECM proteins such as fibronectin and collagen via their integrin receptors activates mitogen-activated protein kinase (MAPK) pathways (Boudreau and Jones, 1999). These signal transduction pathways provide a common route to transcriptional regulation of genes important for cell growth and differentiation. As well, there is evidence that signalling through MAPK and ERK (extracellular signal-regulated kinase) can upregulate the transcription of genes encoding integrin receptors and ECM proteins so that these signalling pathways affect the cell surface and ECM architecture; a process known as inside-out signalling (Boudreau and Jones,

1999).

1.4.3 Extracellular Composition

As discussed earlier, the composition of the ECM is not static; different ECM proteins are more, or less, prominent in different cell types and tissues and the composition of the ECM may change in response to external factors, such as injury. Cell - matrix interactions can be regulated through post-transcriptional modifications of ECM molecules, such as glycosylation. The amount of calcium bound by an ECM molecule can affect cell adhesion, and interactions between various matrix molecules can affect their affinity for other ECM proteins and cell surface receptors (Adams and Watt, 1993). Degradation of the ECM is controlled by a collection of secreted and cell surface proteinases that cleave one or more components of the ECM, which will be discussed in the following section (Matrisian, 1992).

1.5 Remodelling of the ECM: Extracellular Proteinases

As discussed in previous sections, the ECM is involved in biological processes such as development, wound repair and bone growth. Underlying the different functions involving ECM components are the cell - cell and cell - matrix interactions that are important in order for the cell to interpret and respond to its environment. Inherent in these biological processes is the

dynamic character of the ECM, which differs between tissues and during various stages of development. The various roles played by the ECM in normal biological processes require controlled remodelling through both the synthesis of individual ECM components and through their selective degradation (Hay, 1991). The necessity for controlled and regulated degradation of ECM components is evident in several pathological conditions, notably tumour invasion and metastasis, when the levels of activity of matrix degrading enzymes are perturbed (Krane *et al*, 1990; Liotta *et al*, 1991; Stetler-Stevenson *et al.*, 1993). Tumour cells can become metastatic; they detach from the primary tumour, migrate through the surrounding tissue and invade the circulatory system. Carried by the circulatory system, they travel to a distant location where they extravasate into a tissue and proliferate as a secondary tumour (Liotta *et al*, 1991; Stetler-Stevenson *et al.*, 1993; Hanahan and Weinberg, 2000). A characteristic of metastatic tumour cells is their ability to degrade basement membranes which facilitates their invasion of various tissue types. Many types of tumour cells overexpress extracellular serine proteinases and matrix metalloproteinases (MMPs), downregulate the inhibitors of these proteinases and convert inactive precursors of these proteinases to their active forms (Liotta *et al.*, 1991; Stetler-Stevenson *et al.*, 1993; Hanahan and Weinberg, 2000). Increases in the activity of matrix degrading enzymes are associated with dissociation of a

tumour cell from its surrounding ECM and other tumour cells, invasion of, and subsequent escape from the vascular system, as well as, the production of new capillaries so that the new tumour can grow (Liotta *et al.*, 1991; Stetler-Stevenson *et al.*, 1993; Hanahan and Weinberg, 2000).

Several families of extracellular proteinases have been implicated in the selective degradation of ECM and cell surface components. These include the large family of MMPs, the family of membrane proteins with disintegrin and metalloproteinase domains (ADAMs), astacins and the tissue serine proteinases (Bode *et al.*, 1995/96; Werb, 1997; Streuli, 1999). Collectively, these activities can alter cell - cell and cell - matrix interactions through selective cleavage of particular ECM constituents. It follows that the biological activities of growth factors and other cytokines sequestered within the ECM also change as the composition of the ECM is modified (Streuli, 1999). Consequently, changes to the composition of the ECM, either regulated or unregulated, will alter the signals received by the cell from the extracellular environment, and the cell will respond to those signals (discussed in previous sections). It is clear that when this remodelling occurs in an unregulated fashion disease processes result, providing evidence of the absolute requirement for tightly regulated remodelling processes.

This section will review the families of extracellular proteinases responsible for degradation of ECM components, the multiple levels of

regulation for these activities, and their importance in biological processes.

1.5.1 Extracellular Matrix Proteinases

The MMPs are dependent upon metal ions for activity. A single zinc ion, located at the active site, is required for catalysis, while additional zinc and calcium ions play structural roles (Massova *et al.*, 1998). In addition to their metal-ion requirements, comparison of the primary amino acid sequences of known MMPs has identified several common domains: an amino-terminal domain, a catalytic domain, and a carboxy-terminal domain (Matrisian, 1992; Massova *et al.*, 1998). MMPs contain an amino-terminal pro domain which acts as a signal peptide for secretion (Matrisian, 1992). It is the amino-terminal pro domain which confers latency. It contains the conserved sequence, P(SL)-R(T)-C-G(S)-V(NL)-P(ASYE)-D (letters in parentheses are alternatively found at this site), and it is the cysteine within this sequence which interacts with the catalytic zinc to prevent activity (Massova, *et al.*, 1998). Zymogen activation (of these activities) occurs upon proteolytic removal of the propeptide. All MMPs, with the exception of MMP-23, contain this sequence (Nagese and Woessner, 1999). However, MMP-11 (stromelysin-3) and MMP-14 (MT1-MMP) are secreted in an active form, following intracellular activation by furin (Nagese and Woessner, 1999).

It is the catalytic domain that binds the zinc and calcium ions. This

domain contains a highly conserved sequence, V(AIT)-A(G)-A(TV)-H-E-F(LIV)-G-H-(ALSMV)-L(IM)-G-L(M)-X-H-S(ITV)-XXXXX-L(AFIV)-M, with three well conserved histidines (boldface) responsible for chelating the catalytic zinc (Massova *et al.*, 1998). The conserved methionine residue (boldface) is part of a beta-turn, known as the methionine-turn, which is important to the structure of the active site (Bode *et al.*, 1995/96; Massova *et al.*, 1998). The structural zinc and calcium ions are proximal to the catalytic zinc, but their binding site sequences are less well conserved. The gelatinases, MMP-2 and MMP-9, contain an additional domain, within the catalytic domain, known as the fibronectin-type II-like domain, which is thought to facilitate substrate binding (Nagase and Woessner, 1999).

The C-terminal domain, implicated in determining substrate specificity, contains sequences similar to the heme binding protein, hemopexin, and the ECM component, vitronectin (Matrisian, 1992; Nagase and Woessner, 1999). This domain is absent from the lowest molecular weight member of the MMP family, matrilysin (MMP-7). Some groups suggest that the hemopexin-like domain may facilitate binding of some MMPs to collagen, while other groups have shown that this domain is important for binding of the inhibitor molecules, known as tissue inhibitors of MMPs (TIMPs), to the latent form of gelatinases (Massova *et al.*, 1998). This C-terminal domain has a role in zymogen activation of MMP-2, a gelatinase,

by MT1-MMP, as well as facilitating binding of MMP-2 to the cell surface through interaction with its integrin receptor (Massova *et al.*, 1998). Differences between the sequences of various C-terminal domains, and specifically within the hemopexin-like domain, may account for the substrate specificities of the different MMPs (Matrisian, 1992; Nagese and Woessner, 1999).

Initially these proteinases were named and divided into families based on their selective utilization of different components of the ECM as substrates, however they are also identified as MMP-X, where X is a number assigned to each member of this family (Matrisian, 1992). For instance, collagenase-1, matrilysin, and MT1-MMP are known as MMP-1, MMP-7 and MMP-14, respectively (Nagese and Woessner, 1999). The interstitial and neutrophil collagenase family, which include MMP-1, -8, -13 and -18, cleave collagen molecules at a specific site within the triple-helical region, although members differ in their preference for fibrillar collagens (Hay, 1991; Welgus *et al.*, 1981). The gelatinase subclass of MMPs employ gelatin (denatured collagen) as their substrates and include MMP-2 and -9, although MMP-2 has overlapping substrate specificities (Matrisian, 1992). Aimes and Quigley (1995) showed that this gelatinase, known to cleave denatured fibrillar collagen as well as the non-fibrillar type-IV and -V collagens, was also capable of cleaving fibrillar type-I collagen. MMP-2, along with MMP-9, were also shown to

possess the ability to degrade elastin (Senior *et al.*, 1991). The stromelysin family, MMP-3, -7, -10 and -11, can digest proteoglycans and glycoproteins, including fibronectin and laminin (Matrisian, 1992). Macrophage metalloelastase, MMP-12, can cleave elastin, but also has overlapping substrate specificity. Gronska *et al.* (1997) studied the substrate specificity of human macrophage elastase; in addition to degrading elastin, it degraded fibronectin, laminin, entactin, chondroitin and heparan sulfate, and all components of basement membranes.

Recently, membrane-bound MMP activities have been identified. These membrane-type MMPs (MT-MMP) are unique in that they contain a transmembrane sequence (Birkedal-Hansen, 1995). MT-MMPs are capable of cleaving components in the ECM, and can activate other MMPs. Using a human placenta cDNA library, Sato *et al.* (1994) succeeded in isolating a clone that encoded a MT-MMP. They showed not only that the gene product was localized to the cell surface of transfected cells, but that it could activate pro-gelatinase A.

ADAMs, a second family of extracellular proteinases, are membrane-anchored cell surface proteins which contain disintegrin and metalloproteinase domains (Wolfsberg *et al.*, 1995). The disintegrin domain of the ADAMs contain tripeptide recognition sequences for integrin receptors. These tripeptides are followed by a C-terminal cysteine residue, similar to

snake venom P-III (Wolfberg and White, 1996). Since ADAMs are membrane-bound molecules, interactions between the disintegrin domain of an ADAM and an integrin receptor on another cell may result in cell-cell adhesion, as well as signalling through their transmembrane domains. The proteinase and cell adhesion domains of ADAMs are similar and possess sequence homology with snake venom metalloproteinases (SVMPs), which are responsible for disruption of blood vessels and inhibition of platelet aggregation resulting in hemorrhage (Wolfsberg and White, 1996). In addition, members of the ADAM family contain fusion and transmembrane domains. The fusion domain can potentially interact and fuse with neighbouring membranes, while the transmembrane domain contains a cytoplasmic tail which may be responsible for signalling activity (Wolfsberg and White, 1996). At their C-terminal end, these proteinases also contain EGF repeats.

The metalloproteinase domain of ADAMs, like that of the MMPs, belongs to the zinc-dependent family of metalloproteinases, the metazincins and contains the characteristic amino acid sequence, HEXXHXXGXXH, at the catalytic site, as well as the conserved methionine residue which is important to stabilization of the active site (Bode *et al.*, 1995/96; Wolfberg and White, 1996). Again, the three histidine residues ligate the zinc, as is the case with the MMPs.

The cytoplasmic region of ADAMs is not well conserved and varies considerably in both length and in sequence. This diversity may reflect the variety of interactions between ADAMs and intracellular proteins. For example, ADAMs with cytoplasmic regions rich in proline may interact with and bind cytoskeletal associated proteins such as talin and SH3 domain-containing proteins such as the protein tyrosine kinases, Src, which are responsible for intracellular signalling (Wolfsberg and White, 1996). The potential for interaction with integrin molecules through extracellular domains, in combination with their potential for intracellular interactions with signalling molecules, creates the possibility for bidirectional signalling through ADAMs.

The ADAMs degrade ECM components and cell surface proteins because of their cell surface localization and metalloproteinase domains. Tumour necrosis factor- α converting enzyme (TACE), also known as ADAM 17, cleaves TGF- α and tumour necrosis factor- α (TNF- α) in the ECM, as well as p75 TNF receptor, syndecan and L-selectins at the cell surface (Werb and Yan, 1998; Peschon *et al.*, 1998). The ADAM kuzbanian, from *Drosophila*, proteolytically cleaves the ectodomain of Notch, a cell surface receptor involved in cell fate determinations, resulting in maturation of the receptor so that it can interact with extracellular ligands (Peschon *et al.*, 1998). The

proteoglycan, aggrecan, is cleaved *in vivo* by several members of the MMP family, including MMP-1, -2, -3, -7, -8, and -9. In addition, Arner *et al.* (1999) have identified an aggrecanase activity from bovine nasal cartilage cultures capable of cleaving aggrecan at a site distinct from cleavage by the MMPs. Based upon initial characterization studies, this group suggests that aggrecanase may be a member of the ADAM family.

The third family of extracellular proteinases, the astacins, named for the first member of the group to be identified, also belong to the zinc-dependent metalloproteinase family, the matrixins. However, aside from the characteristic amino acid sequence at the active site (described above) and the presence of the methionine turn, this family has no similarity to the other metalloendopeptidases (Dumermuth *et al.*, 1991). The astacins do contain an N-terminal propeptide sequence and are synthesized as inactive precursors, however, unlike the MMPs, they do not contain a cysteine switch mechanism of activation. In fact, no cysteine residues are present within the propeptide sequence of the α subunit of meprin, a mammalian astacin (Johnson and Bond, 1997). The propeptides of astacins confer latency through a mechanism different from that of MMPs and ADAMs. Furin-type enzymes have been implicated in the cleavage of the inhibitory, pro-domain of some astacins, although other proteinases are also involved in astacin activation since not all astacins contain the consensus sequence for furin cleavage in their pro-

domain (Johnson and Hersh, 1994). Some astacins contain regulatory and/or interactive domains such as EGF domains, CUB (complementary subcomponent C1r/C1s) repeats or regions which serve as membrane anchors (Kessler *et al.*, 1998).

Astacin, the prototype for this subfamily of metazincins, functions as a digestive endopeptidase in the crayfish *Astacus astacus*, and, unlike other members of this family, contains only a catalytic domain (Geier *et al.*, 1997). Crayfish astacin can cleave the triple helix of type I collagen (Stocker and Zwilling, 1995). The astacin family includes the membrane-bound meprins, found in the small intestine and kidney, which are capable of degrading proteins at the cell surface and are involved in processing of biologically active peptides (Johnson and Hersh, 1994; Johnson and Bond, 1997). Other astacins which play roles in the morphogenetic patterning of both vertebrates and invertebrates have been identified (Weinmaster, 1998). Human bone morphogenetic protein, BMP-1, involved in formation of bone and cartilage, is identical to type-I procollagen C-peptidase (Kessler *et al.*, 1996). Bone morphogenetic protein-1 can cleave the C-terminal domains of procollagen types-1, -2 and -3, required for formation of mature collagen fibrils (Geier *et al.*, 1997; Geier and Zwilling, 1998). In addition, Tolloid, an astacin proteinase, is expressed during the early development of *Drosophila* embryos, while other astacins have been shown to be required for the breakdown of the egg

envelope during hatching of fish and bird embryos (Childs and O'Connors, 1994; Geier *et al.*, 1997).

The fourth class of extracellular proteinases are the tissue serine proteinases which include the plasmin/plasminogen activator (PA) system, thrombin, cathepsin G and leukocyte elastase (Hay, 1991; Werb, 1997). Plasminogen activator converts plasminogen to a broad substrate specificity enzyme, plasmin, by cleavage of a single bond. Plasmin can cleave some pro-MMPs, thus playing a role in their activation, and it had been suggested that the serine proteinases may function as activators of MMPs, not as matrix degrading enzymes themselves (Hay, 1991; Werb, 1997). However, plasmin can cleave ECM components such as fibronectin, tenascin, proteoglycans and TGF- β (Werb, 1997). The plasminogen activators include the tissue type PA (TPA) involved in the clotting process and urokinase-type PA (UPA) active in fibrinolysis (Hay, 1991). In addition, these PAs are also involved in biological remodelling of ECMs and can utilize proteoglycans, fibronectin and hepatocyte growth factor as substrates (Werb, 1997). Tenascin is degraded by both cathepsin G and leukocyte elastase (Imai *et al.*, 1996). Pidard *et al.* (1994) show that neutrophil cathepsin G proteolytically processes a glycoprotein receptor on platelets during aggregation. The ectodomains of cell surface proteoglycans, syndecan-1 and syndecan-4 are cleaved by both plasmin and thrombin (Subramanian *et al.*, 1997).

Vu *et al.* (1997) have isolated a membrane-bound serine protease, hepsin, from mammalian preimplantation embryos. Prior to uterine implantation, the zona pellucida surrounding the blastocyst must be degraded. This proteinase is expressed in the mouse at the two cell stage, is absent at the eight cell stage, but is detected again prior to the blastocyst stage. It is a type-II membrane protein with a C-terminal catalytic domain with the characteristic catalytic triad of trypsin-like serine proteinases (Vu *et al.*, 1997). Hepsin is synthesized as a single chain zymogen which is cleaved to produce a mature two chain disulfide linked proteinase, and is capable of autoactivation.

1.5.2 Physiological Functions of the Matrix Degrading Enzymes

Collectively the matrix degrading enzymes are responsible for degradation and remodelling of the ECM and cell surface proteins, and play a role in regulating the activity of biologically active peptides such as growth factors. As discussed earlier, embryonic processes such as cell adhesion, migration, proliferation and differentiation are controlled, in part, by growth factors and cell - cell and cell - matrix interactions. Since the extracellular proteinases reviewed above affect changes in the composition of the ECM, which in turn can affect interactions between the cell and its environment, it is reasonable to infer that these enzymes are important regulators of early

developmental processes. Their role in numerous biological processes of adult organisms has also been identified.

Blobel *et al.* (1992) identified ADAMs-1 and -2 (PH-30- α and - β), which participate in sperm-egg fusion during fertilization. They proposed that binding of the sperm to the egg occurred first, mediated by the disintegrin domain of the ADAM and the integrin receptors. This was followed by hydrophobic interaction of the ADAM fusion domain with the egg membrane, and finally formation of a fusion pore between the sperm and egg. ADAMs also participate in myoblast fusion through cell - cell and cell - matrix interactions, both in developing muscle cells and adult muscle cells (Wolfsberg and White, 1996).

Astacins play a role in the hatching processes of both vertebrate and invertebrate embryos, and affect dorsal/ventral patterning of *Drosophila*, bone and cartilage formation and collagen deposition (Vu *et al.*, 1997). Tolloid, an astacin found in *Drosophila*, affects specification of dorsal cell fate as shown by mutations of the gene encoding this protein (Childs and O'Connors, 1994). Tolloid mediates its effect through interaction with and processing of Decapentaplegic (DPP), a homolog of TGF- β , to a morphogenetically active form (Childs and O'Connors, 1994). BMP-1, a component isolated from bone extracts and shown to play a role in bone

formation, may also function in morphogenesis through activation of TGF- β , like molecules (Wozney *et al.*, 1988; Kessler *et al.*, 1996).

Serine proteinases, such as the easter and snake gene products of *Drosophila*, affect dorsal-ventral patterning of the embryo, and are important to developmental processes (Smith and DeLotto, 1994). They participate in an activation cascade, in which activation of snake will in turn activate easter, which will cleave a protein creating a bioactive molecule important to the process of dorsal-ventral patterning. As mentioned previously, a membrane-bound serine protease identified in mammalian preimplantation embryos is important for uterine implantation of the embryo (Vu *et al.*, 1997).

