Membrane type-1 matrix metalloproteinase (MT1-MMP) as a target in cancer therapy

by

CANDICE JULIE CROUCH

B.Sc. (HONS.)

Submitted in fulfilment of the

academic requirements for the degree of
Masters of Science
in the
Discipline of Biochemistry
Faculty of Science and Agriculture
University of KwaZulu-Natal
Pietermaritzburg

This thesis is dedicated to the memory of my mother

Beverley Crouch

Preface

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, from January 2006 to August 2007, under the supervision of Dr Edith Elliott and co-supervision of Dr Alain F.V. Boulangé.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

_____

Candice Julie Crouch (candidate).

_____

Dr Edith Elliott (supervisor).

_____

Dr Alain F.V. Boulangé (co-supervisor).

Abstract

Membrane type I-matrix metalloproteinase (MT1-MMP), a member of the highly active extracellular matrix (ECM)-degrading matrix metalloproteinases (MMPs), is known to be involved in connective tissue remodelling and embryogenesis, as well as tumour invasion and metastasis. Positioned on the leading edge of the invading cell, its proteolytic activity is enhanced by activation of proMMP-2 in a complex with tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). The aim of this study was to attempt to produce highly specific and immunoinhibitory antibodies against human MT1-MMP and to test whether such antibodies are able to stop invasion by blocking MT1-MMP activity. As various MMP domains are highly conserved across species, and within specific MMP groups, and human cancer cells were to be used in invasion assays, sequence alignment of human MT1-MMP domains was used to identify the most variant and, hence, the optimal laboratory species for antibody production, and the chicken was subsequently selected. A hemopexin-like or collagen-binding domain, together with the catalytic domain (PEXcat), the hemopexin-like domain (PEX) and the propeptide and catalytic domain (PROcat) were selected as target domains for antibody production. *Escherichia coli* or *Pichia pastoris* expressed PEXcat and PEX domains, respectively, were obtained collaboratively, and an *E. coli* expression system was used to express the PROcat domain. Urea and β-mercaptoethanol successfully solubilised PROcat inclusion body protein, and Q- and S-Sepharose ion exchange chromatography, removed majority of the *E. coli* contaminating proteins, yielding >200 mg/litre expressed PROcat protein. Alum adjuvant and unrenatured soluble PEXcat and PEX proteins, or the less soluble, S-Sepharose purified PROcat protein was used for inoculations of chickens. The PROcat antigen, also injected as a homogenised band in acrylamide, proved to be inferior to S-Sepharose-purified PROcat antigen in alum, as it failed to induce an antibody response. The S-Sepharose-purified PROcat antigen, in alum adjuvant, produced the highest overall response, purified anti-PROcat IgY recognising recombinant forms of MT1-MMP (33 kDa and 50 kDa) and a 63 kDa protein in human blood, concluded to be either latent, soluble MT1-MMP or a non-specific protein. These antibodies, however, failed to detect native human and murine MT1-MMP (43 kDa) in cell line homogenates, suggesting that they possibly did not recognise the zinc-binding site of the

catalytic domain in the 43 kDa processed MT1-MMP. In contrast to purified IgY, crude anti-PROcat IgY preparations recognised renatured PROcat MT1-MMP (29 kDa), indicating possible binding and removal of anti-human MT1-MMP antibodies during PEG purification. Despite this, the purified IgY resulted in higher immunoinhibition of the renatured PROcat antigen than the crude IgY. Anti-PEXcat antibodies had low titre, recognising native MT1-MMP in human cell (43 kDa) and mouse macrophage homogenates, but did not recognise the original recombinant PEXcat MT1-MMP antigen or PROcat MT1-MMP, possibly due to levels of loaded antigen being too low for detection in the western blots. Although these antibodies also did not seem to recognise the catalytic domain in the western blots, the high immunoinhibitory effect induced by these antibodies suggested otherwise. The PEX antigen induced the weakest antibody response, antibodies detecting only recombinant MT1-MMP (50 kDa). The anti-PROcat IgY, overall, produced denser labelling of the MCF10A and MCF10A-neoT cell lines, than the anti-PEXcat IgY, and these antibodies preferentially recognised the PRO domain of proMT1-MMP, focused in lamellipodia of the MCF10A cell line. Comparisons between the normal and cancer cell line, the anti-PROcat IgY labelled the MCF10A-neoT cells weaker than they labelled the MCF10A cells and the labelling was spread along the plasma membrane and the base of the cell. The anti-PEXcat IgY, in contrast, showed slightly more labelling of MT1-MMP in the MCF10A-neoT cells, compared to the MCF10A cells, which may promote the invasive phenotype of this cell line. In the fixed tissue, anti-PEXcat labelled all forms of MT1-MMP, as expected. Although similar labelling was observed with the anti-PROcat IgY, these antibodies were most likely recognising proMT1-MMP. Renaturation of Q-Sepharose purified PROcat antigen, using 0.5 mM $ZnCl_2$ gradient dialysis, produced catalytically active, renatured protein for immunoinhibition assays, although the observed higher Km in this study possibly suggested this procedure was not a successful as a one-step dialysis procedure previously reported. Despite this, immunoinhibition assays revealed a 88%, 70% and 34% inhibition of activity by the anti-PEXcat, PROcat and anti-PEX IgY, respectively, suggesting that the anti-PEXcat IgY would be most useful in invasion inhibition studies.

Acknowledgements

I would like to sincerely thank the following people for their contributions to this study:

Edith Elliott, my supervisor and mentor, for her guidance throughout this study and her constructive, critical assessment of my write-up. All the skills with which you have equipped me with will be invaluable in the lab and in life.

Alain Boulangé, my co-supervisor, for his advice, guidance and assistance in this study, particularly with regards protein expression, and his contributions towards the assessment of my write-up.

Clive Dennison for his infinite wisdom on the subject of biochemistry and for his good sense of humour. You are definitely an inspiration to me.

Celia Snyman for her contributions to my work, particulary in electron and confocal microscopy. Her contagious enthusiasm for science, together with her positive attitude, made working in the lab an absolute pleasure.

Professor Theresa Coetzer for allowing me to use the equipment in her lab. Her assistance is much appreciated.

My collegues Kelvin Lamberti and Derek Van Rooyan in lab 43 for their ideas and support during this study and for the many laughs we shared, which lightened up my day. Derek, in particular, I would like to thank for his assistance in the antibody production.

The administrative and technical staff, Denzil, Yegan and Jessica and the secretaries Charmaine and Robyn.

Proffessor Harald Tschesche, University of Bielefeld, Germany and Professor Itoh, Imperial Collage, London for the PROcat expression construct and the PEXcat and PEX protein products respectively.

Michèle Brillard, Université de Tours, France, who kindly peformed the N-terminal sequencing for me.

David Eades, my fiancé, for his love and support during my masters. Even though his knoweledge of the subject was extremely limited, he always showed keen interest in my work and encouraged me every step of the way.

My father Mike Crouch, sister Tarryn Cox, brother-in-law Graham Cox and my two beautiful nieces, Rachel and Julia for their love, support and prayers.

My Lord and Saviour Jesus Christ, for his unconditional love, guidance and support. I know He is with me every step of the way.

NRF for funding

Table of contents

List of figures

List of tables

List of abbreviations

α	alpha
A_{280}	absorbance at 280 nm
ABTS	2, 2'-Azino-di-(3-ethyl)-benzthiozoline sulfonic acid
AEC	anion exchange chromatography
AMC	7-amino-4-methyl coumarin
AP	alkaline phosphatase
β	beta
BSA	bovine serum albumin
ºC	degrees Celcius
Ca^{2+}	calcium ion
$CaCl_2$	calcium chloride
CEC	cation exchange chromatography
DMSO	dimethyl sulfoxide
$d.H_2O$	distilled water
$dd.H_2O$	double distilled water
E. coli	Escherichia coli
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay

ER	endoplasmic reticulum
g	gram
h	hour(s)
HRPO	horse radish peroxidase
IEC	ion exchange chromatography
IgG	immunoglobulin G
IgY	immunoglobulin Y
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	kilobases
kDa	kilo Daltons
K_m	Michaelis constant
MCF	Michigan Cancer Foundation
MCF10A	Michigan Cancer Foundation (attached)
Min	minutes
mg	milligram
MMP	matrix metalloproteinase
MT1-MMP	membrane-type-1 matrix metalloproteinase
Ni-NTA	nickel nitrilotriacetic acid
PBS	phosphate buffered saline
PEXcat	hemopexin-like and catalytic domains

pI	isoelectric point
PROcat	propeptide and catalytic domains
Q-Sepharose	quaternary amine sepharose
Ras	Rous Sarcoma
rpm	revolutions per minute
RT	room temperature
S-Sepharose	sulphomethyl sepharose
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacylamide gel electrophoresis
TBS	tris buffered saline
TEMED	N,N,N',N', tetramethyl ethylene diamine
TIMP	tissue inhibitor of matrix metalloproteinases
µg	microgram
µl	microlitre
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
Zn^{2+}	zinc ion
$ZnCl_2$	zinc chloride

Chapter 1

Introduction

1.1 Membrane-type and other matrix metalloproteinases

Malignant cancer is characterised by uncontrolled cell proliferation, invasion and metastasis (Stetler-Stevenson *et al*., 1993). This behaviour is assisted by the ability of cancer cells to penetrate body barriers by degrading the components of the extracellular matrix (ECM) (Westermarck and Kahari, 1999; Kim *et al*., 1998; Sabeh *et al*., 2004). The zinc-dependent matrix metalloproteinases (MMPs) have been shown to be the key enzymes involved in ECM breakdown (Nagase and Woessner, 1999).

MMPs were first described in 1962 by Gross and Lapiere in the tail of a bullfrog tadpole. Since then MMPs have been identified in a wide variety of organisms ranging from sea urchins (Lepage and Gache, 1989) to the higher mammals (Forget *et al*., 1999) and plants (Maidment *et al*., 1999). Present in cells, tissue and interstitial fluids, and prevalent at different stages during tissue development, MMPs are directly responsible for critical remodeling of the extracellular matrix (ECM) in normal processes such as embryonic development, wound healing, ovulation, bone remodelling, parturition and breast and uterus involution (Nagase and Woessner, 1999; Birkedal-Hansen, 1993). The degradation of the ECM by MMPs, however, is also associated with many pathological diseases. These include inflammation, rheumatoid arthritis, atherosclerosis, emphysema, pulmonary fibrosis, skin ulcerations, gastric ulcers, neurological disease, liver fibrosis, and tumour invasion and metastasis (Stanton *et al*., 1998; Nagase and Woessner, 1999; Yana and Seiki, 2002).

MMPs also release or process various biomolecules and are, thus, involved in cell signaling (Foda and Zucker, 2001; Folgueras *et al*., 2004). Many of these cell signaling biomolecules are responsible for cell proliferation, migration, differentiation, programmed cell death (apoptosis) and angiogenesis (Werb, 1997; Massova *et al*., 1998; Vu and Werb, 2000).

Other proteinases also associated with tumour invasion, metastasis and ECM breakdown include serine, aspartic and cysteine proteinases (Duffy, 1997). Unlike the MMPs, which are mainly secreted, the cysteine and aspartic proteinases are normally localised to intracellular

vesicles known as lysosomes. In certain cancers, however, these enzymes may be secreted (Nomura and Katanuma, 2005) or become associated with the plasma membrane (Erdel *et al*., 1990). In particular, cathepsins B, H and L are upregulated in many human malignant cancers (Kos *et al*., 1997). These enzymes seem to activate other proteolytic enzymes which promotes ECM breakdown and tumour invasion and metastasis (Nomura and Katunuma, 2005). It should be added that cathepsin B may also reduce tumour invasion by inducing tumour cell apoptosis, suggesting it may adopt a dual role (Foghsgaard *et al*., 2001). Cathepsin D, on the other hand, is also overexpressed in breast cancer and is thought to be involved primarily in tumour growth rather than invasion (Foekens *et al*., 1999).

The cathepsins are considered to be critical in early stage tumour formation and invasion where they are thought to determine the metastatic route (Dohchin *et al*., 2000). In contrast, MMPs seem to be critical at all stages of tumour development (Ou *et al*., 2006). Some MMPs are present on the surface of the cells while others are secreted into the surrounding millieu allowing tumour cells to migrate to distant tissue, promoting tumour metastasis. Previous studies have shown that the absence of MMPs significantly reduces invasion and metastasis (Sabeh *et al*., 2004). This suggests the MMPs are the key effector enzymes in invasion.

To date, 23 human MMPs have been identified and classified into six major classes based on their substrate specificity, primary structure (Figure 1) and cellular localisation (Sounni *et al*., 2003; Itoh and Seiki, 2006). These classes include the collagenases, stromelysins, matrilysins, elastases and membrane-type MMPs (MT-MMPs) (Table 1). Common to most classes of MMPs are three highly conserved domains, namely the propeptide, catalytic and hemopexin-like domains (Figure 1) (Massova *et al*., 1998). The catalytic domain (~170 amino acids) contains a catalytic zinc ion in the active site cleft (Figure 1) and is primarily responsible for the enzyme's activity (Massova *et al*., 1998; Tam *et al*., 2002). The propeptide domain (~80 amino acids) binds to the catalytic zinc ion, via a highly conserved cysteine residue, and maintains the enzyme in a latent form until cleaved. All MMPs, except for the matrilysins, contain a hemopexin-like (C-terminal) domain (~210 amino acids) (Figure 1) joined to the catalytic domain via a proline-rich hinge region (5-50 amino acids).

It is involved in substrate specificity and binding (Tam *et al*., 2002). Common to all MMPs is a signal peptide, which directs the MMPs to the endoplasmic reticulum or to be intergrated into the plasma membrane or secreted into the extracellular matrix (Folgueras *et al*., 2004).

Unique to the membrane-type MMPs (MT-MMP 1-6) is a transmembrane (24 amino acids) and cytoplasmic domain (20 amino acids) (Sato *et al*., 1996) or glycosylphosphatidylinositol linker (Figure 1). These domains function to anchor the MT-MMPs to the cell surface and, thereby, localises their activity to the surrounding cellular microenvironment. This localisation of MT-MMPs on the cell surface promotes cell migration and invasion of tumour cells and, as a result, MT-MMPs have been implicated in malignant tumour progression. The structure of the cytoplasmic domain of MT1-MMP, has diverged from the other MT-MMPs suggesting a difference in activity and regulation (Jiang *et al*., 2001).

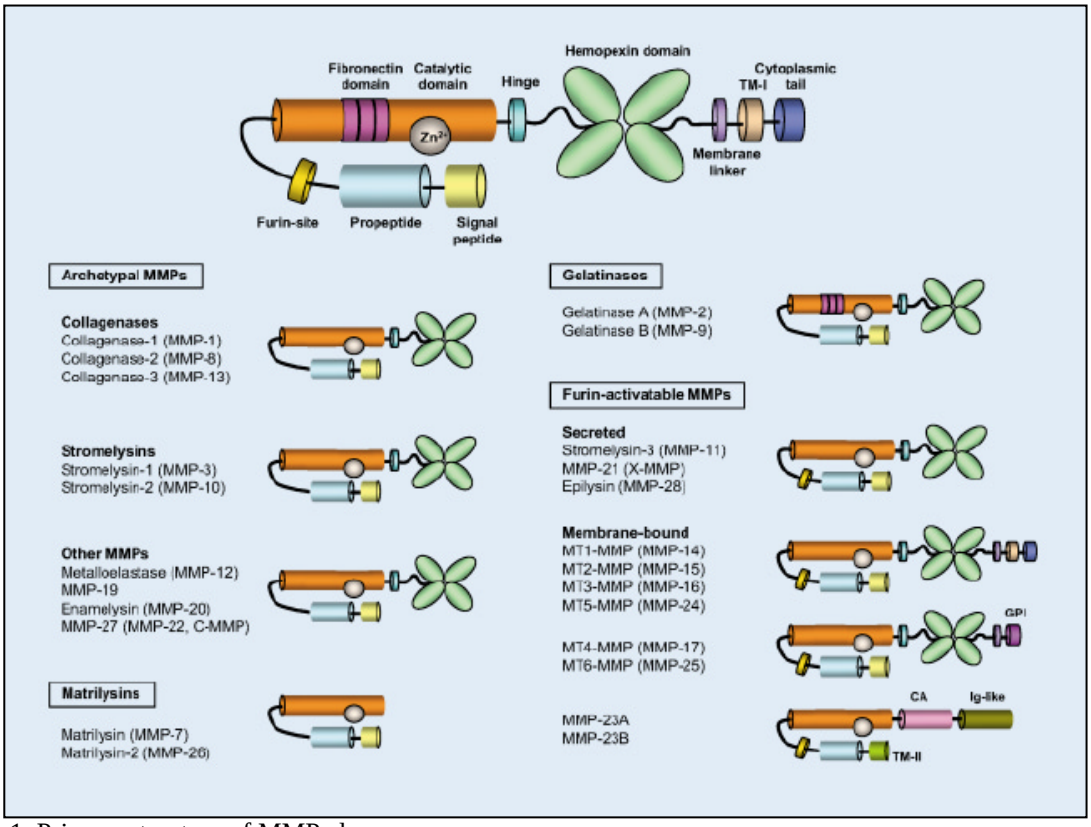

Figure 1. Primary structure of MMP classes.

Common to all MMPs is the catalytic and propeptide domains. All, except the matrilysins, also contain a hemopexin-like domain. The remaining structural features are characteristic of each family of MMPs (Folgueras *et al.*, 2004).

Table 1 Classification, substrate specificity and molecular weights of the matrix metalloproteinase family. (Forget *et al*., 1999; Bigg and Rowan, 2001; Overall, 2002).

MMP family	**Substrates**	**Molecular Weight (kDa)**	
		Latent form	**Active form**
Collagenases			
Collagenase-1 (MMP-1)	collagen I,II,III,IV, X; gelatin; aggrecan; link protein; entactin; tenascin; perlecan	55	45
Collagenase-2 (MMP-8)	collagen I,II, II; gelatin; entactin; aggrecan; tenascin	75	58
Collagenase-3 (MMP-13)	collagen I, II, III; gelatin; entactin; tenascin; aggrecan	60	48
Collagenase-4 (MMP-18)	collagen I, II, III; gelatin	55	42
Gelatinases			
Gelatinase-A (MMP-2)	collagen I, IV, V, VII, X, XI; elastin; fibronectin; laminin-5; aggrecan; brevcin; neurocan; decorin; vitronectin; BM-40	72	66
Gelatinase-B (MMP-9)	Collagen I, IV, V, VII, X, XI; elastin; fibronectin; laminin; aggrecan; link protein; vitronectin	92	86
Stromelysins			
Stromelysin-1 (MMP-3)	collagen II, III, IV, V, IX, X, XI; aggrecan; laminin; fibronectin; gelatin; entactin; perlecan; decorin; tenascin; vitronectin; fibrin; link protein; elastin	57	45
Stromelysin-2 (MMP-10)	gelatin I, III, IV, V; fibronectin; proteoglycan	57	44
Stromelysin-3 (MMP-11)	Fibronectin, laminin, aggrecan	51	44
Stromelysin-4 (MMP-19)	Collagen IV, gelatin, laminin, fibronectin, tenascin, entactin, aggrecan, fibrin/fibrinogen		
Matrilysins			
Matrilysin (MMP-7)	Fibronectin; laminin; collagen IV, V, IX, X, XI; gelatin; aggrecan; entactin; tenascin; vitronectin; fibrin/fibrinogen	28	19
Matrilysin-2 (MMP-26)	Collagen IV, gelatin, fibronectin, fibrin/fibrinogen	28	19
Elastases			
Metalloelastase (MMP-12)	elastin, fibronectin, laminin, proteoglycan, fibrin/fibrinogen	54	45/22
Membrane-type (MT)			
MT1-MMP (MMP-14)	Collagen I, II, III; gelatine; vitronectin, aggrecan, laminin, dermatan sulphate, nidogen, tenascin, fibrin, fibronectin, proMMP-2, proMMP-13, CD44	63	60
MT2-MMP (MMP-15)	Proteoglycans	72	60
MT3-MMP (MMP-16)	collagen III, fibronectin	64	52
MT4-MMP (MMP-17)	gelatin, fibrin/fibrinogen	57	53
MT5-MMP (MMP-24)	fibronectin, proteoglycans, gelatin	63	45
MT6-MMP (MMP-25)	Collagen IV, gelatin, fibronectin, proteoglycans, laminin, fibrin/fibrinogen	63	N/A

MMP activity is highly regulated both at the gene and post-transcriptional level (Figure 2). At the gene level, growth factors, hormones and cytokines control the transcription and translation of MMPs (Nagase and Woessner, 1999). This ensures that these enzymes are only produced on demand. Mutational transformation, cell shape and cellular interaction, certain soluble factors and the ECM also play a role in regulating gene expression of MMPs (Figure 2) (Birkedal-Hansen *et al*., 1993; Nagase and Woessner, 1999). At the post-transcriptional level, MMPs are controlled by their translation, secretion, activation, cellular localisation and trafficking through cellular compartments to the cell surface (Polette and Birembaut, 1998; Sounni *et al*., 2003; Itoh and Seiki, 2006). In particular, MT1-MMP is regulated by the clathrin-dependent and calveolae-dependent pathways (Remacle *et al*., 2003), its trafficking and function being assisted by its cytoplasmic tail (Kridel *et al*., 2002; Yana and Seiki, 2002; Jiang *et al*., 2004). Via these two pathways, the enzyme is endocytosed into vesicles and subsequently degraded or recycled back to the cell surface. This intracellular trafficking could serve as a quick way of localising the enzyme at the leading edge of the cell, a crucial event for cell migration (Remacle *et al*., 2003).

Tissue inhibitors of matrix metalloproteinases (TIMPs) and α2-macroglobulin also regulate MMP activity. Distributed widely in tissue, TIMPs (1-4) are specific inhibitors of MMPs (Henriet *et al*., 1999). They inactivate target MMPs by binding non-covalently, via their N-terminal domain, to the catalytic domain of MMPs at a 1:1 ratio and with a high affinity (Huang *et al*., 1996; Visse and Nagase, 2003). Only TIMPs 2-4, in particular TIMP-2, regulates MT1-MMP activity (Will *et al*., 1996). Binding of the inhibitor to the proform of the enzyme, consequently, delays further enzyme activation and, thus, provides additional regulation of activity (Birkedal-Hansen *et al*., 1993; Massova *et al*., 1998). TIMP-2, is also involved in the activation of MMP-2 (Hernandez-Barrantes *et al*., 2000). The inhibitor, therefore, adopts a dual role in regulating MMP activity. The α2-macroglobulin, on the other hand, is a general protease inhibitor primarily present in plasma and tissue fluids (Folgueras *et al*., 2004; Polette and Birembaut, 1998).

The regulation of MMPs at the gene and post-transcriptional level is aimed at balancing the production of these enzyme under normal conditions. In tumour cells, the regulation of

MMP activity is imbalanced, favouring the activated enzyme and leading to excessive proteolysis of the ECM and the development of various pathological conditions, including tumour invasion and metastasis (Figure 2).

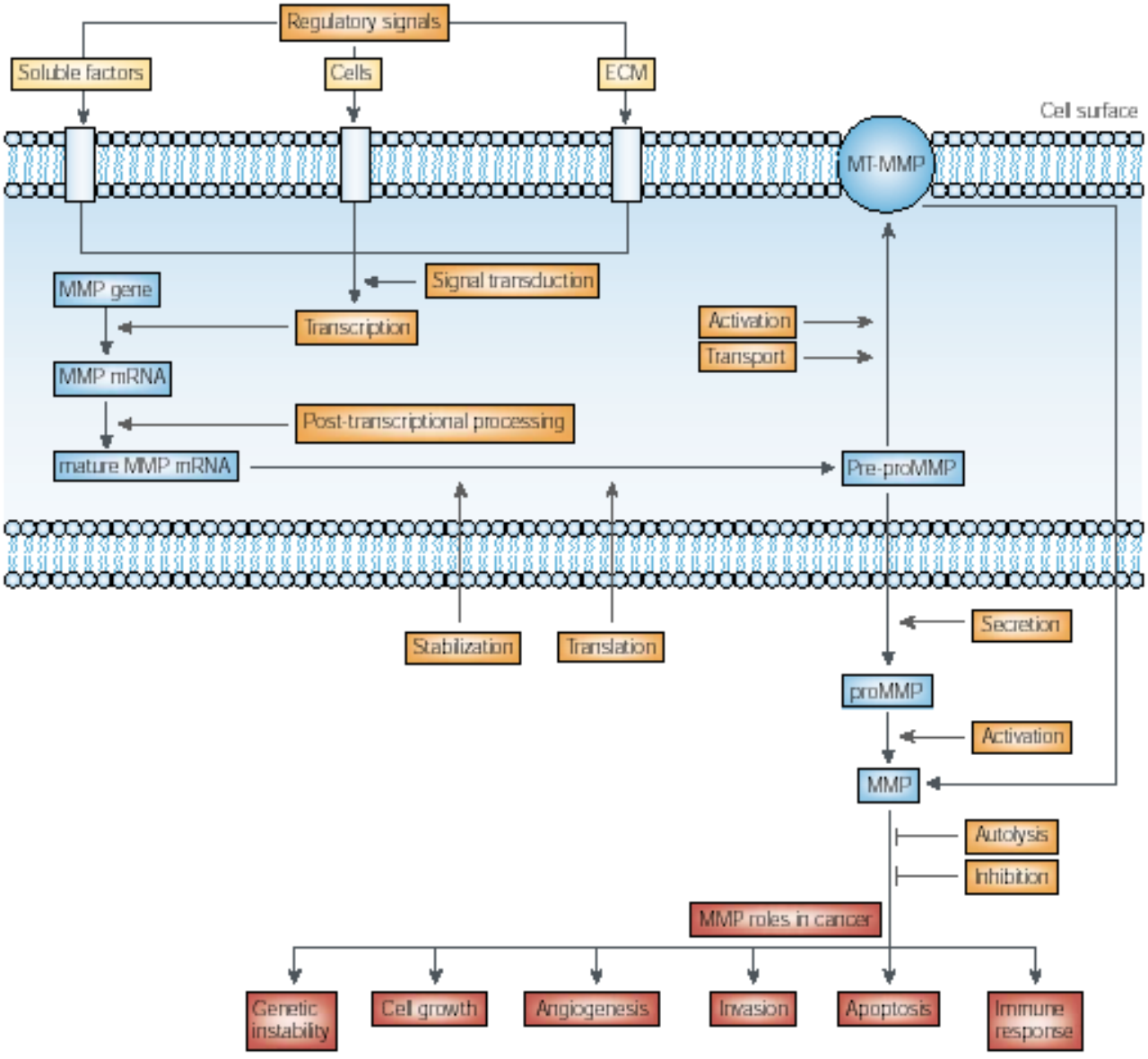

Figure 2. Regulation of MMPs at the gene and post-transcriptional level

(Overall and Lopez-Otin, 2002)

1.2 Activation of proMMPs

Most MMPs are secreted as proenzymes and are activated at the cell surface by proteinases or chemical agents via a step-wise cysteine switch mechanism (Figure 3) (DeClerck, 2000; Visse and Nagase, 2003). This activation involves cleavage/disruption of the Cys-zinc complex in the active site cleft, leading to partial removal of the propeptide domain. This is thought to destabilise the enzyme and, thus, the enzyme is triggered into autoactivation and rest of the propeptide domain is removed (Visse and Nagase, 2003). MMP-11, MMP-23 and MMP-28 and the MT-MMPs contain a furin cleavage site (Figure. 1) (Visse and Nagase, 2003) and are usually activated intracellularly, in the Golgi network, via a furin-like proprotein convertase, which recognises a characteristic Arg-Arg-Lys-Arg^{111} sequence between the propeptide and catalytic domain and cleaves specifically at the Arg^{111}-Tyr^{112} bond (Sato *et al*., 1996). The mature enzyme is subsequently sorted into vesicles and delivered to the cell surface lacking its propeptide domain (Ouchi *et al*., 1997; Massova *et al*., 1998; Remacle *et al*., 2003; Folgueras *et al*., 2004). This is not always the case, however, as MT1-MMP (63 kDa), has been reported to be delivered to the cell surface as a proenzyme, in L_0V_0 cells, where the enzyme may subsequently be activated by soluble/membrane-associated furin or via furin-independent pathways (Table 2) (Yana and Weiss, 2000). Once activated, MT-MMPs, in many cases, are the initiators in the cell surface activation of other MMPs (Figure 4) (Visse and Nagase, 2003).