The process of wound repair necessitates rapid and organized remodelling of the ECM. At the site of injury, interactions between keratinocytes and the ECM change because the cells are now in contact with dermal collagen. These cells must migrate through the fibrin clot to establish epithelial interactions with the basement membrane, healing the wound (Streuli, 1999). Both serine proteinases and MMPs are important in the process of wound repair. Lund *et al.* (1999) showed that mice which have a disrupted plasminogen gene heal wounds more slowly than normal mice. They propose that under normal circumstances, plasmin and MMPs function together during wound repair and that, although slowed, wound repair can occur in the absence of plasmin, due to the activity of MMPs. They showed

that in the absence of MMP and plasmin activity, keratinocyte migration and the process of wound repair did not occur.

Angiogenesis depends upon localized lysis of a vessel wall and basement membrane followed by outgrowth of a population of endothelial cells (Hay, 1991). Expression of both MMP-2 and MT1-MMP, an activator of the pro enzyme form of MMP-2, increase during this process, allowing the putative endothelial cells to migrate and proliferate (Streuli, 1999). Conversely, a decrease in MMP-2 activity is associated with the differentiation of putative endothelial cells and the formation of mature blood vessels (Streuli, 1999).

1.5.3 Regulation of Matrix Degrading Proteinases

The activity of matrix degrading enzymes is controlled at multiple levels: transcriptionally, post-transcriptionally through activation of latent enzymes, and through complex formation with specific inhibitors. The expression of genes encoding matrix degrading enzymes is differentially and developmentally regulated, so that a particular gene is expressed only in certain tissues and only at particular times during development, or in response to cellular signals. Gene expression can be upregulated by growth factors (such as TGF- α , EGF, FGF and PDGF), cytokines (such as IL-1) and chemical agents (such as phorbol esters) or suppressed by other growth factors

(such as TGF- β), retinoic acids and glucocorticoids (Birkedal-Hansen, 1995; Nagase and Woessner, 1999).

These enzymes are also regulated at the post-transcriptional level. Most MMPs, excluding the MT-MMPs and MMP-11, are secreted in their inactive proenzyme form (Nagase and Woessner, 1999). These MMPs, as well as the ADAM family, have their catalytic zinc ion complexed to a cysteine residue in the prodomain, and are activated by what is known as a "cysteine switch" mechanism. Proteolysis of the prodomain results in dissociation of the cysteine from the zinc and activation of the latent enzyme. Both membrane-bound MMP and UPA/plasmin have been implicated as activators of these proenzymes (Nagase and Woessner, 1999). Astacins, although synthesized as proenzymes and containing a zinc-dependent catalytic domain similar to the MMPs and ADAMs, do not possess a cysteine switch mechanism of activation (Johnson and Bond, 1997). Johnson and Hersh (1994) speculate that the pro sequence may be removed by autoactivation or tryptic cleavage followed by trimming of the N-terminus by an aminopeptidase. As well two amino acids are trimmed from the C-terminus by the activity of a carboxypeptidase B, also present in *A. astacus*. Serine proteinases are synthesized as single chain zymogens and require cleavage to generate an active, disulfide-linked two chain form (Hay, 1991; Subramanian *et al.*, 1997).

Regulation of MMP activity also occurs through inhibition by tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs (TIMP 1-4) have been identified in humans with homologous molecules identified in amphibians, fish, insects and *Caenorhabditis elegans* (Brew *et al.*, 2000). The TIMPs can bind to most MMPs forming a 1:1 enzyme-inhibitor complex, however the extent of inhibition varies. For instance, MT-MMPs are inhibited effectively by TIMP-2 and TIMP-3, but not TIMP-1 (Brew *et al.*, 2000). Additionally, TIMPs can bind to the pro-form of MMPs and this interaction can mediate their activation by MT-MMPs. Differences in the expression of MMPs and TIMPs may provide tighter control of ECM degradation.

Although ADAMs contain four characteristic functional domains including the zinc-dependent catalytic domain, there are sequence differences among domains within the ADAM family (Wolfsberg and White, 1996). Therefore, some domains within an ADAM may not be functional, so that a particular ADAM possesses only a subset of possible functions.

Serine proteinases are inhibited by serine proteinase inhibitors (serpins) such as α_2 -macroglobulin, antithrombin III and α_2 -antiplasmin (Subramanian *et al.*, 1997; Riddihough, 1994). These inhibitors interact with the active site of serine proteinases forming stable covalent complexes (Riddihough, 1994). Inhibitor-serine proteinase complexes can interact with

cell surface receptors and lead to internalization, or the serpin can be cleaved and inactivated, and slowly released from this complex (Chen, 1992; Riddihough, 1994).

1.6 The Sea Urchin

1.6.1 The Sea Urchin as a Model System for Developmental Studies

For over one hundred years the sea urchin embryo has been widely used as a system to study development. There are several advantages in using this system to study development. They include the ease in obtaining large numbers of gametes which can be grown as synchronous cultures of embryos for biochemical studies. For many warm water species of sea urchin, gametes can be obtained throughout the year, while gametes of *Strongylocentrotus purpuratus* are available for only a four month season. Some groups switch from species to species to obtain gametes throughout the year. The embryos have a relatively small number of cells in comparison to more complex organisms. The pluteus stage embryos of *Strongylocentrotus purpuratus* consist of approximately 1500 cells (Angerer and Davidson, 1984). Cell lineages, tracing the developmental fate of embryonic cells, have been identified and a number of molecular markers for specific genes and gene products are available to follow the differentiation of cells during development. For instance, the cytoskeletal genes CyIII and Spec

(*Strongylocentrotus purpuratus* ectoderm specific) are expressed by aboral ectoderm cells (Angerer and Davidson, 1984). As well, the utility of the embryo has been enhanced by the ability to produce and culture transgenic embryos (Flytzanis *et al.*, 1985; Cameron and Davidson, 1991).

1.6.2 Extracellular Matrix of the Sea Urchin

The sea urchin embryo has two ECMs: the hyaline layer on the apical surface and the basal lamina on the basal surface of ectoderm cells. Hyaline layer assembly occurs shortly following fertilization, as components of the layer are sequentially released from storage compartments within the egg (Cameron and Holland, 1985; Alliegro and McClay, 1988). Vertebrate-like ECM molecules have been detected and identified in both the hyaline layer and basal lamina of the sea urchin using polyclonal antisera prepared against vertebrate ECM molecules, including laminin, fibronectin, heparin sulfate proteoglycans and various collagen subtypes (Wessel *et al.*, 1984; Alliegro and McClay, 1988). Recent studies have identified genes expressed in the sea urchin embryo, that encode collagens with sequence similarities to vertebrate collagens. Exposito *et al.* (1994) sequenced a vertebrate type IV-like collagen chain from the sea urchin embryo. Reports also confirm the presence of collagen species from the adult tissues of the sea urchin (Burke *et al.*, 1989; Tomita *et al.*, 1994; Robinson, 1997).

Studies have provided evidence that a properly constituted ECM is required for sea urchin development. Benson *et al.* (1991) demonstrated that inhibition of lysyl oxidase, which is necessary for collagen cross-linking and deposition, arrested development at the mesenchyme blastula stage. However, these embryos could be rescued by the growth factors, human PDGF-BB and TGF- α so that they resumed development into the gastrula stage (Ramachandran *et al.*, 1993). Similarly Benson *et al.* (1991) found that inhibition of proteoglycan metabolism in the sea urchin embryo with the inhibitor, alpha-D-xylose, also resulted in arrest of development at the mesenchyme blastula stage. As well, expression of Endo 1, an endoderm specific cell surface protein, did not occur at the blastula stage (Wessel and McClay, 1985). Wessel *et al.* (1989) studied the effect of disrupting collagen deposition within the basal lamina on the levels of RNA for several species. Even though most RNA levels that were studied seemed unaffected, they found that an aboral ectoderm specific protein did not accumulate since the gene encoding this protein was inactive in the absence of collagen in the basal lamina. These experiments provide evidence that a properly constituted ECM is required for sea urchin development, and collectively endorse the utility of the sea urchin embryo as a model system to study the influence of ECM composition on developmental processes.

1.6.3 Remodelling of the Sea Urchin Extracellular Matrix

Several classes of matrix-cleaving proteinases have been identified in the sea urchin embryo. Lepage *et al.* (1992) isolated a cDNA clone which encoded an astacin-like proteinase, BP10 (Blastula Protease 10), expressed during the blastula stage of development in *Paracentrotus lividus*. BP10, a secreted proteinase, is homologous to the human BMP-1 and tolloid from *Drosophila* (Lepage *et al.*, 1992; Lhomond *et al.*, 1996). Reynolds *et al.* (1992) identified the equivalent of BP10, SpAN, in *Strongylocentrotus purpuratus*. The hatching enzyme, responsible for cleavage of the fertilization envelope during hatching of the blastula stage embryo, is a member of the MMP family (Nomura *et al.*, 1997). In addition, several groups have reported the presence of collagen and gelatin cleaving activities in the sea urchin embryo (Karakiulakis *et al.*, 1993; Quigley *et al.*, 1993; Vafa and Nichioka, 1995; Mayne and Robinson, 1996; Robinson, 1997).

1.7 Thesis Focus

Generally, we are interested in studying developmental processes which occur during embryonic development. More specifically, we have been interested in identifying and characterizing components of the ECMs in the sea urchin. Hyalin, the major protein of the hyaline layer, has been characterized (Robinson, 1988; Robinson, 1989; Robinson, 1990; Robinson,

1991; Robinson, 1992; Robinson and Brennan, 1991; Robinson *et al.*, 1992). In addition, we have cloned and characterized a 32 kDa component of the hyaline layer and basal lamina, HLC-32 (Robinson, 1992; Brennan and Robinson, 1994). Immunoelectron microscopy revealed the storage compartment for HLC-32 in the unfertilized egg and developing embryo (Mayne and Robinson, 1998). Recently, our focus has shifted toward defining the functional role(s) of individual components of the ECM during development. Since a properly constituted ECM is required for sea urchin development, we want to develop techniques that could alter its composition. The ability to cleave individual components within the ECMs would afford us the opportunity to study the effects of remodelling on the developing sea urchin embryo.

Initially I employed a qualitative assay, gelatin substrate gel zymography, to detect gelatin-cleavage activities in the egg and embryo. I decided to focus my research on the characterization of the major gelatin-cleavage activity, a 41 kDa species. Several criteria were used to establish that the 41 kDa gelatinase is an invertebrate extracellular matrix-degrading proteinase. Its metal-ion requirements were determined as well as its sensitivity to various classes of proteinase inhibitors (chapter 3). I determined its substrate specificity and identified physiological substrates for this activity (chapter 4). The subcellular localization was determined since vertebrate

matrix-degrading enzymes are synthesized within the cell and secreted into the ECM (chapter 5). In addition, I was interested in investigating the effect of the 41 kDa activity on embryonic development (chapter 6). The detailed biochemical analysis of the 41 kDa activity provided insight into the biological role played by this enzyme. As well the extracellular localization of the 41 kDa activity so that it has access to its substrates, taken together with its demonstrated substrate specificity and kinetically relevant collagen- and gelatin- cleavage activities suggested that the 41 kDa is a biologically significant activity.

CHAPTER 2: MATERIALS AND GENERAL METHODS

2.1 Growth of Embryos

Strongylocentrotus purpuratus (Sp) were purchased from Seacology, Vancouver, Canada. Gametes were obtained by intracoelemic injection with 0.5 M KCl (Fisher). Eggs were filtered through a 300 micron nitex filter and washed four times in millipore-filtered seawater (MFSW; 0.45 mm). Eggs were fertilized with a dilute suspension of sperm, and grown at 10°C - 12°C in a cylindrical chamber with constant aeration. The chambers contained paddles that rotated at 40 rpm. The efficiency of fertilization, determined by microscopic examination of the formation of the fertilization membrane, was typically 98 - 100%, with little to no variation between batches of eggs. Eggs and embryos at various stages of development were harvested by centrifugation at 2000 X g. The eggs and embryos were washed twice in MFSW, centrifuged at 2000 X g, and stored at -70°C.

2.2 Purification of the 41 kDa Collagenase/Gelatinase

2.2.1 Sucrose Density Gradient Ultracentrifugation

Eggs were resuspended to 10% (v/v) in Ca^{2+} -, Mg^{2+} - free seawater [CMFSW; 500 mM NaCl, 10 mM KCl, 2 mM NaHCO_3 and 3 mM Na_2SO_4 (Fisher)] made 50 mM in Tris-HCl, pH 8.0 (Sigma), 20 mM in benzamidine-HCl (Kodak) and homogenized at 0°C. The homogenate was centrifuged at 2000 X g and 4°C for 8 minutes. The supernatant was loaded

onto a 10% - 40% (w/v) sucrose (Bio-Rad) step gradient, followed by centrifugation in a SW 41 rotor at 160,000 X g and 4°C for 18 hours.

2.2.2 Gel Exclusion Chromatography

Sucrose gradient fractions containing the 41 kDa collagenase/gelatinase were pooled and loaded onto a 3 x 35 cm column of the gel exclusion resin, Bio-Gel P-200 (Bio-Rad), equilibrated in 50 mM Tris-HCl, pH 8.0. Sixty drop fractions were collected at a flow rate of 1 drop per 2 minutes.

2.2.3 Ion Exchange Chromatography

Fractions collected from the Bio-Gel P-200 column and containing the 41 kDa collagenase/gelatinase were combined and concentrated 10-fold in an Amicon Centriprep-10 Concentrator. Ion exchange chromatography was performed in a 1 x 4 cm column of DEAE-cellulose (Sigma) equilibrated in 20 mM Tris-HCl, pH 8.0. The column was fractionated with a step gradient of NaCl (0.05-0.45 M; Fisher) in 50 mM Tris-HCl, pH 8.0. The 41 kDa collagenase/gelatinase eluted with 0.30-0.45 M NaCl. All chromatographic procedures were carried out at 4°C.

2.3 Electrophoretic Methods

Electrophoresis was carried out using a Mighty Small II electrophoresis

apparatus (Hoefer Scientific Instruments) and ran on gels (1.5mm thick) containing 10 wells. The electrophoresis buffer contained 25 mM Tris, 200mM glycine (Sigma) and 0.1% (w/v) sodium dodecyl sulfate (SDS; Bio-Rad). Following electrophoresis gels were stained in a solution containing 0.25% (w/v) Coomassie Brilliant Blue R-250 (CBB; Bio-Rad), 45% (v/v) methanol (Fisher) and 10% (v/v) acetic acid (Fisher) for 1 hour at 37°C. Gels were destained in a solution containing 10% (v/v) acetic acid and 7% (v/v) methanol at 37°C. Alternatively, some gels were stained at room temperature using a PlusOne Silver Staining Kit (Pharmacia Biotech).

2.3.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) in 10% (w/v) polyacrylamide [30% (w/v) acrylamide (Bio-Rad); 0.8% (w/v) bisacrylamide (Life Technologies Inc.)] slab gels at a constant current of 30 mA. Samples were precipitated with an equal volume of 20% (w/v) trichloroacetic acid (TCA; Fisher) on ice, for 20 min, and centrifuged in a microcentrifuge (Eppendorf) at 16,000 X g for 10 minutes. Pellets were resuspended in 15 μ L Laemmli solubilizing solution [0.1 M DTT (Sigma), 0.2 M Tris, 2% (w/v) SDS, 32% (v/v) glycerol (Sigma) and 0.01% (w/v) Bromophenol Blue (BPB; Fisher)]. An additional 5 μ L 10%(w/v) SDS was added, the sample boiled for two minutes and loaded onto the gel.

2.3.2 Substrate Gel Zymography

Substrate gel zymography was carried out using the method of Heussen and Dowdle (1980). Aliquots of eggs, embryos or the purified collagenase/gelatinase were incubated for 30 min at room temperature in an equal volume of Laemmli solubilizing buffer from which the BPB and the reducing agent, DTT, had been omitted. Electrophoresis was performed in 10% (w/v) polyacrylamide slab gels, prepared by copolymerizing acrylamide/bisacrylamide [30% (w/v) acrylamide (Bio-Rad); 0.8% (w/v) bisacrylamide (Life Technologies Inc.)] and 0.1% (w/v) gelatin (Bio-Rad) or a test substrate, at a constant current of 8 mA and at 4°C for 4 hours. Following electrophoresis the gels were incubated in a solution containing 2.5% (v/v) Triton X-100 (Sigma) and 50 mM Tris-HCl, pH 8.0, for 1 hour at room temperature. The gels were transferred to a solution containing 50 mM Tris-HCl, pH 8.0 and 10 mM CaCl_2 (Sigma) for 16 hours, stained with CBB and destained, as described above. Clear bands of proteolytic activity were seen against a blue background of stained intact substrate.

2.4 Quantitative Analyses

2.4.1 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) at

16,000 X g for 10 minutes and the supernatants discarded. Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA; Sigma) as a standard. Absorbances were read at 750 nm in a Spectronic 601 spectrophotometer (Milton Roy).

2.4.2 Quantitative Gelatinase Assay

Gelatinase activity was assayed by quantitating the cleavage of gelatin into TCA soluble peptides. Aliquots of collagenase/gelatinase were incubated in a 100 μ L solution containing 50 mM Tris-HCl, pH 8.0, 0.1% (w/v) gelatin and 10 mM CaCl_2 for 60 min at 37°C. Two control reactions were set up. One, to ensure that cleavage of the gelatin was dependent on the collagenase/gelatinase, was identical to the above except that the collagenase/gelatinase was omitted. At the end of the incubations, the samples were precipitated with an equal volume of 20% (w/v) TCA at 0°C for 20 min. The other, a control for measuring the amount of total protein present before hydrolysis by the collagenase/gelatinase contained all the reagents listed above but 20% (w/v) TCA was added at zero time. Samples were centrifuged in a microcentrifuge (Eppendorf) at 16,000 X g for 10 min and the protein concentrations were determined by method of Lowry *et al.* (1951). The percent hydrolysis of the substrate was defined as;

$$\frac{\text{Abs 0 min} - \text{Abs 60 min}}{\text{Abs 0 min}} \times 100$$

Abs 0 min

Absorbance was recorded at 750 nm (Spectronic 601 spectrophotometer) and a unit of gelatinase activity is defined as 0.1 μ g gelatin cleaved/minute at 37°C. Standard gelatinase assays were performed to ensure that the assays described above were measured in the linear range for both gelatinase activity versus time and for gelatinase activity versus concentration of gelatinase.

2.5 Biophysical Characterization

2.5.1 Circular Dichroism

Circular dichroism (CD) spectra in the far-ultraviolet (UV) region, from 190-280 nm, were recorded at room temperature on a model J500-A JASCO spectropolarimeter and processed with a DP-500N data processor. Samples (0.5 mg/ml) of the purified collagenase/gelatinase were dialysed against 50 mM Tris-HCl, pH 8.0 containing 0.1 mM EGTA (Fisher). Spectra were measured in the absence and presence of 10 mM CaCl_2 . The measurements were carried out in a quartz cell with a pathlength of 0.1 cm. Background spectra were recorded for each buffer and were subtracted from the recorded spectra of the protein. Secondary structure was calculated from the CD spectra using the method of Chen *et al.* (1974). Protein concentrations were

determined by the method of Lowry *et al.* (1951).