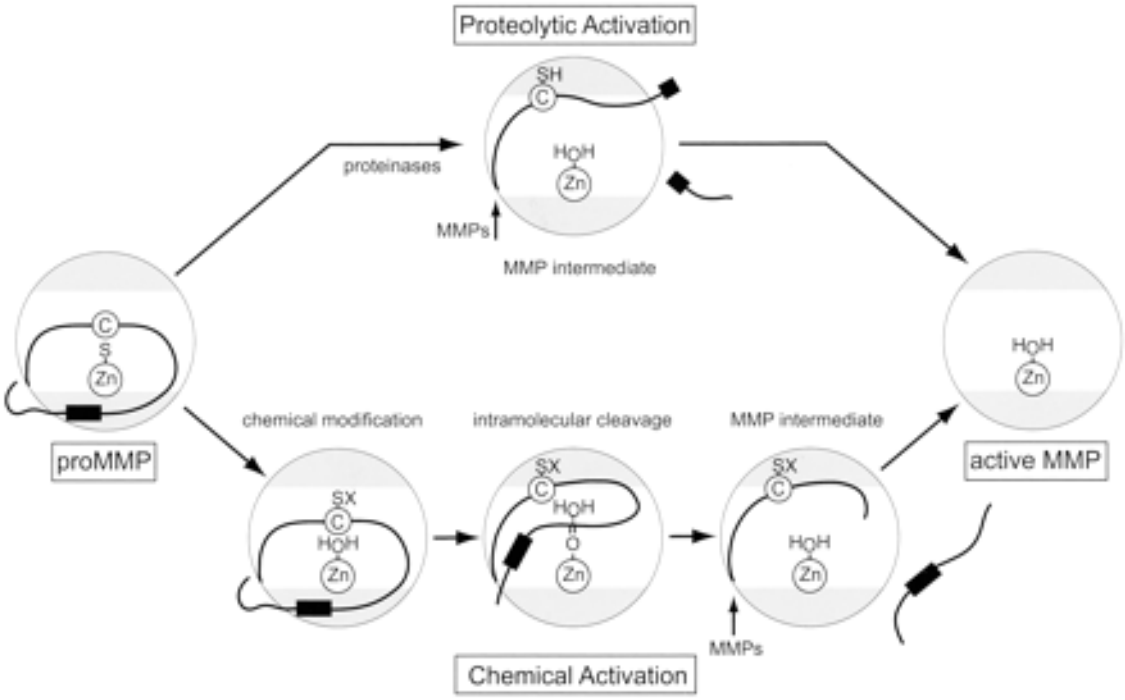

Figure 3. Activation of secreted proMMPs by proteinases or by non-proteolytic agents.

The catalytic domain is in grey and the active site cleft, containing the catalytic zinc ion, is in white. A black line with a black square represents the propeptide domain. Initially the cysteine residue of the propeptide domain is complexed to the catalyitc zinc ion in the active site cleft via a sulfhydryl. Removal of the propeptide domain activates the enzyme. Proteases, such as furin, trypsin, chymotrypsin, thrombin, collgenase I, plasmin, cathepsin B and various MMPs cleave a portion of the propeptide domain, which partially activates the enzyme. Complete activation of the enzyme occurs via an autoactivation process involving intermolecular processing. The Cys-Zn complex can also be disrupted by various chemical reagents including metal ions (i.e silver and mercury), thiol reagents (i.e. N-ethylmaleimide), oxidising agents (i.e. NaOCl), phorbol myristate acetate (PMA), 4-aminophenylmercuric acetate, chaotropic agents and detergents (i.e. sodium dodecyl sulfate), by low pH and high temperatures (Visse and Nagase, 2003).

MT1-MMP is the primary activator of proMMP-2 (progelatinase A) (Sato *et al*., 1994) and, together, these two MMPs trigger downstream activation of a broad range of other soluble MMPs (Figure 4). These include proMMP-9 (progelatinase-B) (Fridman *et al*., 1995; Toth *et al*., 2003) and proMMP-13 (progelatinase-3) (Knauper *et al*., 1996; Cowell *et al*., 1998) (Figure 4), which are also involved in tissue remodelling and cell migration. Cathepsins B (Kobayahshi *et al*., 1991) and L (Goretzki *et al*., 1992) are the primary activators of urokinase plasminogen activator (uPA), a secondary activator of proMMP-2 and proMMP-9 (Figure 4) (Thomssen *et al*., 1995; Baramova *et al*., 1997; Ginestra *et al*., 1997). In fact, before the discovery of MT1-MMP, cathepsin B and MMP-2 were thought to be the primary

enzymes involved in tumour invasion and metastasis (Emmert-Buck *et al*., 1994). Urokinase plasminogen activator converts plasminogen to plasmin, which can also activate MT1-MMP (Baramova *et al*., 1997) (Figure 4). This suggests that MT1-MMP and the cathepsins work together to promote ECM degradation and invasion. The invasiveness of many cancers cells, however, often begin with the activation of MT1-MMP and the up-regulation of other MMPs, in particular MMP-2 (Seiki *et al*., 2003; Sabeh *et al*., 2004). MT1-MMP is, therefore, the primary enzyme involved in malignant tumour cell invasion and is considered a potential target in cancer therapy.

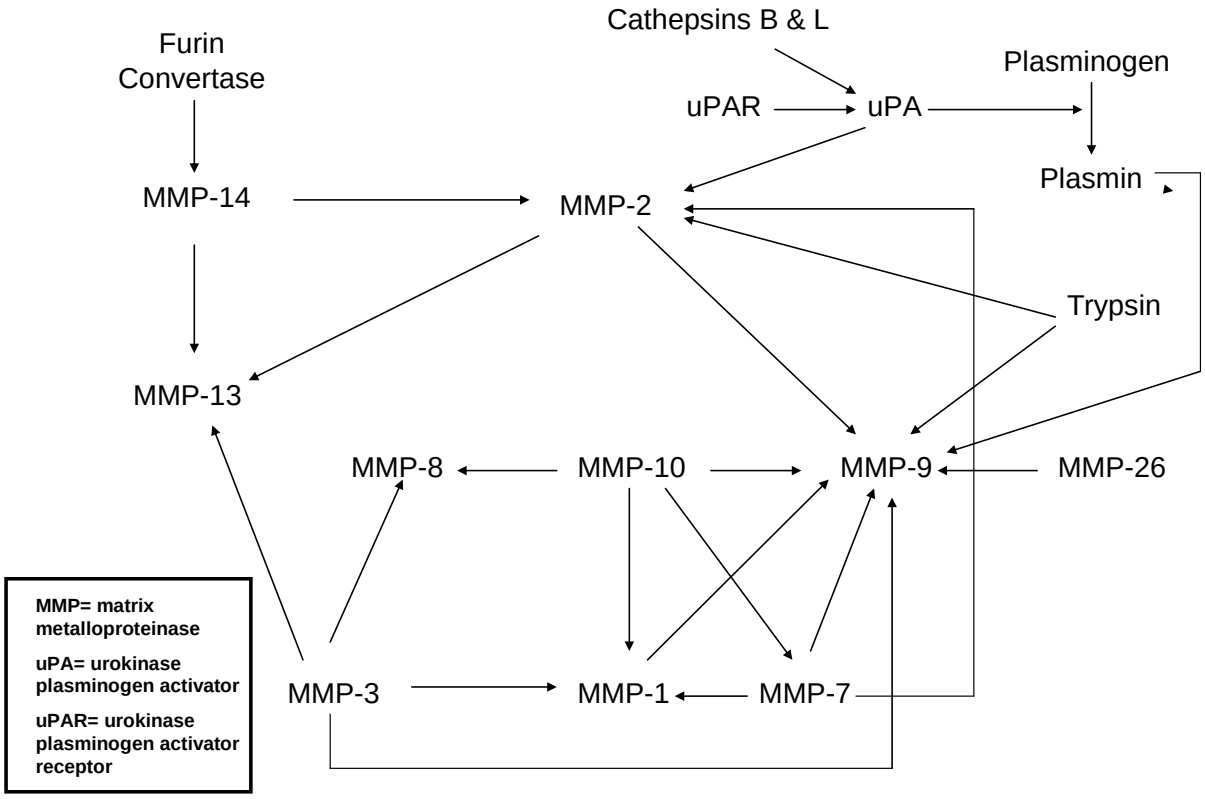

Figure 4. Activation cascades of MMPs triggered by MT1-MMP.

MT1-MMP adopts an initial key role in the activation cascade of MMPs. Cathepsins B and L indirectly activates MMP-2 and MMP-9 via uPA (Adapted from Palosaari, 2003).

The activation of proMMP-2 by MT1-MMP relies on the direct association of MT1-MMP with proMMP-2 in a proMMP-2-MT1-MMP-TIMP-2 ternary complex at the cell surface (Figure 5) (Strongin *et al*., 1995; Will *et al*., 1996). Here TIMP-2 acts as an adapter molecule for proMMP-2 and MT1-MMP interaction. TIMP-2 is also an inhibitor of MMP-2 activity (Will *et al*., 1996; Gomez *et al*., 1997) and, therefore, has a dual role in regulating MMP-2 activity, as previously mentioned (Section 1.1). The catalytic domain of MT1-MMP is critical in the formation of the complex with TIMP-2, and in proteolytic removal of the propeptide domain of proMMP-2. Two molecules of MT1-MMP are, therefore, required to be in close proximity to one another for such an activation to occur (Figure 5). This does not necessarily mean that only mature MT1-MMP is required to activate proMMP-2 as, MT1-MMP delivered to the cell surface as a proenzyme, may also assist in the activation of proMMP-2 (Cao *et al*., 1998). This suggests that the propeptide domain may not necessarily alter binding of TIMP-2 to the catalytic domain of MT1-MMP and that whether there is mature or proenzyme on the cell surface, proMMP-2 activation may occur.

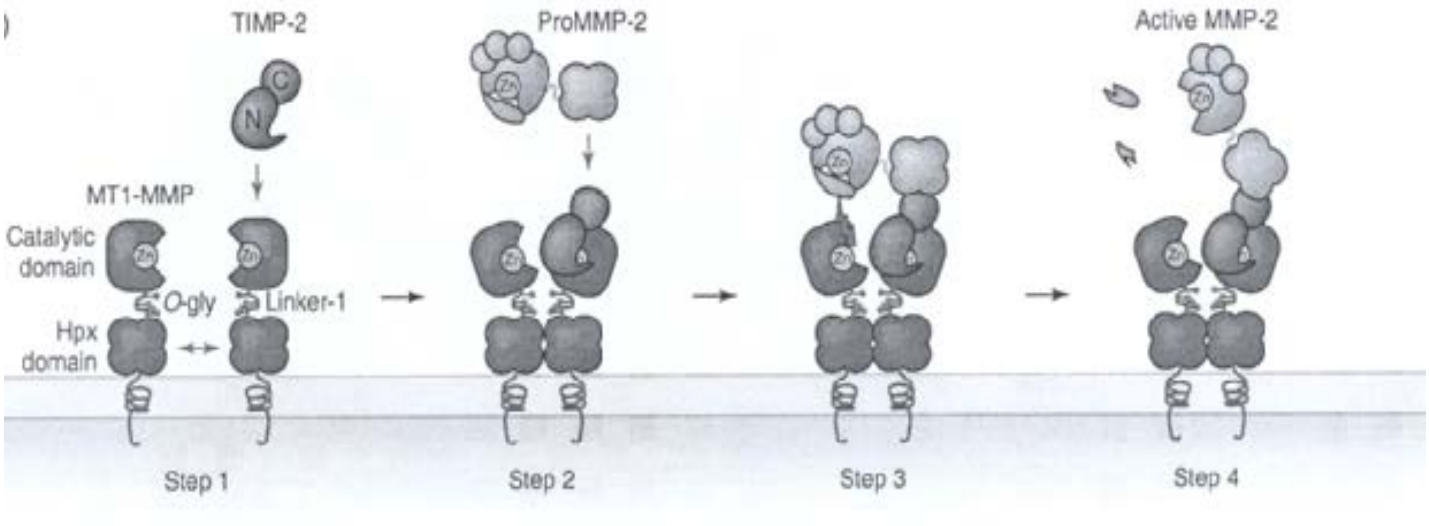

Figure 5. Activation of proMMP-2.

The N-terminal domain of TIMP-2 forms a complex with the catalytic domain of MT1-MMP, which renders the enzyme inactive (step 1) while the C-terminal domain of TIMP-2 binds the hemopexin-like domain of proMMP-2 (step 2) forming a proMMP-2-MT1-MMP-TIMP-2 ternary complex (step 3). A free, active MT1-MMP molecule, in close proximity to the complex, cleaves the propeptide domain of proMMP-2 at the Asn^{37}-Leu^{38} bond converting the 72 kDa proenzyme to the 64 kDa intermediate form, which autocatalytically activates into the 62 kDa mature enzyme (step 4) (Itoh and Seiki, 2006).

Association of MT1-MMP with the CD44 hyaluron receptor molecules, appears necessary for trafficking of MT1-MMP to the migration front and into lamellipodia (Mori *et al*., 2002; Weber *et al*., 2002) where, via its hemopexin-like domain, MT1-MMP forms homodimers, required for the activation of proMMP-2 (Figure 5) and, hence, cell migration and invasion (Deryugina *et al*., 2001). When cells are triggered into migration, the hemopexin-like domain of MT1-MMP, therefore, plays a central role in the activation of proMMP-2, focusing of MT1-MMP and activated MMP-2 at the invasion front. On the surface of invading tumour cells, proMMP-2 has also been reported to colocalise with αvβ3 cell receptor (Brooks *et al*., 1996) and, together with the upregulation of MT1-MMP, αvβ3, thus promotes proMMP-2 activation and cell migration and invasion (Deryugina *et al*., 2001).