2.5.2 Amino Acid Analysis

Amino acid analyses were performed after hydrolysis of the sample for 72 hours in 6M HCl at 110°C. Liberated amino acids were fractionated with a Beckman 121 amino acid analyzer (Amino Acid Facility; Memorial University of Newfoundland). Tryptophan content was not determined.

2.6 Immunological Methods

2.6.1 Preparation of Polyclonal Antisera

Aliquots (50mg) of the purified collagenase/gelatinase were boiled for 2 min in an equal volume of 2% (w/v) SDS. The SDS bound to the collagenase/gelatinase was removed by acetone precipitation and the protein resuspended in 50 mM Tris-HCl, pH 8.0, and an equal volume of incomplete Freund's adjuvant (Cal Biochemicals) added. A New Zealand white rabbit was injected subcutaneously with this sample. The rabbit was twice boosted (with aliquots (50mg) of the purified collagenase/gelatinase prepared as above) at two-week intervals and blood was withdrawn 10 days after the last injection. Prior to injection 10 mL of whole blood was withdrawn and used to prepare preimmune sera.

Preimmune and immune sera were prepared by precipitation of sera

with 50% (w/v) $(\text{NH}_4)_2\text{SO}_4$ (Fisher) on ice for 1 hour, with constant stirring. Samples were centrifuged at 10,000 X g at 4°C for 20 min. Pellets were resuspended in 50 mM Tris-HCl, pH 8.0, and dialysed against 50 mM Tris-HCl, pH 8.0 at 4°C for 2.5 hours. Further purification of preimmune and immune sera were carried out using the Protein-A Purification Kit (Sigma).

2.6.2 Protein Gel Blot Analysis (Western Blotting)

Samples were precipitated in an equal volume of 20% (w/v) TCA on ice, the pellets were solubilized and fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). The protein was transferred onto nitrocellulose membrane (Gelman Sciences) by electroblotting at 60V for 1.5 hours in 25 mM Tris, 0.2 M glycine and 20% (v/v) methanol. The nitrocellulose membrane was blocked overnight at room temperature by incubation in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 3% (w/v) BSA. The nitrocellulose membrane was probed with antisera at 1:200 dilution in washing buffer [50 mM Tris-HCl, pH 7.4, 0.05 % (v/v) NP-40 (Fisher), 0.1% (w/v) SDS, 20 mM NaI (Fisher), 2% (w/v) BSA] for 6 hours at room temperature. The nitrocellulose membrane was washed overnight at room temperature in washing buffer, then incubated with 3 mCi ^{125}I -Protein A (Amersham) in washing buffer for 2 hours at room temperature. The

nitrocellulose membrane was washed for 3 X 15 min in washing buffer, then allowed to air dry. The nitrocellulose membrane was set up for autoradiography with Kodak X-Omat AR film at -70°C.

**CHAPTER 3: CALCIUM BINDING AND ACTIVATION OF A
41 kDa COLLAGENASE/GELATINASE**

3.1 INTRODUCTION

As discussed previously, the ECM plays an important role in regulating interactions between the cell surface and the extracellular environment. Cell surface and extracellular proteinases are collectively responsible for selective remodelling and degradation of the constituent components of ECMs. These ECM-degrading enzymes which include the MMPs, ADAMs, astacins, and serine proteinases modulate cellular processes which are dependent upon the composition of the ECM.

The presence of vertebrate-like ECM molecules and the necessity for a properly constituted ECM during sea urchin embryonic development prompted a search for matrix-degrading activities in the sea urchin egg and embryo. Using the technique of gelatin substrate gel zymography, candidate activities have been identified. Four gelatinase activities of apparent molecular masses of 55, 50, 42 and 38 k were identified in *Arbacia punctulata* at various stages of development (Quigley *et al.*, 1993). Vafa and Nishioka (1995) identified several gelatinase activities from blastula and gastrula stage embryos in *Lytechinus pictus* and *Strongylocentrotus purpuratus*. Employing a collagen-cleavage assay, Karakiulakis *et al.* (1993) identified an activity in blastula stage embryos of *Lytechinus pictus* capable of cleaving both collagen and gelatin. The temporally expressed gelatinase activities in developing *Arbacia punctulata* and *Lytechinus pictus* embryos were sensitive to both the

general divalent cation chelator, EDTA and the zinc-specific chelator, 1,10-phenanthroline.

We have detected a dynamic pattern of gelatinase activities during embryonic development of the sea urchin *Strongylocentrotus purpuratus*. Activities ranging in apparent molecular mass from 27.5 to 86.9 k were seen (Mayne and Robinson, 1996). All these activities were sensitive to inhibition by EDTA, while the 86.9 kDa species was also inhibited by 1,10-phenanthroline (Mayne and Robinson, 1996). We initiated a study of a 41 kDa species which is the predominant gelatinase activity in the egg and early embryo.

3.2 MATERIALS AND METHODS

3.2.1 Reactivation of EGTA-Inhibited Enzyme

The collagenase/gelatinase was dialyzed against 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA overnight at 4°C. Following dialysis, aliquots of the collagenase/gelatinase were incubated with varying concentrations of CaCl_2 . The gelatinase activity was measured using the quantitative gelatinase assay described in chapter 2, section 2.4.2. A control which was not dialysed was assayed in the presence of 10 mM CaCl_2 and this activity was normalized to 100%.

3.2.2 Equilibrium Dialysis

Aliquots (40 or 50 μg) of the collagenase/gelatinase were brought to a final volume of 1 ml in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA. Samples were dialyzed against 100 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA, 20 μCi $^{45}\text{CaCl}_2$ (Amersham; 0.4 mCi/ μmol) and various concentrations of CaCl_2 . Free calcium concentrations were determined using the EQCAL computer program from Biosoft (Cambridge, England). Dialyses were carried out for 72 hours at 4°C. Following dialysis, 50 μl was removed from each dialysis bag along with an equal volume of dialysate and added to 10 ml of Scinti Verse E liquid scintillation fluid (Fisher). The samples were counted in a Beckman model LS 9000 liquid scintillation counter. The counting efficiency was determined to be 85% using known quantities of $^{45}\text{CaCl}_2$. All glassware used during these experiments was soaked overnight in 5 mM EDTA and washed extensively in deionized water. All plasticware was siliconized.

3.2.3 Gel Exclusion Chromatography

A 50 μg aliquot of the purified collagenase/gelatinase was dialysed at 4°C against 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA and fractionated on a Sephacryl S-200 gel exclusion column (1 X 50 cm) equilibrated in 50 mM

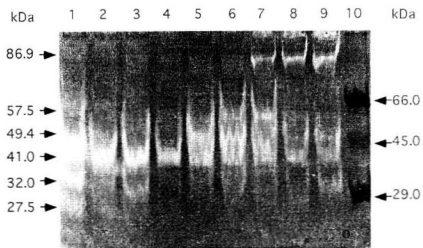
Tris-HCl, pH 8.0. Ten drop fractions were collected at a flow rate of 1 drop per 30 sec. The column was calibrated with the following molecular mass markers: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), yeast alcohol dehydrogenase (150 kDa) and β -amylase (200 kDa) (Sigma). Collagenase/gelatinase activity was assessed using the quantitative gelatinase assay described in chapter 2, section 2.4.2.

3.3 RESULTS

3.3.1 Preliminary Identification of Gelatin-Cleaving Activities in Sea Urchin Egg and Embryo

We employed gelatin substrate gel zymography to visualize the profile of gelatinase activities present during sea urchin embryonic development (Mayne and Robinson, 1996). Figure 3-1 shows a reproduction of a developmental zymogram. The gelatinase species ranged in apparent molecular mass from 27.5 - 86.9 k. The 41 kDa gelatinase was present in the egg (lane 1) and in diminishing amounts throughout embryonic development, while an 86.9 kDa gelatinase appeared at the gastrula stage (lane 7) and increased during subsequent development. In addition, a 49.4 kDa activity appeared at the blastula stage (lane 5), decreasing during subsequent development, while a 57.5 kDa activity was present only during early and late gastrulation (lane 6 and lane 7).

Figure 3-1. Gelatin substrate gel zymography of eggs and embryos. Fifteen microgram aliquots of protein from eggs and various stage embryos were solubilized for 30 minutes at room temperature in an equal volume of solubilizing solution (Laemmli, 1970) from which the bromophenol blue and dithiothreitol had been omitted. Electrophoresis was performed in a 10% (w/v) polyacrylamide slab gel prepared by copolymerizing acrylamide and 0.1% (w/v) gelatin. Following electrophoresis the gel was processed in the presence of 10 mM CaCl_2 as described in chapter 2, section 2.3.2. Lanes 1-9 contain unfertilized eggs, 1, 9.5, 21.5, 27, 32, 46, 53 or 70.5 hour old embryos, respectively. Lane 10 contained molecular mass markers (Sigma Chem Co.); bovine serum albumin, 66 kDa, egg albumin, 45 kDa and carbonic anhydrase, 29 kDa.



The 41 kDa species, the dominant gelatinase activity present in the egg and during early stages of development, was purified from egg homogenate by a combination of sucrose density gradient ultracentrifugation, gel exclusion chromatography and ion exchange chromatography as outlined in chapter 2, section 2.2. Purity was assessed by SDS-PAGE analysis (data not shown; Mayne and Robinson, 1996). Specific activity increased from 13 ± 7.5 units/mg in sucrose gradient fractions to $10,997 \pm 384$ units/mg in fractions from the DEAE-cellulose column, an 839-fold increase in purity (Table 3-1; Mayne and Robinson, 1996). Initial work demonstrated that the 41 kDa species was specific for gelatin as a substrate (Appendix A) and that calcium was required for activity (Appendix B) suggesting that we had purified a candidate matrix-degrading proteinase (Mayne and Robinson, 1996).

3.3.2 Determination of Native Molecular Mass

The collagenase/gelatinase migrated with an apparent molecular mass of 41 k when assessed by either SDS-PAGE or gelatin substrate gel zymography under non-reducing conditions. To determine the native molecular mass we used gel exclusion chromatography, employing a Sephacryl S-200 column, equilibrated in 50 mM Tris-HCl, pH 8.0 (Figure 3-2). The collagenase/gelatinase eluted from the column in a major peak corresponding to an apparent molecular mass of 160 k. The quaternary

TABLE 3-1: PURIFICATION OF THE 41 kDa COLLAGENASE/GELATINASE

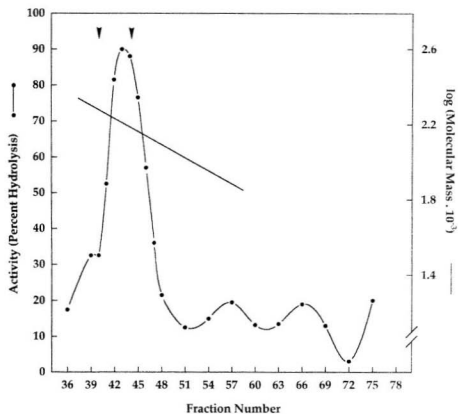
Fraction	Specific Activity (units/mg)	Fold-Purification	Total Units	Percent Yield
Egg Homogenate	ND	ND	ND	ND
Sucrose Density Gradient	13.1 ± 7.5	1	6080 ± 2774	100
P-200 Column	912 ± 287	70	5030 ± 1651	83
DEAE-Cellulose	$10,997 \pm 387$	839	4136 ± 167	68

ND; not determined

Note: Five microlitre aliquots of each fraction was incubated with 0.1% (w/v) gelatin and the cleavage of gelatin measured using the quantitative gelatinase assay as described in chapter 2, section 2.4.2. The fractions from the P-200 column and the DEAE-cellulose column were incubated with gelatin for 60 minutes at 37°C and the sucrose density gradient fractions were incubated with gelatin overnight at 37°C before precipitating the samples with TCA. Activity is expressed as units/mL. One unit of activity is defined as the amount of gelatinase required to cleave 0.1 µg of gelatin into TCA soluble peptides per minute at 37°C. Half of this data was collected by J. Robinson and half was collected by J. Mayne.

Values are means \pm SEM (n=4)

Figure 3-2. Native Molecular Mass Determination of 41 kDa collagenase/gelatinase. Aliquots of 41 kDa collagenase/gelatinase were fractionated in a Sephacryl S-200 gel filtration column equilibrated in 50 mM Tris-HCl, pH 8.0. Fractions eluted from the column were assessed for gelatin-cleavage activity using the quantitative gelatinase assay described in chapter 2, section 2.4.2. The column was calibrated with the following proteins; carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), yeast alcohol dehydrogenase (150 kDa) and β -amylase (200 kDa). Standards were eluted from the column and fractionated on 10 % (w/v) acrylamide gel. Arrows indicate the elution of β -amylase (fraction 40) and alcohol dehydrogenase (fraction 46). The data presented represent the elution profile in the absence of calcium.

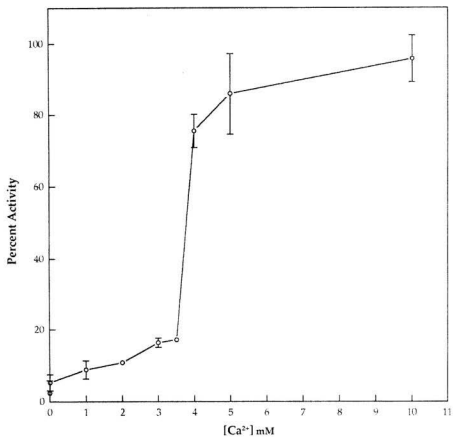


structure of the collagenase/gelatinase was not altered by the presence of 10 mM CaCl_2 (data not shown). This result indicated a tetrameric structure for the native collagenase/gelatinase, although the monomeric 41 kDa subunits are active, as assessed by gelatin substrate gel zymography.

3.3.3 Reactivation of EGTA-Inhibited Enzyme

Studies of extracellular proteinases responsible for degradation of ECM components show that they are dependent upon metal ions for activity. Vertebrate MMPs, ADAMs and astacins require zinc for activity. Our initial studies with the 41 kDa collagenase/gelatinase revealed that zinc was inhibitory, while calcium reactivated the EDTA-inhibited 41 kDa collagenase/gelatinase (Appendix B; Mayne and Robinson, 1996). We have examined the calcium-concentration dependence of reactivation. Addition of 0.1 mM EGTA to the collagenase/gelatinase or dialysis against 0.1 mM EGTA effectively inhibited the enzyme. Figure 3-3 shows that in the presence of 0.1 mM EGTA, the collagenase/gelatinase retains only 2% of its activity. Upon addition of increasing calcium concentrations, the 41 kDa collagenase/gelatinase regained 95.8% activity, when compared to uninhibited enzyme. The apparent dissociation constant for reactivation was 3.7 mM. While the 41 kDa species is dependent upon calcium for activity, the binding of calcium to this enzyme is weak with an apparent binding constant is $2.7 \times 10^2 \text{ M}^{-1}$.

Figure 3-3. Reactivation of the EGTA-Inhibited 41 kDa Collagenase/Gelatinase. The 41 kDa collagenase/gelatinase was dialysed against 50 mM Tris-HCl, pH 8.0 containing 0.1 mM EGTA overnight at 4°C. Following dialysis, 1 μ L aliquots (20 units) of the 41 kDa collagenase/gelatinase were incubated in 80 μ L of 50 mM Tris-HCl, pH 8.0 containing 0.1 mM EGTA and various concentrations of CaCl₂. Samples were incubated on ice for 1 hr and then gelatinase activity was measured using the quantitative gelatinase assay described in chapter 2, section 2.4.2. A control was both dialysed and incubated in the absence of EGTA and assayed in the presence of 10 mM CaCl₂. The activity in this sample was normalized to 100%. Values represent mean \pm SEM (n=5).



3.3.4 Quantitation of Calcium Binding

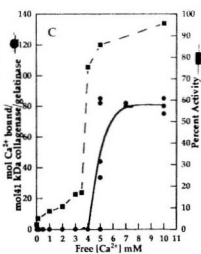
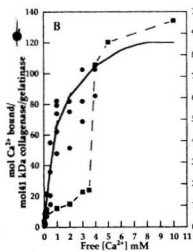
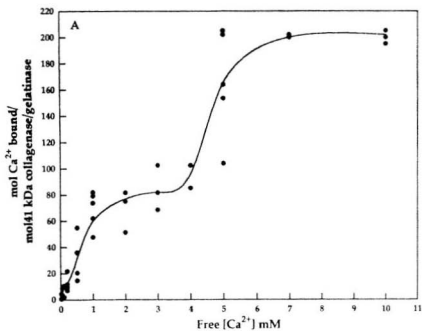
To further characterize the calcium-ion requirement, we quantitated the amount of calcium binding to the collagenase/gelatinase by equilibrium dialysis of the EGTA-inhibited enzyme in the presence of increasing calcium concentrations (Figure 3-4; Panel A). There were two classes of sites which bound calcium ions. To facilitate interpretation of this data we analysed separately the individual binding curves for both classes of sites. The data collected for calcium binding to the collagenase/gelatinase up to 4 mM was interpolated using the equation for a rectangular hyperbola. Calcium binding to the collagenase/gelatinase up to 10 mM was determined using the equation for a sigmoidal curve and subtracting the amount of calcium bound to the first class of sites calculated from the first equation. We determined that 120 mols of calcium bound per mol of the 41 kDa collagenase/gelatinase with a dissociation constant (K_d) of 0.5 mM (Figure 3-4; Panel B), while an additional 80 mols of calcium bound with a K_d of 5 mM (Figure 3-4; Panel C). These results suggested that the 41 kDa collagenase/gelatinase bound a large number of calcium ions with weak affinity since the K_d was in the millimolar range for both classes of sites. As a control, we quantitated the amount of calcium binding to Troponin C using the technique described above. We determined

Figure 3-4. Quantitation of calcium binding to the 41 kDa collagenase/gelatinase.

Panel A: Aliquots of 41 kDa collagenase/gelatinase (40 or 50 μ g) were brought to a final volume of 1 mL in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA and dialysed against 100 mL of 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA, 20 μ Ci $^{45}\text{CaCl}_2$ (Amersham; 0.4 mCi/ μ mol) and various concentrations of CaCl_2 for 72 hrs at 4°C. The data presented are a compilation of 12 independent experiments. The curve is fit to the equation; $\text{Ca bound} = -27.51 + (107.08 / (1 + 10^{\log \text{EC}_{50} - [\text{Ca}]}) + (127.2 * [\text{Ca}]) / (0.4484 + [\text{Ca}])$

Panel B represents the individual analysis of the class of calcium binding sites with a K_d of 0.5 mM (—•—). This data is overlaid with the calcium reactivation curve (---=---) presented in Figure 3-3.

Panel C represent the individual analyses of the class of calcium binding sites with a K_d of 5 mM (—•—). This data is overlaid with the calcium reactivation curve (---=---) presented in Figure 3-3.



that four mols of calcium bound per mol of Troponin C (Appendix C). There were two classes of binding sites, each binding two mols of calcium with K_d s of 1×10^{-7} and 4×10^{-5} M. These results are similar to those documented in the literature (Potter and Gargely, 1975). The data presented in Figure 3-4, panels B and C clearly show that reactivation occurs as the first set of binding sites have been saturated.

3.3.5 Amino Acid Composition

Weak binding of calcium to proteins is often mediated through interactions with oxygen ligands. We determined the amino acid composition of the 41 kDa collagenase/gelatinase (Table 3-2). The amino acid compositional analysis revealed a high content, 24 mol%, of the acidic amino acids and their amide derivatives. Using an average amino acid molecular mass of 115, the 41 kDa collagenase/gelatinase was determined to contain approximately 86 carboxy plus carboxyamide residues. These carboxy and carboxyamide groups, in addition to the carbonyl groups from the peptide backbone and oxygen atoms of alcohol side chains, constitute potential weak calcium binding sites.

TABLE 3-2: The Amino Acid Composition of the Collagenase/Gelatinase

AMINO ACID	RESIDUES PER 1000
Asx	127
Thr	69
Ser	54
Glx	116
Pro	56
Gly	112
Ala	70
Val	78
Cys	0
Met	2
Ile	59
Leu	81
Try	30
Phe	40
Lys	49
His	17
Arg	40

Amino acid compositions were determined following hydrolysis of the 41 kDa collagenase/gelatinase preparations for 72 hrs in 6N HCl at 110°C. Liberated amino acids were fractionated in a Beckman 121 amino acid analyser. Tryptophan content was not determined. Values are presented as amino acid residues per 1000 residues.

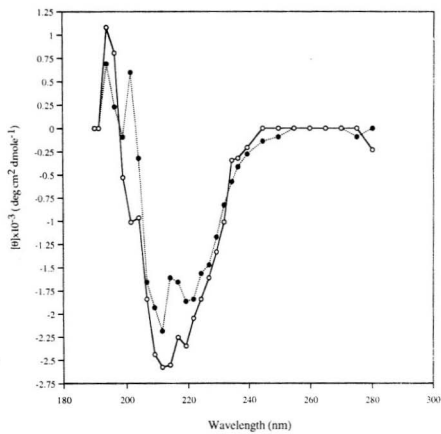
3.3.6 Circular Dichroism

From native molecular mass determinations of the collagenase/gelatinase in the absence and presence of calcium, we concluded that the quaternary structure is unaffected by the binding of calcium. To determine if the calcium-dependent reactivation of the 41 kDa collagenase/gelatinase was mediated through secondary structural changes, the far-UV CD spectrum of the collagenase/gelatinase was recorded in the presence and absence of calcium (Figure 3.5). There was no significant change in either the alpha helical or β -sheet content measured in the presence and absence of 10 mM Ca^{2+} ($6.1 \pm 1.2\%$ vs $7.1 \pm 0.1\%$ and $39.4 \pm 6\%$ vs $40.2 \pm 8\%$, respectively) and suggested that small differences between the spectra may be due to the dilution of the protein by the added calcium. These results suggested that the calcium-dependent activation of the 41 kDa species was not mediated through secondary structural changes.

3.4 DISCUSSION

The 41 kDa collagenase/gelatinase was a calcium-dependent activity present, not only in eggs, but in developing embryos up to the gastrula stage (Figure 3-1). Native molecular mass determination suggests that this enzyme exists as a tetramer although a small amount of the collagenase/gelatinase is

Figure 3-5. Circular dichroic spectra of 41 kDa collagenase/gelatinase in the presence and absence of 10 mM CaCl_2 . Spectra of the 41 kDa collagenase/gelatinase in the presence of 50 mM Tris-HCl, pH 8.0 containing 0.1 mM EGTA, (---•---) and in the presence of 50 mM Tris-HCl, pH 8.0 containing 0.1 mM EGTA and 10 mM CaCl_2 (—o—). Protein concentration was 0.5 mg/ml and the cell pathlength was 0.1 cm.



present as a monomer (Figure 3-2). Interestingly, individual subunits are biologically active.

Matrix metalloproteinases, astacins and ADAMS, as members of the metazincin family of enzymes, all contain a catalytic zinc ion, while the MMPs bind an additional zinc outside the active site (Bode *et al.*, 1995/1996). The role of calcium ions includes structural stabilization of the active site, protection from proteolysis and facilitation of substrate binding. Binding of calcium to human neutrophil gelatinase B stabilizes the active site by forming a bridge between the N- and C-termini of the catalytic domain and is involved in autoproteolytic processing of the C-terminal hemopexin domain (Bu and Pourmotabbed, 1995). Calcium acts to stabilize human collagenase and stromelysin, and prevents autodegradation of active stromelysin (Wetmore and Hardman, 1996). Some serine proteinases also bind calcium, although not at their catalytic sites. Recent research carried out in our laboratory suggests that the 41 kDa activity contains a reactive serine residue (Robinson, 2000). Using both qualitative and quantitative assays in the presence of the serine protease inhibitors, PMSF (phenylmethylsulfonylfluoride) or AEBSF ([4-(2-aminoethyl) benzenesulfonyl]fluoride), resulted in partial inhibition of the 41 kDa gelatinase activity.

The 41 kDa collagenase/gelatinase required calcium for activity and was half-maximally active at a calcium concentration of 3.7 mM (Figure 3-3).

The 41 kDa species was saturated with calcium ions at 10 mM CaCl_2 . These ions bound to two classes of sites, both with K_d s in the millimolar range. The first set of sites with a K_d of 0.5 mM can accommodate 120 mol calcium per mol 41 kDa collagenase/gelatinase. When these calcium binding sites are saturated, the enzyme shifts from an inactive form to an active form (Figure 3-5; Panel B). Sea water contains 10 mM CaCl_2 , and in this context, millimolar dissociation constants are biologically relevant. It is presently unknown whether calcium plays a dynamic or static role in regulating the activity of the 41 kDa collagenase/gelatinase, since at this high calcium concentration the enzyme would be constitutively active. However there may be variations in the local calcium levels in the ECM which provide microenvironments having calcium concentrations significantly different than those found in open sea water. There are components within the ECM of the sea urchin embryo which bind calcium ions. Hyalin, a major component of the apically located hyaline layer can bind 400 mol of calcium per mol of hyalin (Robinson and Brennan, 1991). ECM molecules which bind calcium may act as buffers regulating the amount of calcium bound to the 41 kDa collagenase/ gelatinase. Calcium concentrations in the ECM may vary, and recently an extracellular calcium-sensing receptor has been identified (Brown *et al.*, 1999). These receptors may provide a mechanism to perceive differences in calcium concentration in extracellular compartments. Calcium

gradients in extracellular compartments have been identified in the skin layers and across the placenta. As well, calcium concentrations in the alveolar fluid of fetal sheep increases significantly (3- to 4- fold) after birth (Maurer *et al.*, 1996; Brown *et al.*, 1999).

Many proteins bind calcium. Calcium binding proteins such as calmodulin bind calcium at specific sites known as EF hand repeats (Maurer *et al.*, 1996). These EF hand repeats can be recognized as a sequence of 12 amino acid residues forming the loop of an helix-loop-helix motif, and are often found as pairs. Six residues in the EF hand sequence, along with a water molecule, coordinate specific binding of calcium ions, with K_d s in the range of 10^{-7} M (Maurer *et al.*, 1996). Some calcium-binding proteins contain sites that are rich in aspartate and glutamate, similar to the EF hand repeats, but which lack the helices of the EF hand motif. An example is the cell adhesion molecule, E-cadherin. The globular extracellular domains of E-cadherin bind calcium with a K_d of 10^{-7} M, inducing a conformational change to flexible rods capable of homophilic interactions and cell - cell adhesion (Maurer *et al.*, 1996). Fibrillin and many other ECM proteins contain EGF-like domains that bind calcium at sites which consist of 40 to 50 amino acids, including six conserved cysteines forming three disulfide bonds (Maurer *et al.*, 1996; Reinhardt *et al.*, 1997). However, several reports in the literature state that other calcium binding proteins can bind large amounts of calcium with weak

affinity and that these regions do not consist of a defined sequence. For instance, calcium ions can bind weakly to clusters of glutamates and aspartates, with apparent K_d s in the mM range (Maurer *et al.*, 1996). In this context, the amino acid composition of the collagenase/gelatinase is compatible with the ability to bind a large amount of calcium with weak affinity. The enzyme contained 24 mol% acidic amino acids and their amide derivatives and bound calcium with dissociation constants in the millimolar range.

We were also interested in the relationship between calcium binding and activation of the enzyme. A direct effect of calcium ions at the active site seems unlikely due to the large number of calcium ions required for activation. We were interested in the effect that calcium binding might have in terms of structural changes. Neither the secondary nor the quaternary structure of the collagenase/gelatinase was affected by calcium binding. It is possible that calcium may facilitate reactivation by mediating binding of the substrate, although we have not yet identified the mechanism of reactivation.

**CHAPTER 4: SUBSTRATE SPECIFICITY AND KINETIC
ANALYSIS OF THE CLEAVAGE ACTIVITY OF THE 41 kDa
COLLAGENASE/GELATINASE**

4.1 Introduction

The selective degradation of all ECM components is collectively carried out by the four families of matrix-degrading proteinases. The substrate specificity of these proteinases vary; some degrade only one class of ECM component, while others exhibit overlapping substrate specificities. Neutrophil collagenase (MMP-8) can cleave the three polypeptide chains of fibrillar type I, II and III collagens at a specific sequence (Matrisian, 1992). Stromelysin (MMP-3) can employ several classes of ECM components as substrates, including proteoglycans, fibronectin, gelatin, fibrillar collagens type III and V, as well as the non-fibrillar collagens, types IV and IX (Matrisian, 1992). The proteoglycan, decorin, can be cleaved by gelatinase A (MMP-2), stromelysin-1 (MMP-3) and matrilysin (MMP-7) (Imai *et al.* 1997). The broad substrate specificity of matrilysin also includes the ability to degrade elastin, proteoglycans, fibronectin, casein and gelatins of type I, III, IV and V collagens (Abramson *et al.*, 1995). In addition, Abramson *et al.* (1995) show that matrilysin is involved in the activation of procollagenases, MMP-1 and -13. Ohuchi *et al.* (1997) report that MT1-MMP, an activator of proMMP-2, utilizes type I, II and III collagens, gelatins, as well as proteoglycans, fibronectin, vitronectin and laminin-1 as substrates. The extracellular serine proteinases degrade various glycoproteins and play a role in the activation of latent MMPs (Emonard and Grimaud, 1990). The ectodomains of the cell surface

heparan sulfate proteoglycans, the syndecans, are proteolytically processed by plasmin and thrombin (Subramanian *et al.*, 1997). Other matrix-degrading proteinases are responsible not only for degradation of ECM components, but utilize growth factors, cytokines and cell surface receptors as substrates. An example is the ADAM, tumour necrosis factor- α converting enzyme (TACE), which releases TNF- α and TGF- β from the cell surface so that they can interact with their receptors (Peschon *et al.*, 1998; Streuli, 1999).

As discussed previously, hyalin is a major component of the apical ECM, the hyaline layer, of the sea urchin embryo, although additional ECM components, including laminin, fibronectin, heparan sulfate proteoglycans and various collagen subtypes, have been detected in both the hyaline layer and basal lamina of the sea urchin embryo (Wessel *et al.*, 1984; Alliegro and McClay, 1988; Exposito *et al.*, 1994). Additionally, several groups have isolated collagen species from the adult tissues of the sea urchin (Burke *et al.*, 1989; Tomita *et al.*, 1994; Robinson, 1997).

The 41 kDa collagenase/gelatinase could cleave gelatin, as assessed qualitatively through gelatin substrate gel zymography and quantitatively through the gelatinase assay (Figure 3-1 and Appendix A). Previous results had shown an ability to employ casein as a substrate (although its cleavage was one-third that of gelatin), while it did not utilize either BSA or

hemoglobin as substrates, suggesting it was not a nonspecific proteinase (Appendix A).

To establish the 41 kDa collagenase/gelatinase as an invertebrate matrix-degrading enzyme we searched for a physiological substrate for this activity in the sea urchin. Since gelatin is a denatured form of collagen, and because the presence of vertebrate-like collagen species in the sea urchin had been confirmed, we decided to test collagen extracted from the sea urchin adult tissue, the peristome, as a substrate for the 41 kDa activity. Additionally, we decided to test whether a vertebrate collagen (type I) isolated from rat tail tendon could be cleaved by this enzyme. To initiate comparative analysis with vertebrate collagenases and gelatinases, we determined the kinetic parameters of the 41 kDa collagenase/gelatinase toward native and heat denatured sea urchin collagen polypeptides.

4.2 Materials and Methods

4.2.1 Collagen Preparation

Collagen extractions were carried out using the standard protocol described by Burke *et al.* (1989). Sea urchin adult peristome tissues or rat tail tendons (gift from Dr. J.T. Brosnan) were homogenized in 30 ml of ice-cold, deionized water. The samples were centrifuged at 2000 X g for 30 min and pellets were resuspended and incubated in 30 ml of ice-cold, deionized water for 16 hrs at

4°C. Following incubation, the pellet was harvested by centrifugation at 2000 X g for 30 min, resuspended and incubated in 30 ml of ice-cold, deionized water for 16 hrs at 4°C. This incubation was performed three times. Benzamidine hydrochloride was added to 20 mM, PMSF to 2 mM and EDTA to 5 mM during each resuspension. After the final centrifugation step, the insoluble fraction was resuspended in 20 ml of 0.5 M acetic acid containing 0.5 mg pepsin (Worthington Chemical Co.) and incubated with constant rotation for 6 hours at 4°C. Digestion was stopped by adjusting the pH to 7.6 with NaOH. Undigested material was removed by centrifugation at 2000 X g for 30 min. Sodium chloride was added to the supernatant to a final concentration of 5 M and mixed by rotation for 16 hours at 4°C. Samples were centrifuged at 2000 X g for 30 min, the pellets resuspended in 1 ml deionized water containing 20 mM benzamidine hydrochloride, 2 mM PMSF and 5 mM EDTA and stored at 4°C.

4.2.2 Kinetic Analysis of Collagenase and Gelatinase Activity

Digestions of increasing amounts of peristome collagen were carried out by incubating aliquots at 15°C in 50 mM Tris-HCl, pH 8.0, containing 10 mM CaCl_2 , in a final volume of 100 μl , in the presence and absence of the 41 kDa collagenase. Digestions of peristome gelatin were carried out by preincubating peristome collagen at 60°C for 15 min, to render the collagen into gelatin,

followed by incubations of increasing amounts of the peristome gelatin at 37°C in 50 mM Tris-HCl, pH 8.0, containing 10 mM CaCl_2 , in a final volume of 100 μl , in the presence and absence of the 41 kDa gelatinase. The incubations were terminated by the addition of an equal volume of ice-cold 20% (w/v) TCA. The samples were fractionated by SDS-PAGE [8% (w/v) polyacrylamide] and stained with CBB. The stained bands were excised and the dye eluted overnight at room temperature in 25% (w/v) pyridine. Absorbances were measured at 605 nm to assess substrate cleavage. Background binding of the stain was determined using gel slices that did not contain protein and the appropriate corrections were made.

Substrate cleavage was used to determine the reaction velocities which were linear for the incubation periods. Values of K_m and V_{\max} were determined from both Michaelis-Menten and Lineweaver-Burk analyses using the computer program Graphpad Prism (version 2.0). Values are presented as the mean of both analyses.

4.2.3 Circular Dichroism Analysis of Collagen-Gelatin Transition

Circular dichroism (CD) spectra in the far-UV region from 190-280 nm were recorded at room temperature on a model J500-A JASCO spectropolarimeter and processed with a DP-500N data processor. Samples

(0.10-0.5 mg/ml) of sea urchin peristome collagen and rat tail tendon collagen were dialysed against 100 mM Tris-HCl, pH 8.0 containing 0.15 M NaCl. Spectra were measured at 15°C, 20°C, 25°C, 30°C, 40°C and 60°C for peristome collagen and 22°C, 30°C, 37°C, 40°C and 60°C for rat tail tendon collagen. Samples were allowed to equilibrate at each temperature for 30 min before spectra were recorded. The measurements were carried out in a quartz cell with a pathlength of 1.0 cm. Background spectra were recorded for each buffer at the appropriate temperature and were subtracted from the recorded spectra of the protein. Protein concentrations were determined by the method of Lowry *et al.* (1951).

4.3 Results

4.3.1 Collagen Preparations

We analysed the peristome and rat tail tendon collagens by SDS-PAGE (Figure 4-1). Two species of apparent molecular masses of 140 and 116 k were present in the collagen extract from peristome tissue (Lane 4; Figure 4-1). The rat tail tendon collagen extracts contained two species of apparent molecular masses of 123 and 126 k (Lane 2; Figure 4.1). Migrating at a higher molecular mass than the individual α -chains, are the β -chains, cross-linked dimers of the α -chains (Figure 4.1).

Figure 4-1. Cleavage of peristome and rat tail tendon collagen by the 41 kDa collagenase/gelatinase. Aliquots (15 μ g) of peristome (lanes 1-4) or rat tail tendon (lanes 5-8) collagen in 50 mM Tris-HCl, pH 8.0, were incubated at 37°C (lanes 1, 2, 5 and 6) or 60°C (lanes 1, 3, 5 and 7) for 10 mins. Following this incubation, the samples were incubated in the presence (lanes 1, 3, 5 and 7) or absence (lanes 2, 4, 6 and 8) of 1 μ g of the 41 kDa collagenase/gelatinase at 37°C for 3 hrs. Reactions were stopped by the addition of an equal volume of ice-cold 20% (w/v) TCA. Aliquots (5 μ g) of BSA were incubated at 37°C for 3 hrs in the absence and presence of the 41 kDa collagenase/gelatinase (lanes 9 and 10, respectively). The samples were fractionated in an 8% (w/v) polyacrylamide gel (Laemmli, 1970). The gel was stained with CBB.

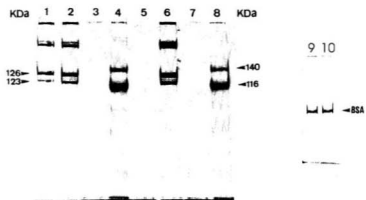


Table 4-1 shows the amino acid composition of peristome and rat tail tendon collagens. Analyses revealed that both preparations have an amino acid composition characteristic of collagen molecules; one-third of their amino acid residues are glycines, approximately one-tenth are proline residues and they contain hydroxyproline and hydroxylysine residues.

4.3.2 Substrate Specificity of the 41 kDa Collagenase/Gelatinase

We were interested in determining the ability of the 41 kDa collagenase/gelatinase to cleave collagen polypeptides from peristome tissue and rat tail tendon. Figure 4.1 shows SDS-PAGE analysis of peristome collagen incubated in the presence (lane 3) or absence (lane 4) of the 41 kDa activity at 37°C. The 41 kDa collagenase/gelatinase cleaved both the 140 and 116 kDa polypeptides. Similarly, we tested whether the 41 kDa activity could cleave the collagen polypeptides isolated from rat tail tendon. Figure 4-1 shows SDS-PAGE analysis of rat tail tendon collagen incubated in the presence (lane 1) or absence (lane 2) of the 41 kDa activity at 37°C. The 41 kDa collagenase/gelatinase could not cleave intact rat tail tendon collagen.