The activation of proMMP-2 may, therefore, be regulated in a number of ways. Since the formation of the proMMP-2-MT1-MMP-TIMP-2 ternary complex depends on levels of TIMP-2 and proMMP-2, as well as the availability of free MT1-MMP (Sato *et al*., 1996), the levels of each are critical in regulating the formation of the complex. The binding of TIMP-2 to MT1-MMP before the formation of the complex, may similarily prevent autocatalytic conversion of the active 60 kDa form of MT1-MMP into the inactive 45 kDa form (Table 2). Binding of TIMP-2 at this stage ensures a continuous supply of active MT1-MMP for the activation of proMMP-2 (Toth *et al*., 2000). Remacle *et al*. (2003) also reported that MT1-MMP can be internalised together with TIMP-2 into vesicles and these vesicles can be transported to other regions of the cell. This possibly allows for continuous exposure of the MT1-MMP-TIMP-2 complex to proMMP-2, promoting proMMP-2 activation where needed at the invasion front (Kridel *et al*., 2002; Yana and Seiki, 2002; Jiang *et al*., 2004).

The activation of proMMP-2 also relies on MT1-MMP being membrane-associated rather than soluble, as soluble MT1-MMP, i.e. MT1-MMP cleaved off the membrane (55 kDa) (Table 2) (Pei and Weiss, 1996) , is not capable of activating proMMP-2 (Jiang *et al*., 2001; Wang *et al*.; 2004). The transmembrane domain and cytoplasmic tail of MT1-MMP, therefore, also contribute towards the formation of MT1-MMP homodimers and proMMP-2 activation (Cao *et al*., 1995). The activation of MMPs by MT1-MMP (in particluar MMP-2),

is a key step associated with tumour migration and invasion in many cancers. This suggests that MT1-MMP may be an effective target in cancer therapy.

Table 2. Different molecular forms of MT1-MMP

MT1-MMP forms	**Constituent domains**	**Molecuar weight**
Proenzyme (membrane-bound/soluble)[1,2]	Propeptide, catalytic, hemopexin-like and transmembrane	63 kDa
Mature enzyme (membrane-bound)[1,3]	Catalytic, hemopexin-like and transmembrane	60 kDa
Soluble mature enzyme[2,4,5]	Catalytic and hemopexin-like	55 kDa
Inactive processed enzyme[1,6,7]	Hemopexin-like domain and zinc-binding site of catalytic domain	45 kDa

1. Yana and Weiss, 2000
2. Kazes *et al*., 1998
3. Strongin *et al*., 1995
4. Pei and Weiss, 1996
5. Li *et al*., 1997
6. Toth *et al*., 2002
7. Maisi *et al.*, 2002

1.3 MT1-MMP as a target in cancer therapy

MT1-MMP was the first MT-MMP discovered (Sato *et al*., 1994) and due to its critical involvement in skin and breast cancer metastasis, has dominated recent research on MMPs. MT1-MMP seems to trigger initial morphogenic changes required for cancer cell behaviour. It is also involved in many developmental processes, including ECM remodeling, growth and cell migration, and absence of the enzyme leads to abnormal development. Knock-outs have shown that MT1-MMP is critical in skeletal development, soft tissue organisation and cartilage remodelling. Mice lacking these enzymes, therefore, show evidence of dwarfism, osteopenia, arthritis and fibrosis of soft tissue even in the presence of other MMPs (Holmbeck *et al*., 1999; Boudreau and Weaver, 2006). In addition, the collagenolytic activity of MT1-MMP has been shown to be crucial for the development and differentiation of adipose tissue in mice (Boudreau and Weaver, 2006). This has been shown to require indirect activation of proMMP-9 by MT1-MMP and may result in pathological diseases, such as obesity (Bouloumie *et al*., 2001). The activation of MMP-2 by MT1-MMP is also critical in regulating mammary gland branching (Wiseman *et al*., 2003). MT1-MMP, MMP-2 and MMP-9 play a fundamental role in the remodelling and degradation of elastin in the aortic wall, leading to persistent inflammation in mice, also known as abdominal aortic aneurysim, associated with cardiovascular disease (Shapiro, 1999; Eagleton *et al*., 2000). In angiogenesis, MT1-MMP is also critical (Langlois *et al*., 2005; Basile *et al*., 2007). Angiogenesis is required for wound healing and tissue development. Uncontrolled angiogenesis, however, may facilitate tumour progression (Sounni *et al*., 2003). This angiogenesis is usually assisted by the release of semaphorin 4D, a membrane-bound protein usually used in regulating blood vessel development. This is released by MT1-MMP and can also promote tumour-induced angiogenesis (Basile *et al*., 2007).

MT1-MMP is, therefore, involved in a number of key processes and, in developing human and mouse embryos, the enzyme is expressed in cells of connective tissue, including osteoblasts, osteoclasts, fibroblasts, perichondrial cells, muscle cells (i.e. airway smooth muscle cells) and tendon fibres (Sato *et al*., 1994; Lang *et al*., 2003; Wu *et al*., 2005). The expression of MT1-MMP generally decreases with age. In tumour cells, however, its expression is enhanced regardless of age (Sato *et al*., 1996).

1.3.1 MT1-MMP cleaves ECM and non-matrix-like components

MT1-MMP and MMP-2 cleave a large repertoire of ECM components. This is critical for ECM remodelling and development (Table 1). MT1-MMP preferentially degrades Type I, II and III collagen, gelatin, fibrin, fibronectin, vitronectin, laminin 1 and 5 and other proteoglycans e.g. aggrecan (Table 1) (Ohuchi *et al.,* 1997; Yana and Seiki, 2002; Sounni *et al*., 2003; Itoh and Seiki, 2006) and MMP-2 primarily breaks down Type I, IV, V, VII, X and XI collagen, lamin, fibronectin and proteoglycans (Aimes and Quigley, 1995; Forget *et al*., 1999; Big and Rowan, 2001; Overall, 2002) (Table 1). The collagens (Type I, II, III, IV and V) constitute a major part of the ECM (Figure 6) (Wolfe, 1995) and, hence, together these enzymes are able to digest most of the components of the ECM.

Type I collagen is the most prevalent as it is the primary constituent of blood vessels, skin and bone and is responsible for supporting and maintaining cellular architecture and integrity (Itoh and Seiki, 2006). Type IV collagen is largely found in the basement membranes. Degradation of these components by MT1-MMP and/or MMP-2 is critical in morphogenesis (Hasebe *et al*., 2007). Such degradation also opens channels facilitating primary tumour cell migration to distant tissue (Foda and Zucker, 2001; Visse and Nagase, 2003; Wang *et al*., 2004; Cao *et al*., 2004; Rozanov *et al*., 2004). Degradation of Type IV collagen by MMP-2, in particular, allows tumour cells to enter into the lymphatic system facilitating tumour dissemination and also promotes angiogenesis during tumour development (Langlois *et al*., 2005).

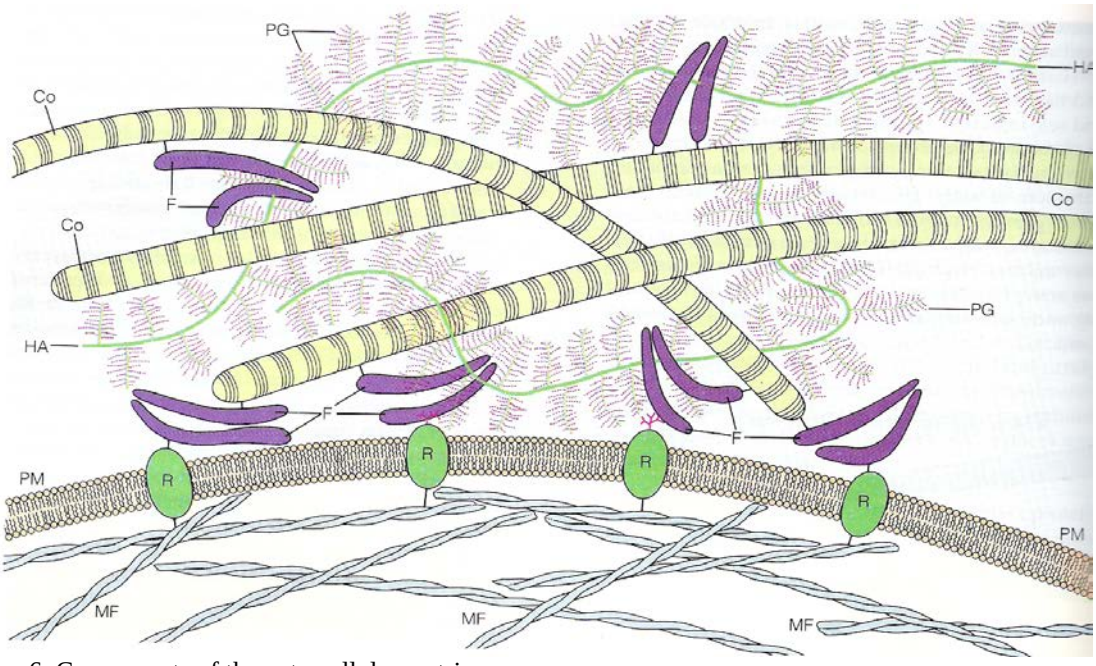

Figure 6. Components of the extracellular matrix.

Co, collagen fibres; PG, proteoglycans; HA, hyaluronic acid; F, fibronectin; R, surface receptors; PM, plasma membrane; MF, microfilaments (Wolfe, 1995).

Fibronectin, laminin, vitronectin and proteoglycans are cross-linked to the collagen fibres to form a network (Figure 6) (Wolfe, 1995). The proteoglycans or proteoglycan-hyaluronic acid complexes are responsible for maintaining cell shape, rigidity and elasticity and keep the cells hydrated (Wolfe, 1995). The fibronectin and laminin connect the collagen in the extracellular matrix to cell surface receptors (i.e. integrins), and form part of the basal lamina (Wolfe, 1995). Both the receptors and such ECM components are critical in cell signalling and cell adhesion (Wolfe, 1995). Cleavage of laminin and fibronectin by MT1-MMP and MMP-2 effects cell morphology and assists in tumour cell migration and invasion (Koshikawa *et al*., 2000).

The specificity of MT1-MMP for the different ECM components is influenced by its catalytic domain. Due to their hydrophobic nature, amino acid residues with long aliphatic (i.e. leucine or isoleucine) or aromatic side chains, in the P1' position, are preferentially cleaved by MT1-MMP as, at this position, the amino acid residues interact easily with the S1' specificity pocket in the active site cleft of MT1-MMP (Kridel *et al*., 1999; Ohkubo *et al*., 1999; Murphy *et al*., 2002). MT1-MMP also preferentially cleaves substrates with

proline in the P3 position or arginine in the P4 position, but not simultaneously, as proline forms a kink in the peptide bond. This cancels out any interaction between the methylene group of P4 arginine and the active site cleft of MT1-MMP (Murphy *et al*., 2002). Therefore, MT1-MMP cleaves two types of substrates, both containing hydrophobic residues in the P1' position and either an arginine at the P4 position or proline in the P3 position. P3 proline is also cleaved by MMP-9 and MMP-2 (Murphy *et al*., 2002). This possibly accounts for the similar ECM components cleaved by MT1-MMP and other MMPs. Cleavage specificity seems also to be associated with structural similarity between members of MMPs (Figure 1). Substrates cleaved by MT1-MMP, therefore, consists primarily of proline, leucine and glycine amino acids. Synthetic peptide substrates, comprising such amino acids, have frequently been used to measure enzyme activity of MT1-MMP *in vitro* (Lichte *et al*., 1996; Roderfeld *et al*., 2000; Koo *et al*., 2002; Kridel *et al*., 2002; Oku *et al*., 2003).

The ECM not only serves as a supporting matrix for cells, but also serves as a scaffold for cell signalling, growth and differentiation. In addition to ECM components, MT1-MMP also cleaves growth factor receptors, growth factor binding proteins, chemokines, cytokines, cell adhesion molecules (e.g. CD44), apoptotic ligands, E-cadherin, the precursor of integrin αV subunit and tissue transglutaminase (Sounni *et al*., 2003; Folgueras *et al*., 2004; Rozanov *et al*., 2004). Cleavage of these non-matrix-like substrates by MT1-MMP is thought to regulate cellular migration but can also contribute towards tumour invasion and progression. Activation of the vascular endothelial growth factor (VEGF), via MT1-MMP, leads to the activation of many other MMPs involved in angiogenesis and promotes neovascularisation at distant sites (Foda and Zucker, 2001; Tetu *et al*., 2006;). Fibroblast growth factor (FGF)-2, and VEGF work synergistically to upregulate MT1-MMP expression and angiogenesis, both of which are essential in cancer progression (DeClerck, 2000). .

MT1-MMP and activated MMP-2 usually work in concert to degrade the ECM components. In normal cells, the action of MT1-MMP and MMP-2 is highly regulated. In tumour cells, however, MT1-MMP is over-expressed. This results in increased levels of MMP-2 activation and the excessive ECM proteolysis, conducive to cancer progression.

1.3.2 MT1-MMP in invasive migration

Knock-outs have shown that MT1-MMP plays a critical role in tumour migration and invasion (Ueda *et al*., 2003), even in the presence of other MMPs and cathepsins (Sabeh *et al*., 2004). MT1-MMP is also able to suppress tumour cell apoptosis and diminish responses associated with the immune system, facilitating tumour progression (Folgueras *et al*., 2004). MT1-MMP and MMP-2 are upregulated in many human malignant tumours, including skin, breast, colon, thyroid, cervical, ovarian, gastric, head, neck, brain and lung cancer (Sato *et al*., 1996; Rosenthal *et al*., 1999; Sounni *et al*., 2003; Wu *et al*., 2004; Seiki, 2004). The aggressiveness of these tumours is related to the increased expression of MT1-MMP and the associated proMMP-2 activation (Deryugina *et al*., 2001; Foda *et al*., 2001). This is particularly observed in hypoxic breast cancer tissue and is associated with tumour cell survival, invasion and angiogenesis (Munoz-Najar *et al*., 2006). Many of these pathological conditions, particulalry tumour invasion, cannot proceed without MT1-MMP, even in the presence of MMP-2.