We also tested the ability of the 41 kDa collagenase/gelatinase to cleave the collagen polypeptides once they had been heat denatured to render them as gelatins. Following heat denaturation at 60°C for 15 min, the polypeptides were incubated in the presence or absence of the 41 kDa activity at 37°C.

Table 4-1: The Amino Acid Composition of Rat Tail Tendon and Sea Urchin Peristome Collagen Preparations

Amino Acid	Rat Tail	Peristome
Asx	57	81
Thr	26	47
Ser	46	77
Glx	75	96
Pro	111	81
Hy-Pro	68	48
Gly	309	281
Ala	104	68
Val	32	36
Cys	1	2
Met	5	9
Ile	21	30
Leu	36	45
Tyr	9	17
Phe	18	16
Lys	25	9
Hy-Lys	6	4
His	5	6
Arg	46	47

Amino acid compositions were determined following hydrolysis of the collagen preparations for 72 hrs in 6N HCl at 110°C. Liberated amino acids were fractionated in a Beckman 121 amino acid analyser. Tryptophan content was not determined.

Values are presented as amino acid residues per 1,000 residues.

Figure 4-1 shows rat tail gelatin incubated in the presence (lane 5) or absence (lane 6) of the 41 kDa activity, and peristome gelatin incubated in the presence (lane 7) or absence (lane 8) of the 41 kDa activity. Upon heat denaturation both the peristome gelatin and rat tail tendon gelatin were accessible as substrates for the 41 kDa collagenase/gelatinase. The 41 kDa species possessed collagenase activity toward sea urchin collagen and had gelatinase activity toward both sea urchin and rat tail tendon gelatins. Figure 4-1 also shows BSA incubated in the presence (lane 9) or absence (lane 10) of the 41 kDa collagenase/gelatinase at 37°C, confirming that the 41 kDa species was not a non-specific protease.

Collagenase activity is limited to the ability to cleave native, triple-helical collagen. The rat's body temperature is 37°C while the sea urchin, *S. purpuratus*, lives in an environment at a temperature of 10-15°C. We measured the circular dichroic spectra of both the rat tail tendon collagen and peristome collagen as a function of temperature, to assess the transition temperature for denaturation. Rat tail tendon collagen exhibited a sharp transition in its circular dichroic spectra between 37°C and 40°C (Figure 4.2; a and b). However peristome collagen underwent the collagen to gelatin transition between 22°C and 30°C (Figure 4.3; a and b).

Figure 4.2 (a). Circular dichroic spectra of rat tail tendon collagen polypeptides recorded at various temperatures. Spectra of the rat tail tendon polypeptides in 100 mM Tris-HCl, pH 8.0 containing 0.15 M NaCl were measured at 22°C (—), 30°C (---), 37°C (— · —), 40°C (----), and 60°C (— · — · —). Samples were allowed to equilibrate at each temperature for 30 min before spectra were recorded. Protein concentrations were 0.40 mg/ml (22°C), 0.38 mg/ml (30°C), 0.21 mg/ml (37°C), 0.11 mg/ml (40°C), and 0.10 mg/ml (60°C) and the cell pathlength was 1.0 cm.

Figure 4.2 (b). Molar Ellipticity of rat tail tendon collagen polypeptides measured at 232 nm by circular dichroism at the following temperatures: 22°C, 30°C, 37°C, 40°C, and 60°C.

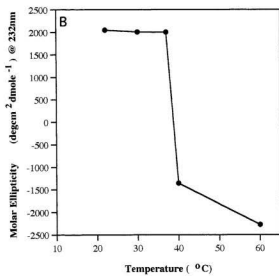
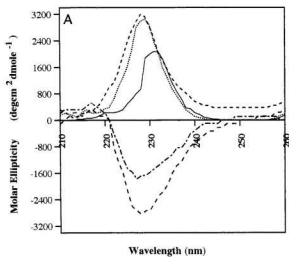
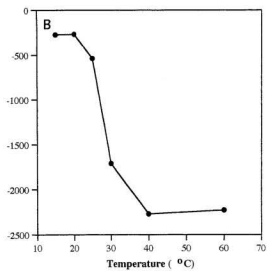
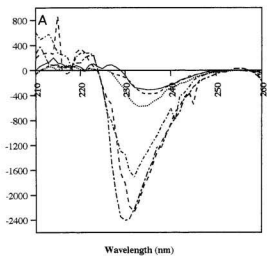


Figure 4.3 (a). Circular dichroic spectra of peristome collagen polypeptides recorded at various temperatures. Spectra of the peristome collagen polypeptides in 100 mM Tris-HCl, pH 8.0 containing 0.15 M NaCl were measured at 15°C (—), 20°C (---), 25°C (— · —), 30°C (— · — · —), 40°C (— · — · — · —) and 60°C (— · — · — · — · —). Samples were allowed to equilibrate at each temperature for 30 min before spectra were recorded. Protein concentrations were 0.43 mg/ml (15°C), 0.40 mg/ml (20°C), 0.48 mg/ml (25°C), 0.21 mg/ml (30°C), 0.20 mg/ml (40°C) and 0.20 mg/ml (60°C) and the cell pathlength was 1.0 cm.

Figure 4.3 (b). Molar Ellipticity of peristome collagen polypeptides measured at 232 nm by circular dichroism at the following temperatures: 10°C, 15°C, 20°C, 25°C, 30°C, 40°C and 60°C.



The previous data (Figure 4-1) measured cleavage of rat tail tendon and peristome polypeptides by the 41 kDa enzyme at 37°C, following preincubation of these substrates at 37°C and 60°C. Data from circular dichroic spectra indicated that at 37°C the peristome collagen was in fact denatured and in its gelatin state. Therefore assays carried out at 37°C were in fact measuring gelatinase activity toward the polypeptides from peristome preparations. To further address this issue, quantitative assays were performed at 15°C following preincubation of the polypeptides at 15°C, 37°C and 60°C (Appendix D; Robinson, 1997). When preincubated at 15°C and 37°C, approximately 3 and 14%, respectively, of rat tail tendon collagen polypeptides are cleaved, while preincubation at 60°C resulted in greater than 90% cleavage. In contrast, when preincubated at 15°C, the peristome collagen polypeptides are cleaved approximately 35%, which increases to approximately 55% upon preincubation at 37°C. Furthermore, preincubation at 60°C did not increase the amount of cleavage measured at 37°C. This data, along with circular dichroic spectra, confirms that at 15°C both the rat tail tendon and peristome collagen polypeptides are in their native collagen forms. Preincubation at 37°C results in loss of the native triple helical structure for the peristome collagen polypeptides, while the rat tail tendon collagen remains in its native form. At 60°C however, the rat tail tendon collagen polypeptides were denatured as indicated by the sharp transition in the circular dichroic spectra

(above 40°C) and its susceptibility to cleavage by the 41 kDa activity. These experiments demonstrated that the 41 kDa species possesses an echinoderm-specific collagen cleavage activity, in addition to a non-specific gelatinase cleavage activity.

4.3.3 Kinetic Analysis of Collagenase and Gelatinase Activities of 41 kDa Enzyme

To further analyse the collagen and gelatin cleavage activities of the 41 kDa enzyme, the kinetic parameters, K_m , V_{max} and the ratio k_{cat}/K_m , were measured for both the native and heat denatured 140 and 116 kDa constituent polypeptides of peristome collagen (Table 4-3). Lineweaver-Burk analyses of the data are presented in Figure 4-4. The K_m s we report for the 41 kDa activity toward both peristome collagen (1.0 and 0.39 μ M for the 140 and 116 kDa polypeptides, respectively) and peristome gelatin (0.9 and 2.6 μ M for the 140 and 116 kDa polypeptides, respectively) are similar suggesting similar binding affinities. The values for V_{max} are two orders of magnitude higher for the 41 kDa gelatinase activity (10^{-6} μ mol.min⁻¹) compared with the 41 kDa collagenase activity (10^{-8} μ mol.min⁻¹), reflecting the difference in temperature at which these two assays were carried out (37°C versus 15°C, respectively). The ratio k_{cat}/K_m was used as a measure of substrate specificity. The ratio

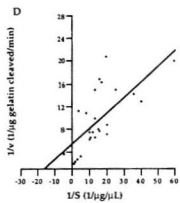
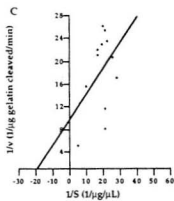
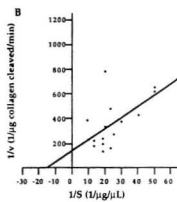
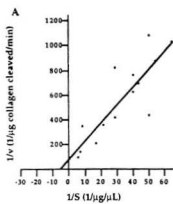
Table 4-2: Kinetic Parameters; V_{\max} , K_m and k_{cat} for the 41 kDa Collagenase/Gelatinase

Cleavage Activity	41 kDa Collagenase		41 kDa Gelatinase	
Substrate	140 kDa polypeptide	116 kDa polypeptide	140 kDa polypeptide	116 kDa polypeptide
K_m (μM)	1.0 ± 0.50	0.39 ± 0.06	0.09 ± 0.70	2.6 ± 1.9
V_{\max} (μmolmin^{-1})	$8.1 \pm 1.7 \cdot 10^{-8}$	$5.29 \pm 0.13 \cdot 10^{-8}$	$1.43 \pm 0.83 \cdot 10^{-6}$	$5.4 \pm 3.0 \cdot 10^{-6}$
k_{cat} (min^{-1})	0.07	0.04	1.17	4.38
k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)	0.07	0.11	1.33	1.66

Sea urchin peristome collagen was used as the substrate for the 41 kDa collagenase activity and heat-denatured peristome collagen was used as the substrate for the 41 kDa gelatinase activity.

The values for K_m and V_{\max} represent the mean of the Michaelis-Menten and Lineweaver-Burk analyses \pm range.

Figure 4-4: Double reciprocal plots of initial velocity versus substrate concentration for the 41 kDa collagenase activity (panels A and B) and the 41 kDa gelatinase activity (panels C and D). Cleavage of the 140 kDa polypeptide was quantitated in panels A and C; cleavage of the 116 kDa polypeptide was quantitated in panels B and D. Non-linear regression analysis using the Michaelis-Menton equation was performed on data collected from several independent experiments. The values for K_m and V_{max} from non-linear analysis were used to fit the Lineweaver-Burk plots.



for the 116 and 140 kDa collagen polypeptides differ by a factor of less than two. This was not sufficiently different to conclude that one polypeptide was used preferentially by the 41 kDa collagenase. Likewise, the ratio k_{cat}/K_m for the 41 kDa gelatinase toward the denatured 116 and 140 kDa polypeptides were similar. These results indicated that neither of the two polypeptides were cleaved in a preferential fashion by the 41 kDa activity in either their native collagen state or as denatured gelatins.

4.4 Discussion

The 41 kDa collagenase/gelatinase degraded both invertebrate collagen and gelatin from the adult peristome tissue of the sea urchin. Several members of the family of MMPs possess both collagen and gelatin cleavage activities. MMP-2, a gelatinase, cleaves not only gelatin, but type I, IV and V collagens (Aimes and Quigley, 1995). In comparison, the gelatinase MMP-9, did not exhibit collagenase activity against type I collagen. Human fibroblast collagenase (MMP-1) can degrade human type I, II and III collagens, although it cleaves type III collagen most efficiently and type II collagen least efficiently (Welgus *et al.*, 1981). Neutrophil collagenase (MMP-8) degraded type I collagen preferentially, in comparison to its cleavage rate against type II and type III collagens, while collagenase-3 (MMP-13) preferred type II collagen (Knauper *et al.*, 1996). Interestingly, Welgus *et al.* (1981) also demonstrated

that the collagenolytic activity depended upon species of origin; human fibroblast collagenase cleaved type I collagen from human, calf, pig and rat; type II collagen from human, calf and pig, but not rat; and human and pig type III collagen, but not that of calf or pig. However, upon heat denaturation, gelatins from all species were degraded equally.

In comparison to its dual activity toward invertebrate collagen and gelatin, the 41 kDa enzyme displayed only gelatinase activity toward vertebrate gelatin from the rat tail tendon. Therefore the collagen substrate specificity of this enzyme was dependent upon the species from which the substrate originated. As mentioned previously, the 41 kDa activity contains a reactive serine residue (Robinson, 2000). The ability to cleave native, triple helical collagen has not been observed for vertebrate extracellular serine-dependent proteinases. Several invertebrate serine proteinases have been identified which are capable of cleaving collagen, although the function of these enzymes is limited to food digestion (Roy, 1996; Tsu and Craik, 1996; Tsu *et al.*, 1997). Also, unlike ECM collagenases, these activities possess broad substrate specificities. The 41 kDa collagenase/gelatinase is unique as an extracellular serine proteinase capable of cleaving native collagen.

The kinetic parameters we report for cleavage of invertebrate collagen and gelatin are similar to those reported in the literature for vertebrate collagenase and gelatinase activities. Vertebrate collagenases and gelatinases

require micromolar concentrations of substrates for half-maximal activity with k_{cat} values which vary with substrate. The 41 kDa collagenase and gelatinase was half-maximally active in the micromolar range which suggested that we were looking at physiologically relevant activities. Maximal velocity was lower for collagenase versus gelatinase activity due to the different temperature of the assay, 15°C versus 37°C, respectively. The k_{cat}/K_m ratio as a measure of substrate specificity for the 41 kDa activity toward the 140 and 116 kDa collagen polypeptides differed by a factor of less than two. This was not significantly different to conclude that one polypeptide was cleaved preferentially by the 41 kDa collagenase. Likewise, the ratio for the 140 and 116 kDa gelatin species were similar to one another. Therefore the 41 kDa activity did not cleave preferentially the 140 or 116 kDa polypeptides in either their native collagen or denatured gelatin states. Moreover, the ability to degrade both invertebrate collagen and gelatin and the micromolar substrate concentrations required for half-maximal activities suggests a role for the 41 kDa collagenase/gelatinase in the remodelling of the sea urchin ECM during embryogenesis.

**CHAPTER 5: LOCALIZATION OF A 41 kDa COLLAGENASE/
GELATINASE IN THE SEA URCHIN EGG AND EMBRYO**

5.1 Introduction

As discussed previously, ECM-degrading enzymes are a collection of secreted and cell surface proteinases, localized to the ECM so that they have access to its components which they selectively degrade. We identified the storage compartment(s) for the 41 kDa collagenase/gelatinase within the egg and its localization in various stage embryos to determine its ability to access the ECMs of developing embryos.

Research has described several storage compartments in the sea urchin egg whose components are destined to be exported into the apical and/or basal laminae during embryonic development. The well characterized cortical granules lie along the periphery of the egg, and secrete their contents onto the apical surface of the egg shortly following fertilization (Hylander and Summers, 1982; McClay and Fink, 1982; Anstrom *et al.*, 1988). Hyalin, a major component of the hyaline layer, is housed in the cortical granules, along with other hyaline layer components such as ovoperoxidase and mucopolysaccharides (Schuel *et al.*, 1974; Hylander and Summers, 1982; Alliegro and Schuel, 1988). As well, several types of non-cortical, cytoplasmic storage granules whose contents become components of the apical and/or basal surfaces have been described. Some cytoplasmic storage granules contain proteins which are transported bidirectionally, to the apical and basal laminae, while other types of cytoplasmic granules export proteins

unidirectionally, to either the apical or basal lamina (Wessel *et al.*, 1984; Alliegro and McClay, 1988; Fuhrman *et al.*, 1992; Matese *et al.*, 1997; Mayne and Robinson, 1998). For instance, apical laminar, echinonectin and cadherin-containing vesicles store components that are transported unidirectionally to the hyaline layer following fertilization (Matese *et al.*, 1997). This research has been largely descriptive in nature and involved immunofluorescence analysis. High resolution immunogold labelling was not used in these studies and, in addition, none of these storage vesicles have been isolated and biochemically characterized. Our laboratory used high resolution immunogold labelling of unfertilized eggs and embryos to show that HLC-32 was stored within yolk granules of unfertilized eggs and was transported bidirectionally to both the hyaline layer and basal lamina (Mayne and Robinson, 1998).

The egg has a complicated intracellular trafficking mechanism for recruiting differentially compartmentalized proteins, as well as a regulated sequence of secretion following fertilization. Matese *et al.* (1997) studied the spatial and temporal recruitment of five different storage compartments for proteins recruited onto the surface of the egg. They found that cortical granules and basal laminar vesicles release their contents into the hyaline layer thirty seconds following fertilization. However, once the embryo becomes polarized, basal laminar vesicles are transported onto the basal

surface as well. Five minutes following fertilization, apical laminar vesicles begin to exocytose their contents onto the apical surface of the embryo, followed by echinonectin vesicles at fifteen minutes postfertilization; cadherin-containing vesicles transport their contents onto the apical surface of the embryo at the two cell stage.

We utilized immunofluorescence and immunoelectron microscopy to determine the storage compartment(s) for the 41 kDa collagenase/gelatinase in the unfertilized egg and to map its ontogenic movements during development.

5.2 Materials and Methods

5.2.1 Preparation of Yolk Granules

Yolk granules were isolated from unfertilized eggs as described by Yokota and Kato (1988). Eggs were washed in MFSW, followed by CMFSW and finally in 0.55 M KCl containing 1 mM EDTA, pH 7.0 and homogenized in a hand-held Dounce homogenizer at 0°C. The homogenate was centrifuged at 400 X g for 4 min at 4°C. The supernatant was recentrifuged at 2400 X g for 10 min at 4°C. The pellet was resuspended in 0.55 M KCl containing 1 mM EDTA, pH 7.0, centrifuged at 400 X g for 4 min at 4°C. The supernatant was centrifuged at 2400 X g for 10 min at 4°C. The final pellet was designated as the yolk granule pellet and was used in protein gel blot analyses.

5.2.2 Isolation of Blastocoelic Fluid

Blastocoelic fluid was isolated from mid-blastula stage embryos as described by Kiyomoto and Tsukahara (1991), except that centrifugation was at 16000 X g for 6 min. Embryos were grown to 30 hr postfertilization, washed three times in CMFSW, and concentrated suspensions centrifuged at 16000 X g for 6 min. The supernatant was retained and dialysed against 50 mM Tris-HCl, pH 8.0, for 1.5 hr at 4°C. Fifteen microlitre aliquots were solubilized in the absence of reducing agent and BPB and fractionated in a gelatin substrate gel as described in chapter two, section 2.3.2.

5.2.3 Dissociation of Collagenase/Gelatinase from the Extraembryonic Hyaline Layer

As described previously, one hour old embryos were prepared from eggs treated with DTT (Robinson, 1991). Unfertilized eggs were dejellied by titration to pH 5.5 with HCl, incubated for two min and the pH returned to 8.0 by the addition of 1 M Tris-HCl, pH 8.0. After extensive washing in ice-cold MFSW, the eggs were resuspended in MFSW to 10% (v/v) and their vitelline layer disrupted by treatment with 10 mM DTT. The embryos were washed twice in MFSW, followed by washing for 2 X 10 min in CMFSW containing 5 mM EDTA. The CMFSW washes were combined, cleared by centrifugation at 20,000 X g for 15 min at 4°C, and the supernatant made 100 mM in CaCl₂ to

precipitate hyalin (Robinson, 1988). An aliquot of the hyalin precipitate was analysed by gelatin substrate gel zymography as described in chapter two, section 2.3.2.

5.2.3 Fixation and Embedding of Eggs and Embryos for Electron Microscopy

Eggs and various stage embryos were fixed as previously described by Spiegel *et al.*, (1989). Fixation was carried out by adding aliquots of eggs and various stage embryos to an equal volume of the stock fixative containing 4% (v/v) glutaraldehyde and 1% (w/v) paraformaldehyde in 75% MFSW buffered with 0.15 M sodium cacodylate (Sigma), pH 7.8, without osmium postfixation. Eggs and embryos were rinsed in 85% MFSW containing 0.1 M sodium cacodylate, pH 7.6, three times for 30 min at room temperature. Samples were then rinsed three times for 30 min in 0.1 M sodium cacodylate, pH 7.6, followed by distilled water three times for 30min. Samples were then dehydrated in an ethanol series and embedded in Spurr's resin overnight at room temperature in an evacuated container. The blocks were cured overnight at 70 °C.

Thin sections (150 nm) of eggs and embryos were placed on nickel grids. Grids were incubated on drops of a solution containing 1% (w/v) BSA, 0.01 M phosphate buffered saline (PBS), consisting of 0.01 M phosphate in 0.15 M NaCl, pH 7.4, and 0.5% (v/v) Tween-20 three times for 15 min at room

temperature. Grids were then transferred to a drop of diluted anti 41 kDa collagenase/gelatinase antibody (1:200 dilution in 0.01 M PBS, pH 7.4, containing 1% (w/v) BSA and 0.5% (v/v) Tween-20) for two hours at room temperature. After antibody incubation the grids were rinsed five times for 5 minutes on drops of 0.01 M PBS, pH 7.4, containing 0.5% (v/v) Tween-20, then incubated on drops of 0.01 M PBS, pH 7.4, containing 1% (w/v) BSA and 0.5% (v/v) Tween-20 three times for 5 min. Grids were incubated on drops of protein A-gold (Sigma Co.; 10 nM colloidal gold) diluted 1:50 in a solution of 0.01 M PBS, pH 7.4, containing 1% (w/v) BSA and 0.5% (v/v) Tween-20 for 60 min at room temperature. Following several rinses in a solution containing 0.01 M PBS, pH 7.4, and then distilled water, the grids were allowed to air dry. Double immunogold labelling was carried out sequentially using the method described above except that sections were probed with the second antibody and labelled with different size protein A-gold particles on the reverse side of the grid.

Sections were stained on a drop of 2% (w/v) uranyl acetate in 50% ethanol for 20 min at room temperature, then rinsed with 50 % ethanol, followed by 25 % ethanol and finally distilled water, and stained on a drop of Reynold's lead citrate for 10 min at room temperature. Sections were rinsed with distilled water, allowed to air dry and viewed in a Zeiss EM 109 transmission electron microscope at 80 kV.

5.3 Results

5.3.1 Detection of the 41 kDa Collagenase/Gelatinase in the Hyaline Layer and Basal Lamina

Initially, we carried out an experiment to determine whether or not the 41 kDa collagenase/gelatinase was present in the apical and basal ECMs of developing sea urchin embryos (Figure 5.1). Hyalin was isolated from one hour old embryos, as described in section 5.2.3. When an aliquot of hyalin was analysed by gelatin substrate gel zymography, an activity migrating with an apparent molecular mass of 41 k was detected (Lane A). Blastocoelic fluid (isolated from mid-blastula stage embryos) contained a gelatinase species (Lane B) that comigrated with an aliquot of purified collagenase/gelatinase (Lane C). These results suggested that the 41 kDa species was present at both the apical and basal surfaces of the sea urchin embryo. This preliminary data suggested that the 41 kDa collagenase/gelatinase may be involved in remodelling of both the hyaline layer and the basal lamina.

5.3.2 Protein Gel Blot Analysis Using an Anti-41 kDa Collagenase/Gelatinase Antibody

We prepared polyclonal antiserum against the purified 41 kDa collagenase/gelatinase for immunolocalization studies and tested its specificity by developmental protein gel blot analysis (Figure 5.2). A single

Figure 5.1: Gelatin substrate gel zymography of the hyalin fraction dissociated from one hour old embryos and the blastocoelic fluid isolated from mid-blastula stage embryos. An aliquot (5 μ g) of the hyalin fraction (lane 1), an aliquot (15 μ l) of blastocoelic fluid (lane 2), or an aliquot (1 μ g) of the purified 41 kDa collagenase/gelatinase (lane 3) were analyzed by gelatin substrate gel zymography as described in chapter 2, section 2.3.2. The gelatinase activity in lane 1 comigrated with the purified 41 kDa collagenase/gelatinase (data not shown). Arrows indicate the 41 kDa activity.

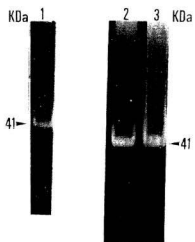
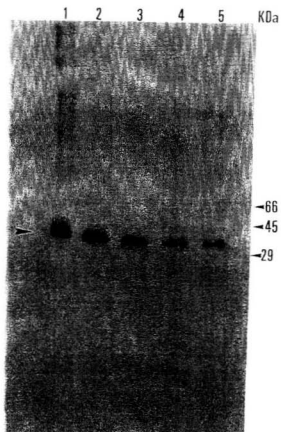


Figure 5.2: Western Blot analysis of eggs and various stage embryos using the anti-41 kDa collagenase/gelatinase antiserum. Aliquots (15 μ g) of eggs and various stage embryos were fractionated in a 3-12% (w/v) polyacrylamide gradient gel and transferred to nitrocellulose membrane. Lanes 1-5 contain eggs, 9.5, 32, 53 and 70.5 hour old embryos, respectively. The nitrocellulose membrane was probed with the antibody at a dilution of 1:200 (v/v). The relative migrations of molecular mass markers are shown; BSA (66 kDa), ovalbumin (45kDa) and carbonic anhydrase (29 kDa).



antigen was detected in eggs, 9.5, 32.5, 53 and 70.5 hour old embryos (Lanes 1-5, respectively). The antigen migrated with an apparent molecular mass of 32 k. We had shown previously that the 41 kDa collagenase/gelatinase migrates with an apparent molecular mass of 32 k upon reduction with DTT (Mayne and Robinson, 1996). The decrease in the 41 kDa collagenase/gelatinase detected in the developmental Western blot mirrored the pattern of decreasing 41 kDa gelatinase activity seen by gelatin substrate gel analysis of eggs and various stage embryos (Figure 3.1).

5.3.3 Immunolocalization of the 41 kDa Collagenase/Gelatinase in the Egg and Various Stage Embryos

Low-resolution immunofluorescence analysis was carried out by Dr. J. Robinson to map the ontogeny of the 41 kDa collagenase/gelatinase during development (Appendix E). The dispersed immunostaining pattern throughout the cytoplasm, as well as an intense layer of immunofluorescence along the periphery of the egg (which is the region occupied by the cortical granules) suggested the existence of at least two storage compartments for the 41 kDa activity in the unfertilized egg (Panel A). Stratification of unfertilized eggs by ultracentrifugation, followed by immunofluorescence analysis, showed that the cytoplasmic labelling was displaced toward the centrifugal pole while the immunostaining pattern along the cortex of the egg was not disrupted

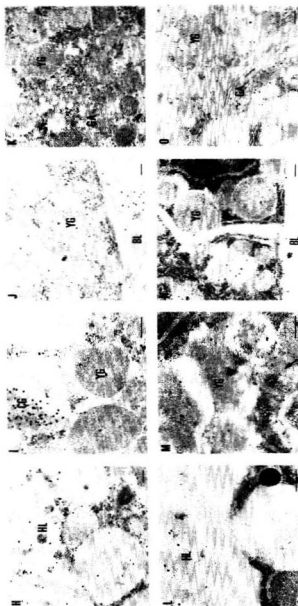
(Panel E). The inability to disrupt labelling associated with the cortex of the egg suggested that the cortical granules were one of the storage compartments for the 41 kDa activity since they are known to be tethered to the surface of the unfertilized egg. The fraction of the immunofluorescence which was displaced during centrifugation suggested that the portion of the 41 kDa collagenase/gelatinase found throughout the cytoplasm of the unfertilized egg was stored in a different compartment which was readily displaced by centrifugation. Immunofluorescence analysis of embryos up to the gastrula stage of development showed that the 41 kDa collagenase/gelatinase was transported in a bidirectional fashion and was present in both ECMs, the hyaline layer and basal lamina (Panels B, C and D represent one hour old embryos, blastula and gastrula stage embryos, respectively).

We employed high resolution, immunogold labelling analysis to further define the storage compartment(s) for the 41 kDa collagenase/gelatinase in eggs and various stage embryos (Figure 5.3). In unfertilized eggs, the electron dense, spiral lamellar regions of cortical granules were heavily labelled (Panel A). Labelling was also evident in yolk granules (approximately 0.75 μm in diameter; Panels A, B and C) and yolk granule-like structures (Panel B; arrow) dispersed throughout the cytoplasm of the egg. The yolk granule-like structures differed from recognizable yolk granules

Figure 5.3: Immunogold labelling of eggs and various stage embryos. Panels A-D represent sections of unfertilized eggs probed with the anti-41 kDa collagenase/gelatinase antiserum. Panels E-G represent sections of one hour old embryos probed with the anti-41 kDa collagenase/gelatinase antiserum. Panel E represents the apical surface of a one hour old embryo, while panels F and G represent the cytoplasm of a one hour old embryo. Antiserum was at a dilution of 1:200 (v/v). Scale bar = 0.25 μ m. CG, cortical granule; YG, yolk granule; M, mitochondrion; HL, hyaline layer; CS, cell surface; BL, blastocoel; LV, lipid vacuole; GA, Golgi apparatus; arrow, yolk granule-like structure. Figure 5.3, panels H-O appear on following page.



Figure 5.3 continued: Immunogold labelling of various stage embryos. H-K represent sections of blastula stage embryos probed with the anti-41 kDa collagenase/gelatinase antiserum. Panels H and J represent the apical and basal surfaces of blastula stage embryos, respectively, while panels I and K represent the cytoplasm of blastula stage embryos. Panels L-O represent sections of gastrula stage embryos probed with the anti-41 kDa collagenase/gelatinase antiserum. Panels L and N represent the apical and basal surfaces of gastrula stage embryos, respectively, while panels M and O represent the cytoplasm of gastrula stage embryos. Antiserum was at a dilution of 1:200 (v/v). Scale bar = 0.25 μm . CG, cortical granule; YG, yolk granule; M, mitochondrion; HL, hyaline layer; CS, cell surface; BL, blastocoel; LV, lipid vacuole; GA, Golgi apparatus.



which were larger and had different internal morphology. In addition, the area of the Golgi apparatus was labelled (Panel D). Other organelles such as mitochondria and lipid vacuoles were unlabelled, confirming specificity of the immunogold labelling (Panels B and C). In control experiments, using preimmune serum, no labelling was detected in cortical granules, yolk granules, yolk granule-like structures or the area of the Golgi apparatus (data not shown).

One hour following fertilization, the 41 kDa collagenase/gelatinase was detected in the apically located hyaline layer (Panel E). As well, sparse granules at the apical surface were labelled (Panel F; arrow), while yolk granules and yolk granule-like structures retained a portion of their label (Panel G).

In blastula stage embryos, the 41 kDa collagenase/gelatinase was present in both ECMs, the hyaline layer (Panel H) and basal lamina (Panel J), while a subpopulation of yolk granules and yolk granule-like structures within the blastomeres retained their label (Panels H-J). Panel I contains a cortical granule which failed to exocytose following fertilization and retained its label into the blastula stage of development, providing a positive internal control. The area of the Golgi apparatus was also labelled (Panel K).

Although the amount of labelling had decreased from that seen in earlier stages of development, the hyaline layer (Panel L) and basal lamina

(Panel N) were labelled in gastrula stage embryos. A subpopulation of yolk granules (Panels M-O) were labelled, as well as the area of the Golgi apparatus (Panel O). Unseen at earlier stages of development, small dense granules located at the apical surface of the gastrula stage embryo were heavily labelled (Panel L; arrow) and lysosomal-like granules (Panel M; arrow) were labelled. Collectively, these results provide evidence for the bidirectional movement of the 41 kDa collagenase/gelatinase into both ECMs of the embryo. The cortical granules secrete the 41 kDa species onto the apical surface of the embryo following fertilization, while the yolk granules and/or yolk granule-like structures export the 41 kDa species to the apical and/or basal surfaces following fertilization.

Quantitation of the immunogold labelling clearly identifies the cortical granules, yolk granule and yolk granule-like structures as storage compartments for the 41 kDa collagenase/gelatinase (Table 5.1). Levels of background labelling were quantitated from the cytoplasm and this value subtracted from the values in Table 5.1 accordingly.

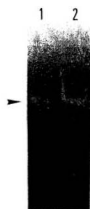
To confirm the presence of the 41 kDa collagenase/gelatinase in yolk granules, we fractionated yolk granules from unfertilized eggs and aliquots were analysed by gelatin substrate gel zymography (Figure 5.4). A 41 kDa species was detected (Figure 5.4).

Table 5.1: Quantitation of Immunogold Labelling

Organelle	<u>Developmental Stage</u>			
	Egg	1HPF	21HPF	48HPF
Cortical Granules	289 (100)	----	----	----
Yolk Granules	10.5 (80)	10.3 (38)	4.3 (58)	4.7 (61)
Yolk Granule-Like Structures	68.8 (100)	N.D	N.D	11.8 (25)
Lysosomal-Like Structures	N.D	N.D	N.D	39.5 (100)
Mitochondria	0	0	0	0
Lipid Vacuoles	0	0	0	0
Area of Golgi Apparatus	8.1 (67)	N.D	7.3 (100)	10.0 (100)
Nuclei	0	0	0	0

Values Represent means. Quantitation was based on counting the number of immunogold particles in a $0.25 \mu\text{m}^2$ area. Values are corrected for background binding by subtracting the number of immunogold particles in the same area of cytoplasm. Values in parentheses represent the percentage of structures labelled above background. HPF=Hours Post Fertilization; ND=Not Determined.

Figure 5.4: Gelatin substrate gel zymography of yolk granules. An aliquot (1 μ g) of the purified 41 kDa collagenase/gelatinase (lane 1) or an aliquot (15 μ l) of yolk granules (lane 2) were analyzed by gelatin substrate gel zymography as described in chapter 2, section 2.3.2. The gelatinase activity in lane 2 comigrated with the purified 41 kDa collagenase/gelatinase (lane 1; arrow).



Yolk granules had previously been identified as the exclusive storage compartment for a 32 kDa hyaline layer protein, HLC-32 (Mayne and Robinson, 1998). HLC-32 stored in the yolk granules of unfertilized eggs was transported bidirectionally to both ECMs of the developing embryo. To compare the storage compartments in the unfertilized egg for HLC-32 and the 41 kDa collagenase/gelatinase we probed sections of unfertilized eggs with both anti 41 kDa collagenase/gelatinase antiserum and anti-HLC-32 antiserum for immunoelectron microscopy (Figure 5.5). The anti-HLC-32 antiserum was labelled with 20 nm gold particles, and the anti-41 kDa collagenase/gelatinase antiserum was labelled with 10 nm gold particles. Cortical granules were singularly labelled with the anti-41 kDa collagenase/gelatinase antiserum (Panel A), whereas yolk granules were labelled with both the anti-41 kDa collagenase/ gelatinase antiserum and the anti-HLC-32 antiserum (Panels C-D). The area of the Golgi was labelled with the anti-41 kDa collagenase/gelatinase antiserum (Panel E). Figure 5.6 is an enlargement of Panel C from Figure 5.5. The yolk granule was clearly labelled with both the anti-41 kDa collagenase/gelatinase antiserum and the anti-HLC-32 antiserum, whereas the smaller yolk granule-like structure (arrow) was labelled only with the anti-41 kDa collagenase/gelatinase antiserum, indicating that these storage compartments are distinct from yolk granules.

Figure 5.5: Double immunogold labelling of eggs. Panels A-F represent sections of eggs probed with the anti-41 kDa collagenase/gelatinase antiserum and labelled with 10 nm gold particles, then with the anti-HLC 32 antiserum and labelled with 20 nm gold particles. Sections were incubated with a 1:200 dilution of the anti-41 kDa collagenase/gelatinase antiserum and a 1:50 dilution of the anti-HLC 32 antiserum. Bars represent 0.25 μ m. CG, cortical granule; YG, yolk granule; M, mitochondrion; LV, lipid vacuole; GA, Golgi apparatus; arrow, yolk granule-like structure.

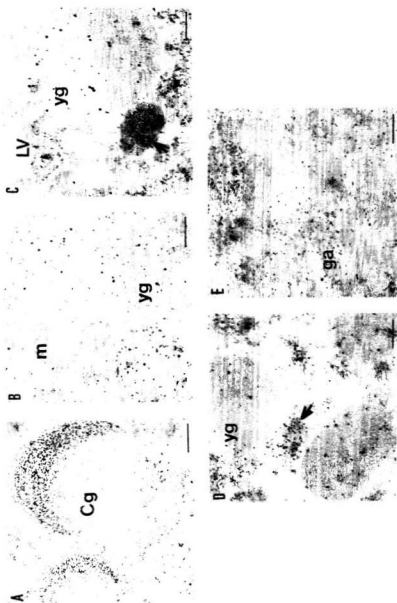
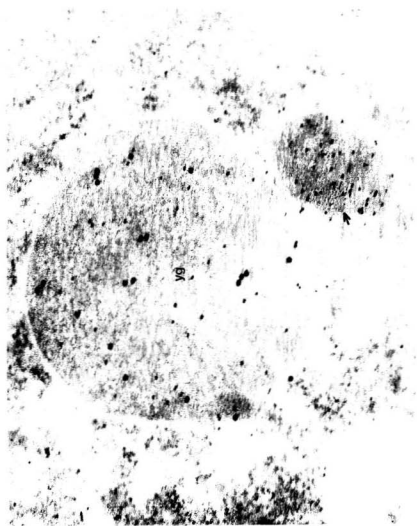


Figure 5.6: Double immunogold labelling of eggs. This is an enlargement of Panel C from Figure 5.5 in which sections of eggs were probed with the anti-41 kDa collagenase/gelatinase antiserum and labelled with 10 nm gold particles, then with the anti-HLC 32 antiserum and labelled with 20 nm gold particles. Sections were incubated with a 1:200 dilution of the anti-41 kDa collagenase/gelatinase antiserum and a 1:50 dilution of the anti-HLC 32 antiserum. YG, yolk granule; arrow, yolk granule-like structure.