MT1-MMP has been shown to be responsible for shape change or epithelial to fibroblast transition required for invasion (Hotary *et al*., 2002). MT1-MMP, MMP-1, MMP-3 and MMP-13 are largely expressed in breast carcinoma cells although MT1-MMP is expressed at much higher levels (Knauper *et al*., 1996). This suggests MT1-MMP is primarily involved in the invasive behaviour of these cells and could also serve as a marker for the prognosis of breast cancer (Tetu *et al*., 2006).

When tumour cells are triggered to migrate, MT1-MMP accumulates in lamellipodia at the leading edge of the invasive tumour cells, facilitated by the hemopexin-like domain of MT1-MMP, as previously mentioned (Section 1.2), and it degrades the ECM in a forward direction in the surrounding microenvironment. Also mentioned (Section 1.2), MT1-MMP, proMMP-2 and $\alpha v\beta 3$ receptor colocalise at the surface when the cells become migratory (Brooks *et al*., 1996; Deryugina *et al*., 2001). This accelerates tumour cell migration and invasion into surrounding stroma in breast cancer cells (Nagase and Woessner, 1999; Yana and Seiki, 2002; Cao *et al*., 2004; Rozanov *et al*., 2004). Under metastatic conditions, there is also an increase in the interaction between the tumour cells and the ECM components and

a decrease in cell to cell adhesion. This allows tumour cells to detach from the primary tumour and invade foreign tissue. Focal adhesion kinase (FAK), which is generally involved in normal cell growth, is over produced. This causes cell growth to escalate and promotes MT1-MMP cell surface expression and associated ECM degradation, facilitating the migration of the tumour cells into the surrounding stroma (Wu *et al*., 2005). Although ECM degradation is the primary way in which MT1-MMP-associated tumour invasion takes place, the tumour cells themselves can also become ameboid-like in morphology and able to migrate by squeezing through small spaces (Sabeh *et al*., 2004). The critical role of MT1-MMP in tumour invasion, therefore, suggests it is a good target for suppression of tumour invasion and metastasis.

1.4. Ha-*ras*-transfected MCF10A cell line

The MCF10A- and its Val-12 c Ha *ras*-transfected counterpart, the MCF10A-neoT cell line, are two human breast epithelial cell lines with a normal and derivative, premalignant phenotype (Zantek *et al*., 2001). These have been frequently used to study the behaviour of malignant cancer cells. The MCF10A cell line is a diploid breast epithelial cell line initially provided by the Michigan Cancer Foundation (MCF). It was derived from mastectomy tissue from a patient suffering of fibrocystic breast disease. A derivative cell line underwent spontaneous immortalisation in culture and grows attached (A = attached) in the presence of low calcium, or floating (F = floating) in the absence of calcium (Soule *et al*., 1990). Originating from the diploid mortal MCF10M (M = mortal) cell line, the MCF10A is non-tumourogenic in nude mice and morphologically resembles normal breast epithelial cells (Tait *et al*., 1990; Sloane *et al*., 1994) and grows in culture as a monolayer (Tait *et al*., 1990). The MCF10A cells, however, have a p53 mutation that makes this cell line immortal.

Co-transfection of the MCF10A cell line with a neomycin-T4 resistence gene and plasmid containing a mutated human c-Ha-*ras* oncogene, resulted in the production of a premalignant MCF10A-neoT cell line. This cell line is invasive and mimics early stage proliferative, premalignant breast cancer (Sloane *et al*., 1994; Premzl *et al*., 2003; Haupt *et al*., 2006). *Ras* GTPases are normally involved in cell proliferation, differentiation, motility and survival (Kim *et al*., 2003). The Harvey (H)-*ras* is responsible for the invasive

phenotype of the c-Ha-*ras*-transfected MCF10A cell line. The oncogene activates the *Rac*-MKK3/6-p38 pathway, which also leads to the upregulation of MMP-2 (Shin *et al*., 2005), though MT1-MMP is the major MMP expressed in the untransfected cell line (Gilles *et al*., 2001). The expression of this enzyme is enhanced when the cells become migratory, suggesting that MT1-MMP is primarily associated with the invasive phenotype of the MCF10A-neoT cell line (Gilles *et al*., 2001). The phenotype of this invasive cell line includes a reduction in adhesion of the cells to the basement membrane and a loss of stress fibres. As such fibres are critical in cell adhesion to the basement membrane, both modifications increase cell mobility (Gadea *et al*., 2002).

The MCF10A-neoT cell line has a mutated p53, a transcription factor that normally acts as a tumour suppressor (i.e. during hypoxia and DNA damage) by inducing cell apoptosis (Thomas *et al*., 2000). It also regulates cell spreading and migration by indirectly preventing filapodia formation by the Rho-GTPase protein, CDC42. In tumour cells it is usually mutated (Thomas *et al*., 2000; Gadea *et al*., 2002). Mutation of p53, consequently, allows filopodia formation, encourages cell migration and invasion, and the cells become immortal. As the cells migrate, the Golgi apparatus is unable to re-orientate in the direction of the movement. This may affect cell polarity and organelle distribution (Gadea *et al*., 2002). This suggests a p53 mutation in the MCF10A cell line could contribute to an altered distribution of enzymes compared to that in normal cells. This, however, was not seen until the MCF10A cell line was transfected with the cHa-*ras* oncogene (Sloane *et al*., 1994).

The cHa-*ras*-transfected MCF10A cell line was, therefore, considered useful for assessing the effect of cHa-*ras* transfection on the localisation and distribution of MT1-MMP in normal and premalignant tissues in this study. Although MMP-2 is also prevalent in this cell line, MT1-MMP, unlike MMP-2, is cell associated, and so it seems it may form a more appropriate target than MMP-2 for immunotherapeutic or protease inhibition to prevent invasion of tumour cells, as the target enzyme would be cell-specific.

The MCF10A, and its *ras* transfected counterpart, therefore, seem to constitute an ideal model system for studies on both the role of MT1-MMP in invasion and to investigate whether immunotherapeutic or protease inhibitors may prevent MT1-MMP-associated

tumour invasion. In addition, the model seems to allow the effect of the *ras* oncogene and any therapeutic approach to be assessed against both the cancerous and normal cell lines. This model, therefore, is unique, allowing assessment of the selectivity and selective toxicity of such approaches.

To check whether antibodies or protease inhibitors may be used to prevent invasion, it is necessary to have a model system that replicates, as far as possible, the ECM and vascular system into and out of which cancer cells invade. Such a system was first described by Davison and Solomon (1980). This is a chicken embryo *in vivo* model system that closely mimics the vascular system of humans and, thus, is frequently used to study tumour invasion and metastasis (Dani and Espindola, 2002). It has also been used by Testa *et al*. (1999) to investigate the importance of membrane-associated enzymes in the migration and invasion of murine tumour cells using antibodies, and by Sabeh *et al*. (2004) to show the critical *in vivo* role of MT1-MMP in the invasive and metastatic behaviour of mutated fibroblasts.

The upper region of the vascular system, known as the chorioallantoic membrane (CAM), of the developing embryo is innoculated with cancer cells, i.e. premalignant MCF10A-neoT cells. These cells migrate easily through the underlying mesenchyme of the CAM and form secondary tumours at distant sites (Dani and Espindola, 2002). As the antibodies, produced by a hen laying fertile eggs, are passed into the egg yolk and subsequently into the vascular system of the developing embryo, the chicken embryo model system, thus, gives rise to a suitable model system for assessing immunotherapeutic or protease inhibitor strategies for preventing invasion. Before attempting such an approach, however, it is important to identify target domains of MT1-MMP involved in ECM degradation and invasion against which specific, and possibly immunotherapeutic, antibodies may be raised to stop invasion.

1.5. MT1-MMP domain targets for immunoinhibition studies

The generation of specific antibodies against cell-associated MT1-MMP may produce both MT1-MMP-specific antibodies for immunolabelling of cancer cell lines, but also such antibodies may have immunoinhibitory properties preventing invasion. This may especially be the case if antibodies to domains near to the catalytic domain are targeted. Instead of raising antibodies against the whole enzyme, it, therefore, was decided to target those

regions of the enzyme primarily involved in proteolysis and, hence, possible invasion. This also seemed a good strategy for producing MT1-MMP-specific antibodies, as identification of non-homologous, smaller domains in MT1-MMP, compared to other MMPs, would also be possibly easier and necessary for production of both MT1-MMP-specific as well as immunoinhibitory antibodies, considering the high level of homology between various MMPs across species. Since the catalytic domain is primarily responsible for the enzyme's activity, it is possibly the most suitable domain to target for the prevention of MT1-MMP-associated tumour cell migration and invasion.

The crystal structure of the catalytic domain of MT1-MMP in complex with TIMP-2 shows that the catalytic domain divides into upper and lower sub-domains, separated by an active site cleft, containing the catalytic zinc ion (Figure 7a) (Fernandez-Catalan *et al*., 1998). The upper subdomain comprises a twisted five-stranded (sI,-sV) β-pleated sheet (Figure 7b) (Lehti *et al*., 2000). Four of the five twisted β-pleated sheets are arranged parallel and the strand closest to the active site cleft, sIV, is arranged anti-parallel and is linked to sIII via the S-shaped binding loop, which is supported by one structural zinc and one or two β-sheet-bound calcium ions (Figure 7b) (Visse and Nagase, 2003). Together, the S-shaped binding loop and sIV are crucial for the binding of substrates and inhibitors to the active site cleft and, therefore, are vital for the enzyme's activity (Bode *et al*., 1999; Lang *et al*., 2004). The abundance of β-pleated sheets in the upper subdomain, however, suggests that they too play a critical part in the enzyme's activity. The secondary and tertiary structure of the catalytic domain of MT1-MMP is almost identical to other MMPs (Fernandez-Catalan *et al*., 1998) and, if the primary structure is also conserved, it may be difficult to specifically target this MT1-MMP domain. Antibodies against this domain may, therefore, cross react with many different MMPs. Sequence homology of the catalytic domain of MT1-MMP, therefore, needed to be assessed and is reported in Chapter 3 (Section 3.1).

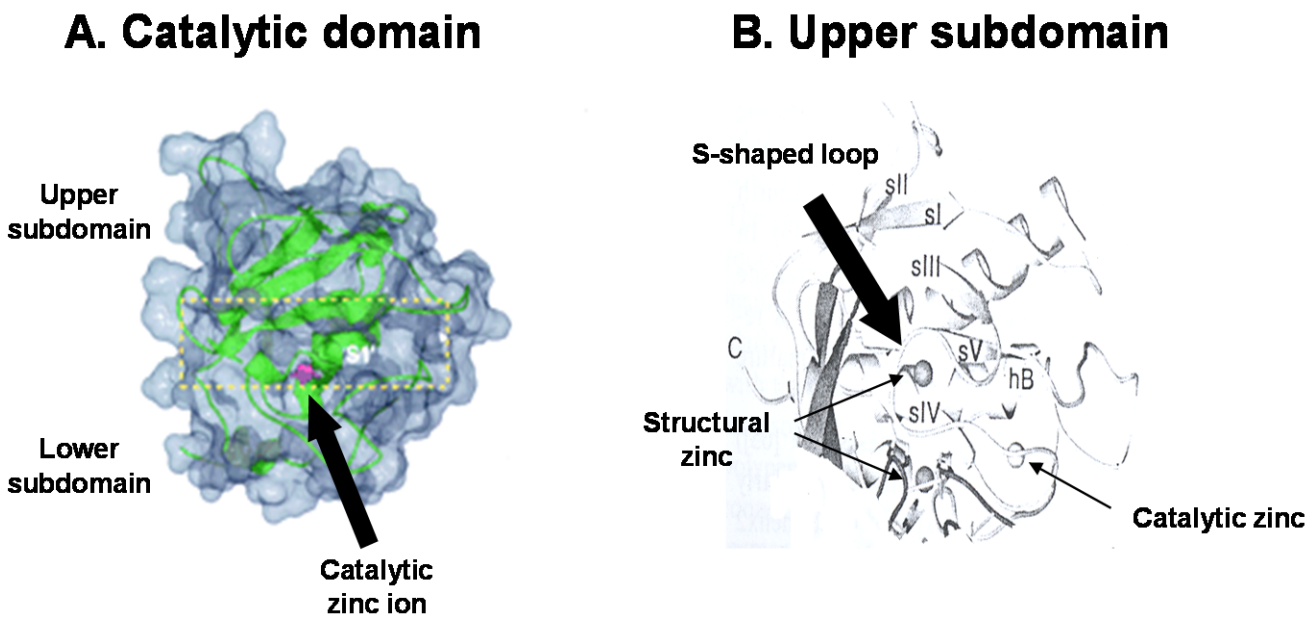

Figure 7. Structure of the catalytic domain of MT1-MMP.

A. The active site is represented by a ribbon structure and the dashed line outlines the active site cleft. The arrow indicates the catalytic zinc ion positioned on the left of the S1' pocket (Visse and Nagase, 2003). B. Here the upper subdomain of the catalytic domain is illustrated. Five β-pleated sheets are shown as is the two structural zinc ions (dark circles), the catalytic zinc ion (light circle) and the S-shaped loop (Bode *et al*., 1999).

The hemopexin-like domain influences substrate specificity and binding (i.e. it is critical in recognising and binding to the collagen substrate) (Murphy and Knauper, 1997; Tam *et al*., 2002) and is involved in proMMP-2 activation and tumour cell migration (Deryugina *et al*. 2001), as mentioned previously (Section 1.2 and Section 1.3.2). This suggests that the catalytic domain of MT1-MMP also requires the hemopexin-like domain to break down ECM components and invade into the surrounding milieu. The hemopexin-like domain, therefore, could also serve as a good target and, if targeted together with the catalytic domain, may possibly stop tumour invasion and metastasis.

The X-ray crystal structure of the hemopexin-like domain has only been reported for MMP-1, MMP-2, MMP-9, MMP-13 and more recently MMP-19 (Bode *et al*., 1999). Like the catalytic domain, the hemopexin-like domain is also composed of β-pleated sheets and these sheets are organised into four blades (I-IV) and arranged perpendicular to a central

tunnel (Figure 8) (Bode *et al*., 1999; Lehti *et al*., 2002). Each blade is connected to each other at two regions by a smaller strand at the rim of the blade and a longer loop at the centre of the blade and the first and fourth blade are connected by a conserved disulfide bond, which stabilises the structure (Bode *et al*., 1999; Visse and Nagase, 2003). The central tunnel contains several calcium and chlorine ions involved in the recognition of substrates (Lichte *et al*., 1996). The abundance of β-pleated sheets in both the catalytic and hemopexin-like domains suggests that they are critical to the enzyme's activity and function and, if a unique domain in MT1-MMP could be identified, these sheets could be specifically targeted to inhibit MT1-MMP-associated invasion. Sequence homology across various MMPs containing a hemopexin-like domain, and across species, therefore, needed to be carried out (Chapter 3, Section 3.1).