5.4 Discussion

The immunolocalization results identify the storage sites for the 41 kDa collagenase/gelatinase in the unfertilized egg and provide evidence that the 41 kDa collagenase/gelatinase is present in both ECMs of the developing sea urchin embryo. The enzyme was colocalized to the cortical granules with several other ECM proteins such as hyalin. Upon exocytosis from the egg, at the time of fertilization, these proteins form the apical ECM, the hyaline layer. The presence of the 41 kDa collagenase/gelatinase in the ECM suggests that it may be involved in remodelling of the hyaline layer in early stage embryos. The presence of the 41 kDa collagenase/gelatinase in the blastocoel and basal lamina is indicative of a role in formation and remodelling of the basal lamina in blastula and gastrula stage embryos.

The unfertilized egg acts as a reservoir for the components for embryonic development. It contains the information and building blocks necessary for cell growth following fertilization. During oogenesis, components that allow the egg to support embryonic development are both made within the oocyte and/or are transported to the oocyte where they are packaged into vesicles and granules. The egg can support embryonic development since the contents of these storage compartments can be utilized. This recruitment is controlled both spatially and temporally. As mentioned in the introduction to this chapter, several storage compartments

for ECM components have been described in the sea urchin egg: cortical granules, and at least two types of cytoplasmic granules. One set of cytoplasmic granules are deposited into both the hyaline layer and the basal lamina, while the contents of the second set are deposited solely into the hyaline layer (Wessel *et al.*, 1984; Alliegro and McClay, 1988; Fuhrman *et al.*, 1992). We identified cortical granules, yolk granules and yolk granule-like structures as storage compartments for the 41 kDa collagenase/gelatinase.

The identification of yolk granules as a storage compartment for proteins destined for export from the developing embryo is significant. In several systems, including the sea urchin, yolk granules (which comprise nearly one-third the volume of the unfertilized egg) were viewed as a storage compartment for nutrients required by the developing embryo. Recently, several groups have begun to challenge the view of the yolk granule as a benign storage compartment. Scott *et al.* (1990) showed that *Strongylocentrotus purpuratus* at the feeding larvae stage did not consume yolk granule proteins as an alternative food source under starvation conditions, disputing the notion that yolk granules contain nutrients which could be used by the embryo. In fact, they showed that it was fed larvae that utilized these proteins, suggesting that yolk granules may contain components necessary for growth. Several groups have shown that although proteolytic cleavage of yolk granule proteins occurs during development, the

total composition of the yolk granule does not change, also refuting the hypothesis that yolk granules contain nutrients utilized during early embryogenesis. Yakota and Kato (1988) employed SDS-PAGE to analyse the profile of yolk granule proteins under acidic conditions. In both *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina*, the major yolk granule protein (180-200 kDa) was degraded, while the intensity of several lower molecular weight species increased. Acidification of yolk granules occurs *in vivo* during embryonic development which activates a cathepsin B-like thiol protease present within the granule (Yakota and Kato, 1988). Mallya *et al.* (1992) showed that the total composition of the yolk granule remained unchanged during embryonic development of *Strongylocentrotus purpuratus* and *Lytechinus pictus*. In addition, Yakota (1993) observed no changes in the number of yolk granules, even though the electrophoretic profile of yolk granule proteins changed during development. In the starfish, *Piaster ochraceus*, Reimer and Crawford (1995) showed that the molecular composition of the yolk granule changed during early development but they did not detect loss of yolk granule proteins until the larval stage.

Other groups have shown that yolk granule proteins can become components of the cell surface or are exported and incorporated into the ECM of developing embryos. For instance, Gratwohl *et al.* (1991) showed that toposome stored within the yolk granules of sea urchin eggs becomes

incorporated into the cell surface following fertilization. This glycoprotein is proteolytically processed within the yolk granule during development from a hexamer of 160 kDa subunits to several fragments of lower molecular weight. Using immunoelectron microscopy, this processed form of toposome was detected in all plasma membranes of developing embryos. In *Xenopus laevis*, Outenreath *et al.* (1988), identified both cortical granules and yolk granules as the storage compartments for a cell surface lectin. Liao and Wang (1994) identified yolk granules of the bullfrog (*Rana catesbeiana*) oocyte as the storage compartment for ribonuclease. Mayne and Robinson (1998) showed that a protein component of both the hyaline layer and basal lamina, HLC-32, is stored in yolk granules. The results of immunolocalization studies (presented earlier) employing the anti-41 kDa collagenase/gelatinase antiserum showed that this enzyme, found at the apical and basal surfaces of developing embryos, was housed in yolk granules of unfertilized eggs. These results collectively establish the yolk granule as a dynamic storage compartment for cell surface components, ECM components, enzymes responsible in ECM remodelling, as well as enzymes involved in the degradation and processing of RNA.

Research in several systems has begun to identify yolk granule interactions with several components of trafficking and export pathways in the cell. Komazaki and Hiruma (1999) identified a role for endosomes during

yolk granule degradation in amphibian embryos. They found that the number of endosomes increased during early development. At the gastrula stage, fusion of the endosome and yolk granule promoted degradation of yolk granule proteins. This result showed the ability of yolk granule membranes to participate in fusion reactions. In the fleshfly, *Neobellieria bullata*, vitellogenic yolk granules are involved in protein export from the ovary to the gut (Bylemans *et al.*, 1996). Neb-TMOF (a trypsin modulating oostatic factor) regulates trypsin biosynthesis in the gut of late vitellogenic females and is synthesized in follicle cells. Immunolocalization studies employing polyclonal antisera against Neb-TMOF showed accumulation of the antigen in the yolk granules. However, the amount of label within the yolk granule compartment decreased from mid-vitellogenesis on and coincided with the appearance of Neb-TMOF in the hemolymph and gut. Annexins are calcium-dependent phospholipid binding proteins capable of membrane fusion events and which have been implicated in the process of exocytosis. Annexins have been localized to yolk granules in oocytes of both *Xenopus laevis* and *C. elegans* suggesting that this organelle can participate in membrane fusion and exocytotic reactions (Creutz *et al.* 1996; Srivastava *et al.*, 1996).

These results establish the yolk granule as a dynamic rather than a benign storage compartment. Components necessary for development can be compartmentalized and proteolytically processed within the yolk granule,

and can be exported from the yolk granule as required by the embryo. Components of these storage compartments now need to be identified and fully characterized. As well, research should be directed toward understanding the process through which components are sorted into the yolk granule in the unfertilized egg, and how they are transported to their final destinations in developing embryos.

**CHAPTER 6: INVESTIGATION OF THE EFFECT(S) OF THE
41 kDa COLLAGENASE/ GELATINASE AND THE ANTI-41 kDa
COLLAGENASE/ GELATINASE ANTIBODY ON EMBRYONIC
DEVELOPMENT**

6.1 INTRODUCTION

The composition and organization of the ECM influences many developmental processes including cell adhesion, migration, differentiation and proliferation (reviewed in chapter 1). The roles that individual components of the ECM play during these cellular events have been defined using several different experimental approaches: (1) by identification and characterization of naturally occurring mutations and by the introduction of mutations into ECM proteins and/or their receptors and determining the functional consequences of these mutations, (2) disruption of ECM component - ECM component and/or ECM component - cell surface receptor interactions employing specific antibodies and peptides, and (3) experiments which utilize cell culture systems to investigate the instructive role of particular ECM components on cell adhesion, migration, growth or differentiation have all shown the importance of ECM molecules during development.

Following fertilization, assembly of the hyaline layer requires the sequential deposition of individual components onto the apical surface of the sea urchin embryo. Employing immunofluorescence analysis, Matese *et al.* (1997) followed the temporal deposition of several ECM components from five individual storage vesicles in the egg (discussed in chapter 5). Storage vesicles containing basal lamina components were transported onto the basal

surface while components of the hyaline layer were exocytosed onto the apical surface. Although some ECM components are common to both the hyaline layer and basal lamina, other ECM components are particular to either the hyaline layer or basal lamina. For instance, hyalin is a component of the hyaline layer, whereas HLC-32 is a component of both the hyaline layer and basal lamina (Hylander and Summers, 1982; Brennan and Robinson, 1994). Some molecules are spatially restricted within the ECM. A basal lamina protein of *Lytechinus variegatus*, ECM 3, is deposited throughout the blastocoel of developing embryos, except at (the basal lamina at) the animal pole (Wessel and Berg, 1995). Berg *et al.* (1996) identified an ECM molecule, ECM 18, which accumulated in the basal lamina of ectoderm cells during early gastrulation but was absent from the vegetal plate, and was not associated with the ECM of primary mesenchyme cells. However by late gastrulation ECM 18 was found throughout the basal lamina.

During sea urchin embryogenesis, the composition of the ECMs influences several developmental processes including gastrulation, primary mesenchyme cell (PMC) migration and spiculogenesis. Addition of agents which affect the biosynthesis and post-translational modifications of specific ECM components such as collagens, proteoglycans and glycoproteins have been shown to arrest gastrulation (Kinoshita and Saiga, 1979; Butler *et al.*, 1987; Wessel and McClay, 1987; Adelson and Humphreys, 1988). As well the

addition of antibodies, prepared against ECM components, has been shown to affect sea urchin embryogenesis (Adelson and Humphreys, 1988; Burke *et al.*, 1991; Yokota *et al.*, 1994; Berg *et al.*, 1996).

We have characterized a 41 kDa collagenase/gelatinase stored in the unfertilized egg of the sea urchin which is exported to both the hyaline layer and basal lamina following fertilization. Given the evidence that a properly constituted ECM is required throughout development, we were interested in the role which the 41 kDa collagenase/gelatinase might play during development. We therefore examined the effects on embryonic development of (a) the addition of the 41 kDa collagenase/gelatinase and (b) the presence of the anti-41 kDa collagenase/gelatinase antiserum to cultures of developing embryos.

6.2 MATERIALS AND METHODS

6.2.1 Growth of Embryos in the Presence of the 41 kDa Collagenase/Gelatinase or Anti-41 kDa Collagenase/Gelatinase Antiserum

Gametes were collected and fertilized as described in chapter two, section 2.1. Embryos were resuspended to 1% in MFSW containing 40 µg/ml gentamycin sulfate (Sigma Co.). One hour following fertilization 20 ml aliquots of this suspension were then cultured with agitation at 10°C in the presence of 2 µg/ml 41 kDa collagenase/gelatinase or 20 µg/ml anti-41 kDa

collagenase/gelatinase antiserum or 20 $\mu\text{g/ml}$ of preimmune serum (Concentrations of antiserum and preimmune serum were reflective of those used in the literature). At various times samples were removed and the embryos viewed in a Nikon Eclipse E600 microscope equipped for photography.

6.3 RESULTS

6.3.1 Growth of Embryos in the Presence of the 41 kDa Collagenase/Gelatinase

The 41 kDa collagenase/gelatinase was added to embryos one hour following fertilization and the development of these embryos monitored until control embryos, grown in the absence of collagenase/gelatinase, reached the pluteus stage of development. Figure 6.1 depicts representative control embryos at gastrula (52 hours post fertilization (HPF); Panel A), late gastrula (74 HPF; Panel C), prism (96 HPF; Panel E) and pluteus (120 and 144 HPF; Panels G and I, respectively) stages of development. Control embryos reached the hatched blastula stage at 24 HPF, while embryos grown in the presence of the collagenase/gelatinase reached the hatched blastula stage at 28 HPF (data not shown). Embryos grown to the hatched blastula stage in the presence of the collagenase/gelatinase did not differ in appearance from control embryos (data not shown). Addition of the collagenase/gelatinase

Figure 6.1: Effect of the 41 kDa collagenase/gelatinase on embryonic development of *Strongylocentrotus purpuratus*. Top row (Panels A, C and E) shows representative control embryos. Bottom row (Panels B, D and F) shows representative embryos grown in the presence of 2 $\mu\text{g}/\text{ml}$ of the 41 kDa collagenase/gelatinase added one hour following fertilization. Panels A and B depict embryos 52 HPF, Panels C and D depict embryos 74 HPF, and Panels E and F depict embryos 96 HPF. Archenteron, star; primary mesenchyme cells, arrow.

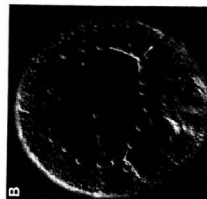
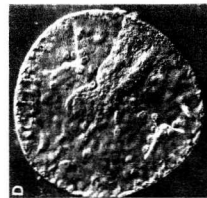
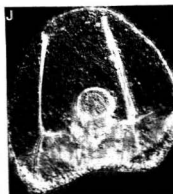
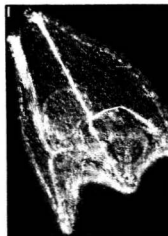


Figure 6.1 (continued): Effect of the 41 kDa collagenase/gelatinase during embryonic development of *Strongylocentrotus purpuratus*. Top row (Panels G and I) shows representative control embryos. Bottom row (Panels H and J) shows representative embryos grown in the presence of 2 $\mu\text{g}/\text{ml}$ of the 41 kDa collagenase/gelatinase added one hour following fertilization. Panels G and H depict embryos 120 HPF, and Panels I and J depict embryos 144 HPF. Oral and anal arms, stars.



prior to fertilization did not affect formation of the fertilization membrane and the collagenase/gelatinase did not possess cleavage activity for the fertilization membrane (data not shown).

Fifty-two hours following fertilization control embryos were at the gastrula stage of development (Panel A). Primary mesenchyme cells (arrow) had ingressed and formed the mesenchymal ring and spicule formation had started. The archenteron (star) had invaginated more than three-quarters into the blastocoel in 70% percent of these embryos (Panel A), and more than one-quarter in 95% of the embryos. In comparison, at 52 HPF only 10% of the embryos grown in the presence of the collagenase/gelatinase had gastrulated so that the archenteron had invaginated more than three-quarters into the blastocoel. Eighty percent of embryos examined were either at the mesenchyme blastula stage, or the archenteron had invaginated less than one-quarter into the embryo (Panel B). However primary mesenchyme cells migrated into the blastocoel, formed a mesenchymal ring and spicule growth had begun in the absence of significant gut formation (Panel B).

The retarded development of embryos grown in the presence of the collagenase/gelatinase continued throughout development. Seventy-four hours following fertilization, 60% of control embryos had fully gastrulated, spicules forming the body rods were elongated and embryos were becoming

prism shaped (Panel C). In comparison, 70% of collagenase/gelatinase treated embryos were at the mid-gastrula stage of development (Panel D).

Ninety-six hours following fertilization, greater than 90% of control embryos were prism-shaped (Panel E), while embryos grown in the presence of the collagenase/gelatinase for 96 hrs (Panel F) more closely resembled 74 HPF control embryos (Panel C). Although gut formation had occurred and spicules were elongating, embryos grown in the presence of the collagenase/gelatinase for 96 hrs (Panel F) had not become prism-shaped.

Control embryos were at the early pluteus stage 120 hours following fertilization (Panel G). The gut had compartmentalized and spicules forming the body rods were fully elongated. The oral and anal arm spicules were elongating and oral and anal arm development was occurring (Panel G; stars). In comparison, embryos grown in the presence of the collagenase/gelatinase for 120 hours were at the late gastrula-prism stage of development (Panel H).

When control embryos reached the pluteus stage, 144 hours following fertilization (Panel I), only 10 % of treated embryos were plutei. The remainder of the collagenase/gelatinase treated embryos had gastrulated and become prism-shaped, but no arm development occurred (Panel J). Interestingly, if the collagenase/gelatinase was removed from cultures of embryos at 52 hours following fertilization, when the delay in development

was apparent then these embryos developed into normal plutei that by 144 hours following fertilization were indistinguishable in appearance from control embryos with well developed anal and oral arm development (data not shown).

6.3.2 Growth of Embryos in the Presence of the Anti-41 kDa Collagenase/Gelatinase Antibody

The anti-41 kDa collagenase/gelatinase antisera was added to embryos one hour following fertilization and the development of these embryos was followed until control embryos, grown in the presence of preimmune serum, reached the pluteus stage of development. Figure 6.2 depicts representative control embryos at gastrula (52 HPF; Panel A), late gastrula (74 HPF; Panel C), prism (96 HPF; Panel E) and pluteus (120 and 144 HPF; Panels G and I, respectively) stages of development. Embryos grown in the presence of the preimmune serum to 52 HPF were at the gastrula stage of development (Panel A). The archenteron (star) had ingressed more than three-quarters into the blastocoel, primary mesenchyme cells (arrow) had migrated and formed the mesenchymal ring and spicules were forming. In comparison, at 52 HPF the majority of anti-41 kDa collagenase/gelatinase antibody treated embryos were at the mesenchyme blastula (80%) and very early gastrula stages (10%) (Panel B). In some cases primary mesenchyme cells had ingressed and

the archenteron was beginning to invaginate, however spicule formation had not begun (Panel B).

The delay in development of antibody treated embryos compared with control embryos continued throughout development. Seventy-two hours following fertilization, embryos grown in the presence of the preimmune serum were at the late gastrula to prism stages of development (Panel C) while antibody treated embryos were at the early to mid-gastrula stages of development (Panel D). The archenteron had invaginated one-quarter to one-half the distance into the blastocoel for antibody treated embryos and spicule formation had begun (Panel D).

Ninety-six hours following fertilization the delay in development for antibody treated embryos remained evident. Antibody treated embryos were at the gastrula stage of development (Panel F) while embryos treated with preimmune serum were prism-shaped with mature gut development and well formed spicules (Panel E).

One-hundred and twenty hours following fertilization, preimmune treated embryos had reached the pluteus stage of development as oral and anal arm spicules continued to elongate and arm rudiments had formed (Panel G; stars). Embryos grown in the presence of the antibody for 120 hours were at the prism stage of development (Panel H) and resembled control embryos grown in the presence of preimmune for 96 hours (Panel E).

Figure 6.2: Effect of the anti-41 kDa collagenase/gelatinase antibody during embryonic development of *Strongylocentrotus purpuratus*. Top row (Panels A, C and E) shows representative control embryos grown in the presence of 20 $\mu\text{g}/\text{ml}$ of preimmune serum added one hour following fertilization. Bottom row (Panels B, D and F) shows representative embryos grown in the presence of 20 $\mu\text{g}/\text{ml}$ of the anti-41 kDa collagenase/gelatinase antibody added one hour following fertilization. Panels A and B depict embryos 52 HPF, Panels C and D depict embryos 74 HPF, and Panels E and F depict embryos 96 HPF. Archenteron, star; primary mesenchyme cells, arrow.