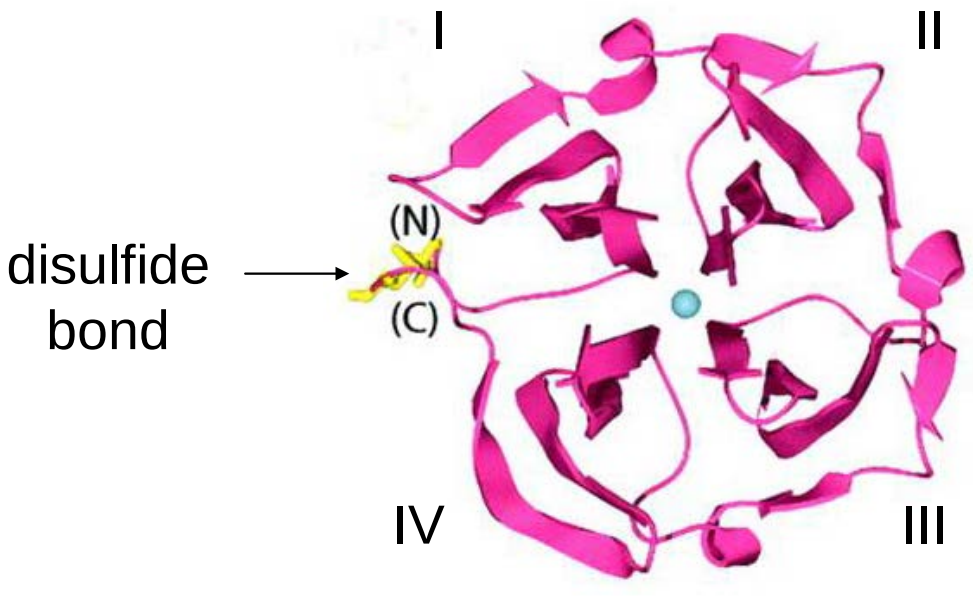

Figure 8. Hemopexin-like domain of MMP-1.

The four blades, comprising the propeller, are labelled I-IV. The conserved disulfide bond is positioned between blades I and IV. The central tunnel is shown as a blue sphere (Visse and Nagase, 2003).

As mentioned previously, MT1-MMP is activated either intracellularly by furin, losing its propeptide domain before it reaches the cell surface (Sato *et al*., 1996), or the proenzyme may be activated at the cell surface via furin-dependent of furin-independent pathways

(Yana and Weiss, 2000). Similar results were shown in MT1-MMP transfected COS-1 cells (Cao *et al*., 1998). Here the propeptide domain was shown to facilitate the activation of proMMP-2, and subsequently may assist in tumour cell invasion and metastasis (Cao *et al*., 1995; Cao *et al*., 1998).

The propeptide domain, as determined for MMP-1, MMP-2, MMP-3 and MMP-9 (Holmbeck *et al*., 1999; Visse and Nagase, 2003), is composed of 3 α-helices (Figure 9) and, although this configuration seems to play no significant part in the catalytic activity of the enzyme (unlike the β-pleated sheets, which are crucial for enzyme activity), it contributes to the enzyme's activating function. The propeptide domain is reported to be highly conserved amongst MMPs (Folgueras *et al*., 2004) and since soluble MMPs retain their propeptide domain outside the cell, it was reasoned that such antibodies may cross react with other MMPs and this needed to be checked. It was considered that the propeptide domain, in conjunction with the catalytic domain, may be a more effective target for inhibiting MT1-MMP activity as the two domains together may give rise to distinctive conformational epitope targets, which may be specifically recognised by antibodies against these co-expressed domains.

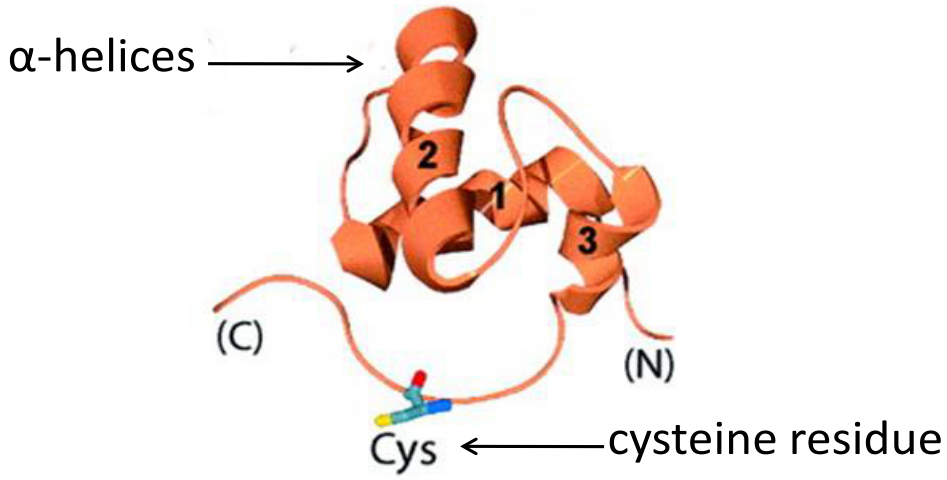

Figure 9. Propeptide domain of MMP-2.

Shown are the α-helices, which are the major components of the propeptide domain, and the cysteine residue, which binds to the zinc ion in the active site cleft, rendering the enzyme inactive (Adapted from Visse and Nagase, 2003).

The catalytic domain, in combination with the propeptide or hemopexin-like domains were, therefore, considered good targets against which to raise MT1-MMP-specific antibodies for immunoinhibition studies. Antibody binding and distortion of the active site cleft may, therefore, inhibit MT1-MMP activity. All possible targets needed to be researched more closely to identify possible non-homologous regions, however. Similarily, the optimum species for raising the antibodies against the target regions needed to be determined (i.e. the species most different from human MT1-MMP).

The chicken is a useful host for raising antibodies against MMPs as they are not only phylogenetically different from humans, but, as previously mentioned (Section 1.4), the hen usually transfers the antibodies to the egg and, hence, the developing embryo to assist in the formation of humoral immunity in the chick (Patterson *et al*., 1962; Grindstaff *et al*., 2003). Antibodies raised against the target antigen (in this case MT1-MMP) in the hen are delivered to the egg yolk and subsequently into the vascular system of the embryo, therefore, the immunoinhibitory response of these antibodies may possibly be tested in a chicken embryo model system, as described in Section 1.4. The immunoinhibitory response of the anti-MT1-MMP antibodies may be measured by their ability to inhibit protease-assisted invasion by

the MCF10A-neoT cancer cells in the chicken embryo CAM system. Here we wished to assess the immunoinhibitory potential of antibodies raised, initially *in vitro*, in an enzyme assay against renatured enzyme, and subsequently *in vivo* in the CAM system, to assess the ability of the immunoinhibitory antibodies *in vivo*.

1.6 Aim of this study

The broad aim of this study was to attempt to raise highly specific and immunoinhibitory antibodies against different MT1-MMP domains for use in electron and confocal microscopy, and chicken CAM invasion studies, to determine the role of MT1-MMP in cancer cell invasion, and to test, *in vitro* and *in vivo*, whether immunoinhibitory antibodies, could be generated against human MT1-MMP and could prevent MT1-MMP-mediated invasion. In order to do this, the antigen needed to be expressed and a domain search needed to be performed to determine which host species and which antigens were suitable for antibody production. The antibodies needed to be characterised to determine whether or not they were specific and inhibitory, *in vitro*, before they would be applied to the *in vivo* model system.

In Chapter 2, the general biochemical techniques used in various sections in this study are discussed and, in Chapter 3, the *in vitro* expression of MT1-MMP is described. In Chapter 4, the purification and renaturation of MT1-MMP is reported while in Chapter 5 the production of antibodies against three MT1-MMP forms in chickens and the subsequent characterisation of these antibodies is described. This chapter also includes the use of the renatured, expressed enzyme in an *in vitro* immunoinhibition assay using the chicken anti-MT1-MMP antibodies. In the final chapter (Chapter 6), the interpretation of the results and application of this study is discussed.

Chapter 2

Materials and methods

2.1 Introduction

A range of commonly used biochemical techniques employed in this study and the principles of these procedures as well as the methods and reagents used will be described in this chapter. More specialised methods are reported in the relevant sections.

2.2. Materials

Most of the chemicals in this study were of analytical grade. The pET11a-MT1-MMP construct was from Prof. Harald Tscheche (University of Bielefeld, Heidelberg, Germany). JM109 (DE3) *E. coli* was from Novagen (WI, USA). Q-Sepharose and S-Sepharose were from Biorad (CA, USA). Zinc chloride, magnesium chloride and glucose were from Saarchem (Gauteng, SA). The Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2.ACOH MMP substrate, polyethylene glycol, amido black, Tween 20, glutaraldehyde, urea and 2-mercaptoethanol were from Merck (Damstadt, Germany). Ethylenediaminetetraacetic acid (EDTA), calcium chloride dihydrate and Triton X-100 were from BDH (UK). Serva Blue G was from Serva Chemicals (Heidelberg, Germany). Gelatin, Brij 35, bovine serum albumin (BSA), dialysis tubing, ampicillin, lysozyme, aluminum sulfate, agar, DMSO, fish skin gelatin, ethidium bromide, yeast extract and rabbit anti-chicken-alkaline phosphatase were from Sigma Chemical Company (MO, USA). Bacto-peptone and Bacto-tryptone were from Becton, Dickenson and Co. (MD, USA). *Bam*HI restriction enzyme was from Fermentas and *Nde*I restriction enzyme was from Promega (WI, USA). Aluminium phosphate “adju-phos” adjuvant was from Brenntag (Műlheim, Germany). Rabbit anti-chicken-horseradish peroxidase was from Jackson Immunoresearch Laboratories (PA, USA). 2, 2’-Azino-di-(3-ethyl)-benzthiozoline sulfonic acid (ABTS) and sodium dodecyl sulfate (SDS) were from Boehringer Mannheim (Mannheim, Germany) and nitro-blue tetrazolium (NBT), bromo-chloro-indolyl phosphate (BCIP) and isopropylthiogalactoside (IPTG) were from Roche (IN, USA). Nunc-Immuno Maxisorp F96 plates were from Nunc Intermed (Roskilde, Denmark), nitrocellulose membrane was from Amersham Biosciences (Buckinghamshire, UK) and Elite fat-free milk was from a local supermarket. MCF10A and MCF10A neoT cell lines

were provided by Dr. Bonnie Sloane (Wayne State University, Detroit, MI.). Nickel grids were from SCI Sciences Services (Munich, Germany) and Protein A-gold was from the Department of Cell Biology, University of Utrecht, Netherlands. MMP-9 antibody was previously raised by Brendon Price and MMP-2 antibodies were raised by Megan Gemmel, both at the Univeristy of KwaZulu-Natal, Pietermaritzburg.

2.3 Separation of proteins on polyacrylamide gels

Polyacrylamide is a synthetic matrix, frequently used to separate biomolecules by gel electrophoresis (Raymond and Weintraub, 1959). By varying the concentration of acrylamide, different sized proteins are sifted through the gel. Gels with low percentage levels of acrylamide (i.e. 7%) have large pore sizes, which allows for larger proteins to pass through the gel. Higher acrylamide percentage gels (i.e. 15%), in contrast, have smaller pore sizes and are able to separate proteins of lower molecular weight as well.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was first introduced by Shapiro *et al*. (1967). This system depends on the interaction of the detergent, SDS, with the hydrophobic domains of a protein at a ratio of 1 SDS molecule per 2 amino acid residues (Bischoff *et al*., 1998). The SDS keeps the protein soluble. When the protein sample is boiled in the presence of SDS, the proteins denature and break down into separate rod-like, polypepetide chains, which allow the detergent to complex with the available binding sites in the polypeptide (Bischoff *et al*., 1998; Dennison, 2003). A reducing agent (i.e. β-mercaptoethanol or dithiothreitol) is usually used in the presence of SDS to assist in breaking down the protein by removing disulfide bonds and subsequently facilitates the binding of SDS to the polypeptide chain. Proteins are generally made up of positive and negative charges and besides breaking up the protein, the SDS disguises their original charge by giving the proteins an overall negative charge, which allows the proteins to migrate in the same direction when an electric field is applied. Therefore the proteins are separated, not by charge, but by molecular weight (Dennison, 2003).

To improve the resolution of the separated proteins on the gel matrix, a discontinuous (disc.) polyacrylamide gel electrophoresis (PAGE) system is usually employed. This system requires a discontinous use of buffers and gels (running and stacking gels) to concentrate the

proteins into narrow bands, making them easy to separate and resolve (Dennison, 2003). Tris-HCl buffer is common to both the stacking and running gel, except that it is used at a different pH in each gel. The running gel, which separates the proteins, is prepared under alkaline conditions (pH 8.8) and contains a relatively high concentration of acrylamide (7%-15%), whereas the stacking gel (poured above the running gel) is more acidic (pH 6.8) and contains a low concentration of acrylamide (3%). As the proteins migrate from the stacking gel to the running gel, the change in the pH and acrylamide concentration causes the proteins to aggregate into narrow bands and these proteins are stacked according to their mobility (Dennison, 2003).

The SDS-PAGE method of Shapiro *et al*. (1967) did not use this discontinuous system. The stacking gel, therefore, was not very effective in concentrating the proteins into thin bands, which is important for improving protein separation and resolution. Laemmli (1970) improved on this SDS-PAGE method by combining the use of SDS with disc. PAGE and was able to identify proteins, including a few unknown proteins, present in bacteriophage T4. SDS-PAGE, adopting the method of Laemmli (1970), is the most frequently used in laboratories today. This method is simple, economical, easy to use and is efficient in identifying a range of proteins in a complex mixture with high resolution and reproducibility (Bischoff *et al*., 1998) and for these reasons was the method of choice for analysing MT1-MMP in this study.

2.3.1 Reagents

Solution A: Monomer solution [30% (m/v) acrylamide, 2.7% (m/v) Bis-acrylamide]. Acrylamide (73 g) and Bis-acrylamide (2 g) were dissolved in 250 ml d.H_2O and stored in brown reagent bottle at 4ºC.

Solution B: 4 x Running gel buffer [1.5 M Tris-HCl, pH 8.8]. Tris (45.37 g) was dissolved in 200 ml d.H_2O, adjusted to pH 8.8 with HCl and made up to 250 ml.

Solution C: 4X Stacking buffer [500 mM Tris-HCl, pH 6.8]. Tris Base (6 g) was dissolved in 80 ml d.H_2O, adjusted to pH 6.8 with HCl and made up to 100 ml. The solution was stored in a plastic reagent bottle at 4ºC.

Solution D: 10% (m/v) SDS. SDS (10 g) was dissolved in d.H_2O (100 ml) with gentle heating if necessary.

Solution E: 10% (m/v) Ammonium persulfate. Ammonium persulfate (0.2 g) was dissolved in 2 ml d.H_2O. The solution is made up just before use.

Solution F: Tank buffer [250 mM Tris-HCl, pH 8.3, 192 mM Glycine, 0.1% (m/v) SDS]. Tris Base (15 g) and glycine (72 g) were dissolved in 4.5 litres d.H_2O, adjusted to pH 8.3 with HCl and made up to 5 litres. Just before use, 10% (m/v) SDS (solution D) (2.5 ml) was added to 250 ml tank buffer.

Reducing treatment buffer [125 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v 2-mercaptoethanol]. Solution C (2.5 ml), solution D (0.4 ml), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were dissolved in 8 ml d.H_2O, adjusted to pH 6.8 with HCl and made up to 10 ml.

Non-reducing treatment buffer [125 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol]. Solution C (2.5 ml), solution D (4 ml) and glycerol (2 ml) were added to 80 ml d.H_2O, adjusted to pH 6.8 with HCl and made up to 100 ml.

Bromophenol blue loading dye (0.1%). Bromophenol blue dye (0.01 g) was dissolved in 10 ml d.H_2O.

2.3.2. Procedure

Table 3 Preparation of running and stacking gel using the Laemlli (1970) SDS-PAGE system

Reagent	Running Gel (ml)			Stacking gel (ml)
	15%	**12.5%**	**10%**	**4%T2.7%C**
A	7.5	6.25	5	0.94
B	3.75	3.75	3.75	
C				1.75
D	0.150	0.150	0.150	0.070
d.H_2O	3.5	4.75	6	4.2
E	0.075	0.075	0.075	0.035
TEMED	0.0075	0.0075	0.0075	0.012

According to the manufacturer's manual, the Hoefer SE 250 Mighty Small II vertical slab electrophoresis unit was assembled as follows: a glass and aluminium plate were cleaned with ethanol, rinsed with d.H_2O, dried and clamped together, separated by two plastic spacers at the edges. Once clamped, the plates were fixed firmly onto a gel caster lined with rubber. The running gel was carefully poured into the space between the aluminuim and glass plate to a depth approximately 3 cm from the top. A small volume (200 µl) of d.H_2O was run over the running gel to allow for even polymerisation and the gel was allowed to set for approximately 1 h. The formation of a definite interface between the d.H_2O and the gel solution indicated the gel was set. The d.H_2O was subsequently poured off and a comb (10 or 15 well) was inserted allowing for a 0.5 cm space between the running gel and the floor of the wells. The stacking gel was poured in up to the notch of the aluminium plate and left to polymerise for 30 min. Once set, the comb was removed and the wells were rinsed with d.H_2O.

The aluminium and glass plates were removed from the gel caster and clamped to the electrode. Tank buffer, containing 0.1% (m/v) SDS, was poured into the upper and lower electrode compartments ensuring all wells were completely immersed in the buffer. The

protein samples were prepared as a 1:1 dilution with reducing treatment buffer, boiled in a water bath for 2 min, cooled and sample loading dye (bromophenol blue) added. For non-reducing SDS-PAGE, the protein samples were prepared at a 1:1 ratio with non-reducing buffer (lacking the 2-mercaptoethanol) and were not boiled (Section 2.5). The protein sample (20 µl) was loaded into the relevant wells and a marker, comprising proteins of known molecular weight, was also loaded (5 µl). The gel unit was connected to a power supply and run (20 mA per gel, 4ºC). After the run, the power supply was disconnected, the plates removed and the glass plate separated from the aluminum plate. The stacking gel was excised and the running gel was peeled off the aluminium plate, briefly rinsed in d.H_2O and soaked in the Coomassie G250 staining solution (section 2.4.).

2.4 Coomassie Brilliant Blue G250

Coomassie brilliant blue (CBB) is frequently employed to quantitatively visualise almost any proteins separated on an SDS-PAGE gel. Compared to silver staining, the CBB stain has a lower sensitivity (500-50 ng). However, the duration of the staining procedure for CBB is in the region of ~2-8 h, as opposed to silver staining, which takes up to 16 h to stain the protein bands, making it a comparatively more laborious method (Fernandez-Patron *et al*., 1995). The reagents required for silver staining are, in addition, expensive and high background can occur if the water is not adequately purified or the gel container is not scrupulously clean (Fernandez-Patron *et al*., 1995). Most importantly, the silver stain procedure has modifying effects, and as a result the protein stained using this method cannot be used for subsequent analysis such as N-terminal sequencing (Fernandez-Patron *et al*., 1995).

Fernandez-Patron *et al*. (1995) developed a double staining approach combining CBB (positive stain) and imidazole-zinc salts (negative stain), which could detect between 1-10 ng of protein. Although this method was relatively quick, the gels were easily susceptible to over-staining with the imidazole-zinc, which could affect the resolution of the protein bands. Once stained, the gels also did not store for very long compared to gels stained with the conventional CBB.

The sensitivity of CBB was also improved by incorporating ammonium sulfate and methanol into the CBB stain (Kang *et al*., 2002). These conditions, however, caused the dye to aggregate into colloidal particles, which reduced sensitivity and resulted in a prolonged staining time. The stain was subsequently modified by replacing ammonium sulfate with aluminum sulfate [$(Al_2\ (S0_4)_3.(H_20)x$, x=14-18] and using ethanol instead of methanol to detect proteins (Kang *et al*., 2002). The aluminum sulfate improved the avidity with which CBB G250 bound to the protein, shortening the staining time and increasing its sensitivity, which was comparable with silver staining. The stain also produced a very low background, requiring little or no destaining and the first bands could be detected within an hour. The aluminum based-CBB G250 dye is simple and highly sensitive, being able to detect as little as 1 ng of protein per band (Kang *et al*., 2002). Therefore, the protocol of Kang and co-workers (2002) was employed in this study.

2.4.1 Reagents

Coomassie Blue G250 [0.02% (m/v) CBB-G250, 2% (w/v) phosphoric acid, 5% (m/v) aluminium sulfate, 10% (v/v) ethanol]. Coomassie Brilliant blue dye (0.2 g) and $(Al_2(SO_4)_3.(H_2O)_{15}$ (50 g) were dissolved in 86% (v/v) phosphoric acid (24 ml) and ethanol (100 ml) and made up to 1 litre with $d.H_2O$ (876 ml). The solution was stirred at room temperature for a few minutes and stored in a glass reagent bottle.

2.4.2 Procedure

Following electrophoresis, the SDS-PAGE gel was soaked (3 x 30 s) in $d.H_2O$ and stained in the CBB G250 dye on an orbital shaker until bands developed (2 h). The gel was briefly destained in $d.H_2O$ before the image was captured on the Versadoc imaging system.

2.5 Gelatin-containing substrate SDS-PAGE

The proteolytic activity of MT1-MMP can be qualitatively measured by separating the enzyme on an SDS-PAGE gel containing a suitable substrate (i.e. zymography) (Heussen and Dowdle, 1980). The activity of the enzyme is identified as a negatively stained band against a darkly stained background. Conventional SDS-PAGE denatures the protein and is not suitable if the enzyme is required in its active form. Heussen and Dowdle (1980), however, developed a method, based on the Laemmli (1970) SDS-PAGE system, but

without boiling the samples or mixing with a reducing reagent, which preserves the enzyme in an active form. During electrophoresis, the enzyme is inactive due to the presence of SDS, but washing the SDS-PAGE gel with a non-ionic detergent (i.e. Triton X-100), removes the SDS and reconstitutes the enzyme's activity (Gabriel and Gersten, 1992; Dennison, 2003).

Following renaturation, the SDS-PAGE gel is incubated in a suitable digestion buffer, which allows the enzyme to digest the substrate at the specific position it occupies on the gel. The degree of substrate breakdown (band intensity) depends on the amount of enzyme loaded on the gel (Kleiner and Stetler-Stevenson, 1994; Leber and Balkwill, 1997). The digestion buffer also contains an activator, which for MMPs is $ZnCl_2$ and $CaCl_2$ (Lichte *et al*., 1996; Koo *et al*., 2002), and ensures specific proteolytic activity.

Zymography is highly sensitive, being able to detect picogram quantities of active enzyme, as well as specific, as the enzyme's activity is confined to a particular molecular weight region. Small sample volumes are required, thereby providing an inexpensive way of determining the abundance of an enzyme in a complex mixture with high resolution (Kleiner and Stetler-Stevenson, 1994; Bischoff *et al*., 1998). Zymography, using gelatin as a substrate, has frequently been used to demonstrate proteolytic degradation by MT1-MMP (Lichte *et al*., 1996; Pei and Weiss, 1996; Baker and Leaper, 2003**;** Choi *et al*., 2006) and was used in this study to determine the activity of the renatured catalytic domain of MT1-MMP.

2.5.1 Reagents

1% (m/v) gelatin stock solution. Gelatin (0.1 g) was dissolved in running gel buffer (10 ml, solution B, Section 2.3.1) with gentle heating.

Non-reducing treatment buffer:[125 mM Tris-HCl buffer, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol]. Tris Base (0.15 g), solution D (0.4 ml) and glycerol (2 ml) was made up to 10 ml with d.H_2O.

Renaturation buffer: [2.5% (v/v) Triton X-100 solution in dH_2O]. Triton X-100 (25 ml) was dissolved in 1 litre d.H_2O.

Digestion buffer: 50 mM Tris-HCl buffer, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2.2H_2O$. Tris (3 g) and NaCl (5.8 g) were dissolved in 450 ml d.H_2O, adjusted to pH 7.6 with HCl,

and $CaCl_2.2H_2O$ (0.34 g) added. The pH was adjusted if necessary and the solution made up to 500 ml with $d.H_2O$.

Staining solution: [0.1% amido black in 30% (v/v) methanol and 10% (v/v) acetic acid]. Amido black (0.1 g) was dissolved in methanol (30 ml) and glacial acetic acid (10 ml) and made up to 100 ml with $d.H_2O$.

Destaining solution: [30% (v/v) methanol, 10% (v/v) glacial acetic acid]. Methanol (300 ml) and glacial acetic acid (100 ml) were made up to 1 litre with $d.H_2O$.

2.5.2 Procedure

The MT1-MMP activity was analysed under non-reducing conditions on a 12.5% SDS-PAGE gel containing 0.1% (m/v) gelatin. This was achieved by adding 1% (m/v) gelatin stock solution (1.5 ml) to the running gel buffer (2.75 ml) and then adding this to the rest of the solution used to cast the SDS-PAGE gel. The gel was poured quickly before the gelatin was allowed to set. The samples (20 µl) were separated on duplicate gels (20 mA per gel, 3 ½ h, 4ºC), the second gel serving as the control. Following the electrophoretic run, the gels were incubated in a renaturation buffer (3 x 30 min) and digestion buffer (37°C, 18 h). The control gel was incubated in a digestion buffer containing 1% (m/v) EDTA. The enzyme activity was visualised by staining the gel (1 h) in amido black followed by destaining with 30% (v/v) methanol and 10% (v/v) glacial acetic acid (overnight with several changes of the destain).

2.6 Determining protein concentration

Quantifying proteins is necessary for many procedures and can be calculated using the extinction co-efficient of the protein at a wavelength of 280 nm (A_{280}). This method of protein determination is quick but is only useful if the protein to be measured is pure and its extinction coefficient known. Otherwise, for a mixture of proteins with unknown extinction coefficient, their concentration can be determined using the Bradford dye-binding assay. These protein determination procedures are discussed in Sections 2.6.1 and 2.6.2.

2.6.1 Bradford dye-binding assay

The Bradford dye-binding assay, first developed by Bradford (1976), is a quick and sensitive procedure, routinely used to quantify proteins. This assay involves the non-specific binding

of CBB G250 dye to the protein, which is measured spectrophotometrically at 595 nm (A_{595}). The CBB G250 dye is usually mixed with phosphoric acid, which reduces the pH of the solution to below 1, and under these acidic conditions, the CBB G250 dye exists as a cationic (red) form, which absorbs light at 465 nm (Dennison, 2003). Upon binding to the protein, the dye is stabilised and is converted to an anionic (blue) form and absorbs at 595 nm. The assay is based on the change in the maximum absorbance (λmax) of CBB G250 between 465 nm and 595 nm when the dye changes from the cationic to the anionic form upon binding to the protein. The colour usually develops within 2 min, with the degree of colour change being directly proportional to the concentration of the protein in the solution.

CBB G250 binds non-specifically to basic amino acids, such as arginine and to aromatic amino acids, such as lysine, tryptophan, tyrosine and phenylalanine (Compton and Jones, 1985). The dye binds via Van Der Waal, hydrophobic and electrostatic interactions (Compton and Jones, 1985).