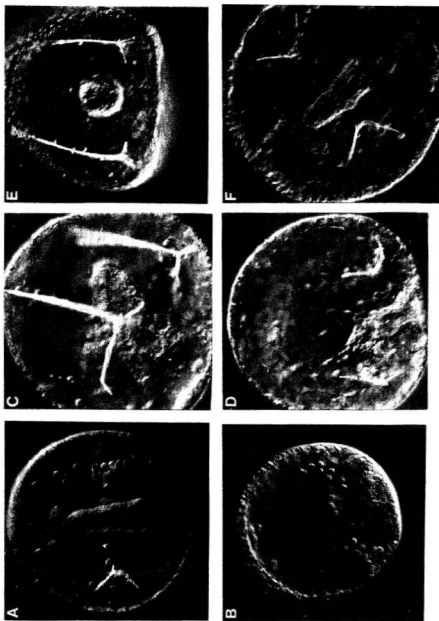
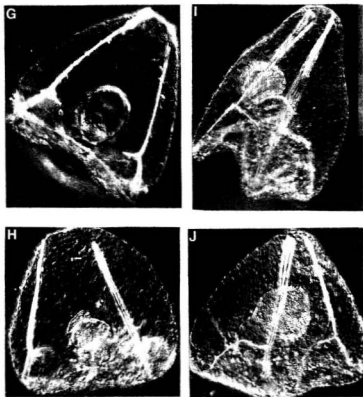


Figure 6.2 (continued): Effect of the anti-41 kDa collagenase/gelatinase antibody during embryonic development of *Strongylocentrotus purpuratus*. Top row (Panels G and I) shows representative control embryos grown in the presence of 20 $\mu\text{g}/\text{ml}$ of preimmune serum added one hour following fertilization. Bottom row (Panels H and J) shows representative embryos grown in the presence of 20 $\mu\text{g}/\text{ml}$ of the anti-41 kDa collagenase/gelatinase antibody added one hour following fertilization. Panels G and H depict embryos 120 HPF, and Panels I and J depict embryos 144 HPF. Oral and anal arms, stars.



Embryos grown in the presence of preimmune serum were at the pluteus stage of development 144 hours following fertilization (Panel I). The oral and anal arms had developed and spicules were fully elongated. Antibody treated embryos failed to develop into plutei with elongated arms following 144 hours of growth (Panel J). In fact, approximately 20% of embryos remained at the late gastrula stage of development.

6.4 DISCUSSION

A properly constituted ECM is required for sea urchin development and studies have begun to establish roles for individual ECM components during embryogenesis. The process of gastrulation, which involves cellular rearrangement, migration and expression of a differentiated phenotype, seems to be particularly sensitive to treatments which disrupt ECM composition. Inhibitors of the biosynthesis and processing of glycoproteins and proteoglycans affect morphogenesis. Kinoshita and Saiga (1979) employed inhibitors against specific steps during proteoglycan synthesis to demonstrate that proper proteoglycan synthesis was required for development beyond the mesenchyme blastula stage. Kabakoff and Lennarz (1990) inhibited the process of spiculogenesis when they interfered with

glycoprotein synthesis by preventing the processing of the oligosaccharide chains.

Several groups have employed antibodies directed against specific ECM components to study the role of these ECM molecules on embryogenesis. Adelson and Humphreys (1988) generated a monoclonal antibody against hyalin. This antibody inhibited blastocoel expansion and prevented gastrulation when added to cultures of embryos. They observed that the presence of anti-hyalin antibodies caused separation of the hyaline layer from the surface of the embryo, suggesting that the antibody interfered with the binding of cells to the hyaline layer. Yokota *et al.* (1994) found that the arms of plutei grown from fertilization in the presence of an anti-Th-nectin antibody (detected in the apical ECM) were shorter than those of normal plutei. Berg *et al.* (1996) identified an ECM molecule, ECM 18, which was expressed by invaginating endoderm cells during gastrulation and accumulated in the basal lamina. Injection of antibodies prepared against ECM 18 into the blastocoel of developing embryos at the early mesenchyme blastula stage resulted in delayed and reduced invagination of the archenteron, and, although primary mesenchyme cells ingressed they did not form a mesenchymal ring. When these antibodies were injected into the blastocoel of developing embryos at primary invagination, during early

gastrulation, invagination of the vegetal plate arrested and secondary invagination was not detected, primary mesenchyme cells ingressed in a disorganized fashion and they did not synthesize an elongated skeleton. However, 12-16 hours following injection of the antibody, embryos began to recover and resumed development. Berg *et al.* (1996) speculated that the antibody effect was lost due to either cellular endocytosis and loss of antibody molecules, and/or through synthesis of ECM 18 which could be transported into the blastocoel and would titrate out the antibody present. Tesoro *et al.* (1998) isolated a 200 kDa protein component of the basal lamina from *Paracentrotus lividus* and *Hemicentrotus pulcherrimus* with binding affinities for type I collagen and heparin. This component was localized to the surface of primary mesenchyme cells. They injected antibodies prepared against this protein into the blastocoel of early blastula stage embryos and followed development into the pluteus stage. Arm development did not occur in these embryos, or was severely reduced, and skeletal malformations were evident.

The cross-linking of collagen molecules which is important for fibril formation and which contributes to the tensile strength of collagen in the ECM, can be blocked by lathrytic agents such as β -aminopropionitrile (BAPN). Butler *et al.* (1987) showed that the activity of lysyl oxidase, which

plays a role in cross-linking collagen molecules, increased six- to seven-fold during gastrulation in both *Strongylocentrotus purpuratus* and *Lytechinus pictus*. Treating late cleavage stage embryos with BAPN prevented the increase in lysyl oxidase activity and inhibited morphogenesis beyond the mesenchyme blastula stage. When BAPN was added to cultures of hatched blastula stage embryos, PMCs ingressed into the blastocoel, but did not migrate properly and the archenteron which formed was flaccid with lateral buckling. Wessel and McClay (1987) reported similar results when they added BAPN to cultures of *Lytechinus variegatus* embryos. When added at fertilization, BAPN prevented development beyond the mesenchyme blastula stage. They also found that the addition of proline analogs (to cultures of embryos) which could be incorporated into collagen molecules during synthesis to prevent the formation of triple helices, inhibited gastrulation. These results indicated that the presence of a properly constituted collagenous matrix was required for development beyond the mesenchyme blastula stage. Furthermore, Wessel and McClay (1987) also determined that the expression of Endo 1 by endoderm at gastrulation required the presence of a properly constituted collagenous matrix.

Previous studies have shown that the 41 kDa collagenase/gelatinase is localized to both the hyaline layer and basal lamina. Additional studies have

shown that it can degrade both collagen and gelatin. To date we have not determined the physiological substrate(s) for the 41 kDa activity. Presumably addition to cultures of embryos of either the 41 kDa collagenase/gelatinase or the antibody prepared against this activity altered the turnover of, as yet, unidentified substrates within the ECMs which affected gastrulation. However, not all processes which occur during gastrulation were affected. Primary mesenchyme cells migrated into the blastocoel and spiculogenesis occurred normally, but gut formation was retarded. These results indicated that the presence of the 41 kDa activity or the antibody prepared against it did not affect either primary mesenchyme cell migration into the blastocoel or spiculogenesis. In contrast, the process of archenteron elongation was retarded, suggesting that the 41 kDa activity and the antibody prepared against it affected ECM components which play a role in directing this process. In addition, treated embryos failed to develop into plutei with well developed arms so that this process was also affected by the perturbation of the ECM by the 41 kDa activity or the antibody prepared against it.

Although delayed, gut formation did occur in most treated embryos. Addition of newly synthesized substrates and their deposition into the ECM may account for the delayed but eventual development of the gut. In fact, Wessel and McClay (1987) showed that four hours following treatment with

BAPN, collagen molecules within the blastocoel had been turned-over. They could no longer detect previously cross-linked collagen within the matrix suggesting that continuous remodelling and deposition of collagen molecules had occurred. Embryos grown in the presence of the 41 kDa collagenase/gelatinase or the anti-41 kDa collagenase/gelatinase antibody remained viable, indicating that neither the 41 kDa activity nor the antibody prepared against it were exhibiting a general toxic effect on these embryos. In fact, the retarded development of embryos grown in the presence of the collagenase/gelatinase was reversible and upon removal of the 41 kDa activity these embryos developed into normal plutei.

We do not know the exact mechanism through which either the 41 kDa activity or the antibody prepared against it affected gut formation and prevented development of plutei with well developed arms. As mentioned above, the collagenase/gelatinase may cleave one or more substrates within the ECMs. Cleavage of ECM molecules may alter the physical organization of the ECMs and/or the interaction of molecules within the ECMs. These

changes may interfere with ECM signalling important during the process of gastrulation and during subsequent development of the oral and anal arms of the pluteus stage embryos. The effect of the anti-41 kDa collagenase/gelatinase antibody may be to alter the activity of the collagenase/gelatinase

within the ECM and therefore alter the turnover of substrates within the ECMs. Alternatively, the antibody may exhibit a steric effect within the ECMs. The presence of the antibody molecules within the ECMs may sterically hinder the interaction of ECM molecules and disrupt the organization of the ECMs, thus affecting development.

CHAPTER 7: FUTURE DIRECTIONS

In the future we want to continue the biochemical characterization of the 41 kDa collagenase/gelatinase. We have measured the kinetic parameters, K_m and V_{max} for this enzyme against peristome gelatin and peristome collagen. Collagenases cut collagen molecules at specific sequences along the molecule to generate 1/4 and 3/4 length polypeptides. Synthetic peptide sequences can be used as substrates to determine if the 41 kDa collagenase/gelatinase cleaves particular peptide sequences more efficiently.

Extracellular matrix proteinases play diverse functions in developing embryos and in adult organisms, in both physiological and pathological conditions. We have begun to investigate the role that the 41 kDa collagenase/gelatinase plays during sea urchin embryogenesis. Raising embryos in the presence of the 41 kDa collagenase/gelatinase or the antibody prepared against it from one hour following fertilization, resulted in delayed development at the gastrula stage and these embryos failed to develop into plutei with well formed arms. Additional studies should be carried out to further define the role of the 41 kDa collagenase/gelatinase during development: what is the effect on embryonic development when increasing concentrations of the 41 kDa activity or the anti-41 kDa antibody are added to cultures of embryos?; what effect would we observe on subsequent development if we removed the 41 kDa activity at the hatched blastula stage, or if we added the 41 kDa activity or the anti-41 kDa antibody after

gastrulation?; can embryos grown in the presence of the 41 kDa activity or the anti- 41 kDa antibody develop into plutei with well formed arms if we follow growth beyond 144 hours post fertilization?; what effect would we observe if we inject the 41 kDa activity or the anti-41 kDa antibody into the blastocoel of developing embryos?

Vertebrate collagenases are inhibited by tissue inhibitors of matrix metalloproteinases. The presence of these molecules in the sea urchin embryo can be detected using the technique of reverse zymography. In this qualitative assay both a substrate, such as gelatin and the matrix metalloproteinase activity are copolymerized in the acrylamide in SDS-PAGE. Samples are fractionated in the gel. The presence of tissue inhibitors of matrix metalloproteinases are visualized as a band of stained intact gelatin against an unstained background. These molecules, if present in the sea urchin embryo could be employed in inhibition studies to investigate the function of this collagenase/gelatinase activity.

The anti-41 kDa antibody can be used to screen expression libraries of the sea urchin to identify the cDNA clone of the collagenase/gelatinase. Sequence data can be used to identify sequence homology with vertebrate extracellular matrix proteinases. A cDNA clone could also be employed in mutational analyses. Do certain mutations affect substrate binding, substrate cleavage? How do mutations in the 41 kDa collagenase/gelatinase introduced

into developing embryos affect development? Is the collagenase/gelatinase only expressed in the embryo or do adult sea urchin tissues express this enzyme? At what embryonic stages is it expressed?

The notion of the yolk granule as a benign storage compartment for nutrients for the developing embryo has been challenged (literature reviewed in chapter 5). Yolk granules in the sea urchin persist throughout development and disappear only at the fed larval stage. My research showed that the yolk granule contains components which are exported into the ECMs of developing embryos. The functional role of the yolk granule during development remains to be fully defined. Components stored within yolk granules should be identified and characterized. How are the protein components within yolk granules exported? Do yolk granules interact with other components of export pathways such as the Golgi apparatus, the plasma membrane or other transport vesicles? Do yolk granules interact with annexins and clathrin, protein markers known to be involved in transport processes? Experiments designed to investigate these possibilities will allow us to further define the functional role of the yolk granule in protein export. The technique of immunogold labelling employing antibodies against transport proteins in combination with electron microscopy is one approach which we can utilize to begin to investigate these interactions.

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Appendix A: Substrate Specificity of the 41 kDa Collagenase/Gelatinase

Substrate specificity of the 41 kDa collagenase/gelatinase was examined by substrate gel zymography, in parallel gels containing 0.1% (w/v) each of three test substrates and using the quantitative assay described in chapter 2. Test substrates included gelatin, casein, BSA and hemoglobin. These experiments were carried out as part of my honours project and are reported in Mayne and Robinson (1996).

Using substrate gel zymography, both gelatin and casein were cleaved by the 41 kDa activity (Figure 1; lanes 1 and 2, respectively), although gelatin was cleaved to the greatest extent. Cleavage of hemoglobin was barely detectable in the zymogen gel, while no cleavage activity was seen in the gel containing BSA as a test substrate (Figure 1; lanes 3 and 4, respectively). These results were confirmed by the quantitative assay (Table 1). Casein hydrolysis was one-third that of gelatin and minimal activity was measured for hemoglobin and BSA.

These results clearly demonstrated that gelatin was the preferred substrate of the 41 kDa collagenase/gelatinase.

Figure 1-A1. Substrate specificity of the 41 kDa gelatinase. Ten microlitre aliquots of the purified gelatinase were solubilized for 30 minutes at room temperature in an equal volume of solubilizing solution (Laemmli, 1970) from which the bromophenol blue and dithiothreitol had been omitted. Substrate gel zymography was performed as described in the Methods section and the gel contained acrylamide copolymerized with 0.1% (w/v) gelatin (lane 1), 0.1% (w/v) casein (lane 2), 0.1% (w/v) hemoglobin (lane 3) or 0.1% (w/v) bovine serum albumin (lane 4).

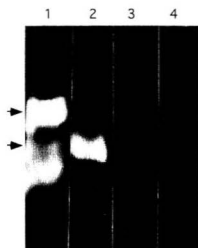


TABLE 1-A1: SUBSTRATE SPECIFICITY OF THE 41 kDa CLEAVAGE ACTIVITY

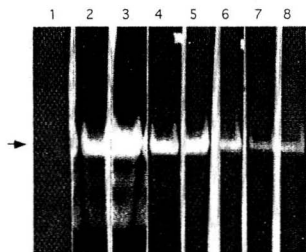
<u>Substrate Tested</u>	<u>Activity</u> <u>(units/mL)</u>	<u>Relative Activity</u>
Gelatin	2040	100
Casein	616	30.2
Hemoglobin	183	8.9
Bovine Serum Albumin	84	4.1

Note: Two microlitre aliquots of the purified gelatinase were incubated with 0.1% (w/v) of the test substrate and the cleavage of the test substrate measured using the quantitative assay as described in the Methods section. Activity is expressed as units/mL. One unit of activity is defined as the amount of gelatinase required to cleave 0.1 μ g of protein into TCA soluble pellets per minute at 37°C. Activity is expressed as a percentage of cleavage of gelatin which was normalized to 100%.

Appendix B: Metal-ion Activation of the 41 kDa Collagenase/ Gelatinase by Various Metal Ions.

As part of my honours thesis gelatin substrate gel zymography was carried out to examine the effects of various metal ions on the activity of the 41 kDa collagenase/gelatinase. Gelatin substrate gel strips were incubated in the presence of the various metal ions at concentrations of 10 mM (Figure 1). The 41 kDa activity had the greatest amount of activity when incubated in the presence of calcium (lane 3). Addition of EDTA to 5mM was an effective inhibitor of the 41 kDa activity (lane 1). Both magnesium and manganese were unable to activate the 41 kDa activity (lanes 4 and 5, respectively), when compared to the activity of the 41 kDa species in the absence of exogenously added metal ions (lane 2). Neither copper, cadmium or zinc were effective in activating the 41 kDa activity (lanes 6, 7, and 8, respectively).

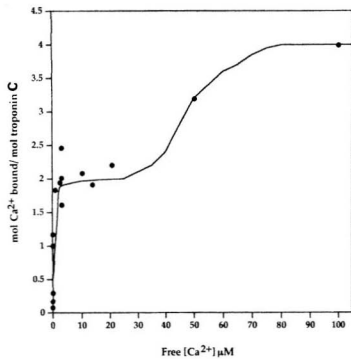
Figure 1-A2. Metal-ion activation of the gelatinase by various metal ions. Fifteen microgram aliquots of eggs were solubilized and fractionated by gelatin substrate zymography as described in Figure 1. Following electrophoresis strips of gel were processed in the presence of 5 mM EDTA (lane 1), in the absence of any exogenously added metal ion (lane 2), in the presence of 10 mM CaCl_2 (lane 3), in the presence of 10 mM MnCl_2 (lane 4), in the presence of 10 mM MgCl_2 (lane 5), in the presence of 10 mM CuCl_2 (lane 6), in the presence of 10 mM CdCl_2 (lane 7) or in the presence of 10 mM ZnCl_2 (lane 8).



AppendixC: Quantitation of Calcium Bindingto Troponin C

As a control for the quantitation of calcium binding to the 41 kDa collagenase/gelatinase, we quantitated the amount of calcium binding to troponin C by equilibrium dialysis of apo-troponin C, in the presence of increasing calcium concentrations. We determined that four mols of calcium bound per mol of Troponin C. There were two classes of binding sites, each binding two mols of calcium with k_{ds} of 1×10^{-7} and 4×10^{-5} M. These results are in agreement with published data (Potter and Gargely, 1975).

Figure 1-A3: Quantitation of calcium binding to troponin C. Aliquots of troponin C (50 μ g) were brought to a final volume of 1 mL in 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA and dialyzed against 100 mL of 50 mM Tris-HCl, pH 8.0, containing 0.1 mM EGTA, 20 μ Ci 45 CaCl₂ (Amersham; 0.4 mCi/ μ mol) and various concentrations of CaCl₂ for 72 hrs at 4°C.



**AppendixD: Percentage Cleavage of Peristome and Rat Tail
Tendon Collagens Following Preincubation at 15°C, 37°C and 60°C**

	Peristome Collagen		Rat Tail Tendon Collagen	
Preincubation Temperature (°C)	140 kDa	116 kDa	126 kDa	123 kDa
15	35.2 ± 4.5	32.4 ± 4.8	3.2 ± 2.4	3.2 ± 1.8
37	55.4 ± 6.3	58.3 ± 7.1	14.9 ± 3.2	13.7 ± 3.8
60	51.2 ± 5.8	58.6 ± 6.2	93.9 ± 3.4	93.8 ± 4.5

Appendix E: Immunofluorescence of Sea Urchin Eggs and Embryos

Figure 1-A5: Indirect immunofluorescence analysis of sea urchin eggs and embryos using the anti-41 kDa antibody. The antibody was used at a dilution of 1:100 (v/v). Panels A - E represent unfertilized eggs, 1 HPF, 18 HPF and 45 HPF, respectively. Panel E depicts a stratified, unfertilized egg. When preimmune serum was used no significant immunofluorescence was detected in the eggs or the embryos (data not shown). Bars=0.25 μ m.