Factors which may interfere with the binding of the dye to the protein include small amounts of detergents, such as SDS and/or Triton X-100, reducing reagents or phenols in the sample. These effects, however, can be reduced by using a suitable buffer blank. CBB G250 dye also varies in its binding capacity with different proteins and since the standard curve is established using other proteins (i.e. ovalbumin, lysozyme or BSA), the validity of the results may be compromised. The ideal situation would be to create a standard curve with the protein under study in its pure form. However, this is impractical and uses too much of the desired protein. Read and Northcote (1981) attempted to reduce variation by replacing CBB G250 with Serva blue G, by increasing the concentration of dye and decreasing the concentration of phosphoric acid used. These modifications increased sensitivity of the assay and reduced the variation in the binding of the dye to a range of proteins. These changes to the method did, however, lead to precipitation of the dye, reducing the reagent's shelf life. In our study, we chose to use the Bradford dye binding assay, using Serva blue G dye instead of CBB G250 at the recommended concentration. To minimise the amount of protein used or to measure small quantities of proteins (1-5 μg/ml), a micro Bradford assay was employed

using the volume of dye reagent suggested by Read and Northcote (1981). Ovalbumin was used to establish the standard curve as it generally responds well to the CBB G250 dye.

2.6.1.1 Reagents

Dye reagent. Serva blue G (50 mg) was dissolved in 88% (v/v) phosphoric acid (50 ml) and 99.5% (v/v) ethanol (23.5 ml) and made up to 500 ml with d.H_2O. The solution was magnetically stirred (30 min), filtered through Whatman No. 1 filter paper and stored in a brown reagent bottle.

Standard protein (ovalbumin) solution. An ovalbumin stock solution (20 mg/ml) was made up in d.H_2O

2.6.1.2 Procedure

The protein ovalbumin standard (0-50 µl of a 20 mg/ml solution) was diluted to 50 µl with d.H_2O in 1.5 ml eppendorf microfuge tubes to obtain protein concentrations ranging from 20 mg/ml to 0.01 mg/ml. Bradford reagent (950 µl) was added, the mixture vortexed and the colour was allowed to develop for approximately 2 min. The A_{595} for each protein concentration was read in 3 ml plastic cuvettes against a blank of d.H_2O. The resultant values were used to construct a protein standard curve of absorbance against known protein concentration.

To determine the concentration of the unknown protein solutions, the protein samples were diluted 1: 1 with d.H_2O to a final volume of 50 µl in 1.5 ml eppendorf microfuge tubes, Bradford reagent (950 µl) added, vortexed and the A_{595} of the protein samples determined using 3 ml plastic micro-cuvettes. The concentration of the unknown protein solution was determined from the protein (ovalbumin) standard curve taking the dilution factor into account.

2.6.2 Protein concentration determination using the extinction coefficient

Quantifying proteins using their extinction coefficient is frequently used as it is more rapid compared to the Bradford dye-binding method. Proteins, however, have specific extinction coefficients and, since different proteins absorb light at different intensities at a wavelength of 280 nm, it is crucial that the protein is pure. It is, therefore, difficult to determine the

concentration of a proten mixture. The concentration of pure MT1-MMP and IgY can be determined at 280 nm using their extinction coefficients of 1.407 m/cm (ExPASy proteomics server) and 1.25 ml/mg/cm (Hudson and Hay, 1980) respectively.

2.6.2.1. Reagents

100 mM sodium phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. NaH_2PO_4 (13.8 g) was dissolved in 800 ml $d.H_2O$ and adjusted to pH 7.6 with NaOH. NaN_3 (0.2 g) was added, the pH adjusted if necessary, and the solution was made up to 1 litre with $d.H_2O$.

2.6.2.2. Procedure:

The protein concentration was established by diluting the sample 1:50 (v/v) in 100 mM Na-phosphate buffer and the A_{280} of the sample determined spectrophotometrically in a 1 ml quartz cuvette. The protein concentration was calculated by rearrangement of the formula:

$$A = \varepsilon \, l \, c$$

to $c = A / \varepsilon l$

where A = absorbance at 280 nm

$\varepsilon^{0.1\%}$ = extinction coefficient [absorbance of a 0. solution

(i.e. 1 mg/ml) in a 1 cm cuvette path length]

c = protein concentration in mg/ml

l = length of light path (1 cm)

The dilution factor was included in the calculation of protein concentration.

2.7. IgY Isolation

Immunoglobulin Y (IgY) can be isolated from chicken eggs using polyethylene glycol (PEG) MW 6000. PEG is a high molecular weight, water soluble polymer, which was first introduced by Polson *et al*. (1963) to precipitate proteins from a complex mixture and was later on used more specifically to isolate IgY from chicken eggs (Polsen *et al*., 1985; Polsen

et al., 1980). PEG, like other polymers (i.e. dextran sulfate) and ammonium sulfate, can precipitate proteins at room temperature without denaturing the protein (Dennison, 2003). PEG precipitation results in clear cut fractions and at 12% (m/v) PEG concentration, IgY antibodies reach their solubility limit and precipitate out of solution with a purity of up to 90% (Goldring and Coetzer 2003). Protein precipitation with ammonium sulfate or dextran sulfate, in contrast, is less specific resulting in purity lower than 80% (Goldring and Coetzer 2003; Polsen *et al*., 1964).

Isolating IgY antibodies with PEG is simple, quick and a cheaper alternative to ammonium sulfate and dextran sulfate, as its high molecular weight means that lower concentrations are required to precipitate proteins. Large quantities of IgY can be isolated using the PEG precipitation procedure, which can subsequently be used in multiple studies including cancer research, diagnostics and therapy (Narat, 2003). For these reasons, IgY was isolated in this study employing the method of Polsen *et al*. (1985) with a few minor modifications.

2.7.1. Reagents

100 mM sodium phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. NaH_2PO_4 (13.8 g) was dissolved in 800 ml d.H_2O, adjusted to pH 7.6 with NaOH and NaN_3 (0.2 g) added and made up to 1 litre with d.H_2O.

2.7.2. Procedure

The chicken yolk was separated from the albumin (egg white) and any adhering albumin was removed from the yolk by rinsing with d.H_2O. The yolk sac was punctured and the yolk emptied into a measuring cylinder, being careful not to include any of the yolk sac. The yolk was diluted with 100 mM Na-phosphate buffer in a volume twice that of the yolk volume and mixed thoroughly. PEG was added to the mixture to a final concentration of 3.5% (m/v) and allowed to dissolve thoroughly. The mixture was centrifuged (4420 x g, 30 min, RT) to separate the vitellin layer from the supernatant, which still contained a layer of lipids. The lipid layer was removed by filtering the supernatant through a loose plug of cotton wool. To the filtrate, PEG was added to a final concentration of 12% (m/v), [8.5% (m/v) PEG was added], and the solution was mixed thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant was discarded and the pellet re-dissolved in 100 mM Na-phosphate buffer

equal to the filtrate volume. An additional 12% (m/v) PEG was added to the solution and centrifuged (12 000 x g, 10 min, RT). The final pellet was dissolved in 100 mM Na-phosphate buffer equal to a 1/6 egg yolk volume and stored at 4ºC. The concentration of IgY was determined as described in Section 2.6.2

2.8. Western blot analysis

Western blotting (immunoblotting) is a useful technique frequently used to detect and characterise a large number of antigens. In this study, western blotting was used to characterise the IgY antibodies. In this procedure, the antigen is separated on an Laemmli (1970) SDS-PAGE gel and immobilised, via transverse electrophoresis, on a supporting medium, such as nitrocellulose membrane, as an exact replica of the original gel pattern. In the gel, the antigen is inaccessible, but once immobilised on the membrane, the antigen is available to various ligands (i.e. antibodies) for detection (Kurien and Scofield, 2006). The antigen, however, is denatured using the Laemmli (1970) SDS-PAGE method and so the antibody, targeting a particular antigen, may not recognise this denatured form of the antigen. In this case, the antigen can be prepared without reduction and boiling in an attempt to preserve the native form of the antigen.

Nitrocellulose is the most frequently used matrix to bind transferred antigens. Kurien and Scofield (2006) describe various methods of transfering the protein onto nitrocellulose of which electroblotting is the most common and results in complete and rapid protein transfer. Here the gel and the nitrocellulose membrane are sandwiched together and, in the presence of a transfer buffer, are subjected to an electric field which initiates the migration of the protein from the gel to the nitrocellulose membrane. As in SDS-PAGE (Section 2.3), the overall charge of the protein is negative and it migrates from the cathode to the anode and, therefore, the nitrocellulose membrane needs to be positioned on the anodic side of the gel (Dennison, 2003). This overall negative charge on the proteins eliminates the possible effect that net charge normally has on protein transfer efficiency. Instead, the factors which affect protein transfer efficiency include, size of the polypeptide chain, as different sized proteins will migrate across at different rates, electric current (voltage) applied and the nature of the polyacrylamide gel used, as proteins will transfer across more rapidly from, for example a

12.5% SDS-PAGE gel than from a 15% SDS-PAGE gel, due to pore size difference (Desai *et al*., 2001; Kurien and Scofield, 2006).

To ensure the primary antibody specifically binds to the antigen, all unoccupied binding sites on the nitrocellulose membrane are blocked with a blocking agent i.e. fat-free milk. Following incubation with the primary antibody, unbound antibody is washed off and the antigen-antibody complex can be immunologically visualised using a detection system that involves an enzyme conjugated to a secondary antibody, specific for the primary antibody. Upon addition of a suitable substrate, the enzyme breaks the chromogenic substrate down to produce a distinctive, coloured product (Dennison, 2003). The nature of the enzyme coupled to the secondary antibody determines the type of substrate used. Horse radish peroxidase (HRPO) and alkaline phosphatase (AP) are two frequently used enzyme conjugates that form coloured products (Smejkal and Kaul, 2001; Kurien and Scofield, 2006). AP catalyses the breakdown of a 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium chloride (BCIP-NBT) substrate, whereas HRPO breaks down ABTS as well as the chromogenic 3, 3'-diaminobenzidine (DAB) substrates. These enzyme conjugate systems are sensitive enough to detect small quantities (ng) of proteins in the presence of low antibody titres (Towbin *et al*., 1979; Dennison, 2003).

Other immunodetection systems used to visualise antibody-antigen complexes include immunoprecipitation, immunofluorescence (i.e. fluorescent tagged antibodies) and immunoradioassays (IRA) (i.e. isotopes attached to the antigen). Compared to enzyme conjugate systems, immunoprecipitation requires large amounts of antigen and antibody to visualise the antigen-antibody complex and, thus, is possibly less sensitive (Dennison, 2003). Although immunofluorescence and IRA are both senstive procedures, immunoassays using enzyme conjugates are comparably cheaper to use and less labour-intensive (Voller *et al*., 1978). Enzymes are also generally more robust and stable compared to the isotopes used in IRA. An enzymes conjugate system was, therefore, considered in this study, to visualise antigen-antibody complexes formed on nitrocellulose membrane.

The method of Towbin *et al*. (1979) was adopted in this study, with a few minor modifications, as it is relatively simple, quick and efficient. It is not only able to transfer

proteins as an exact replica on the SDS-PAGE gel, but the viability and conformation of the proteins is maintained. For the immunoassay, the BCIP-NBT substrate was used as it is senstive and, compared to ABTS, this substrate produces an insoluble coloured product that is easily detected on the nitrocellulose membrane.

2.8.1 Reagents

Blotting buffer.

Tris Base (27.23 g) and glycine (64.8 g) were dissolved in 3 litres of d.H_2O. To this, 10% (m/v) SDS (4.5 ml) and methanol (900 ml) were added and the volume made up to 4.5 litres with d.H_2O.

Ponceau S stain [0.1% (m/v) Ponceau S in 1% (v/v) acetic acid]. Glacial acetic acid (1 ml) was diluted to 90 ml with d.H_2O after which Ponceau S (0.1 g) was dissolved and the solution made up to 100 ml.

Tris buffered saline (TBS), pH 7.4. Tris Base (2.42 g) and NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 800 ml d.H_2O, adjusted to pH 7.4 with HCl and made up to 1 litre.

Blocking agent [5% (m/v) non-fat milk powder in TBS]. Elite non-fat milk powder (5 g) was dissolved in 90 ml PBS and made up to 100 ml.

0.5% (m/v) Bovine serum albumin (BSA) in PBS (BSA-TBS). BSA (0.5 g) was dissolved in 80 ml TBS and made up to 100 ml.

Alkaline phosphatase buffer (50 mM Tris-HCl buffer, 5 mM $MgCl_2$, pH 9.5. Tris Base (0.61 g) and $MgCl_2$ (0.233 g) were dissolved in 90 ml d.H_2O, adjusted to pH 9.5 with HCl and made up to 100 ml.

Bromo-chloro-indolyl phosphate (BCIP) substrate solution [50 mM Bromo-chloro-indolyl phosphate]. Bromo-chloro-indolyl phosphate (5 mg) was dissolved in 100% DMF (100 µl) and stored in a dark reagent bottle at 4ºC.

Nitro-blue tetrazolium (NBT) substrate solution [100 mM Nitro-blue tetrazolium]. Nitro-blue tetrazolium (10 mg) was dissolved in 100% DMF (100 µl) and stored in a dark reagent bottle at 4ºC.

BCIP-NBT substrate solution. BCIP (30 µl) and NBT (30 µl) were added to 10 ml alkaline phosphate buffer. The solution was made up just before use.

2.8.2 Procedure

The protein antigens were separated on duplicate Laemmli (1970) SDS-PAGE gels as described in Section 2.3. A protein solution (25 µg/ml) was prepared with reducing treatment buffer and loaded onto the SDS-PAGE gel (12.5 µg/ml protein per well). The one gel was stained with CBB G250 dye, to illustrate the protein pattern, and the other gel was used for immunoblotting. The gel used for immunoblotting, the filter paper and nitrocellulose membrane were all soaked in blotting buffer before use. The gel was placed over two layers of filter paper and the nitrocellulose membrane, cut to fit the gel, was carefully laid over the gel ensuring no air bubbles formed between the gel and the nitrocellulose membrane. Two more layers of filter paper were placed over the nitrocellulose. The various layers were enclosed between two pieces of Scotch-Brite® (also saturated with blotting buffer) and clamped together between two plastic grids and the entire assembly was inserted into the western blotting apparatus, ensuring the gel was positioned on the cathodic side and the nitrocellulose membrane on the anodic side of the platinum wire electrodes. The chamber of the western blotting apparatus was filled with blotting buffer until the asembly was completely covered and the power supply was connected to the apparatus. Electroblotting (200 mA, 16 h) was conducted on ice and with continuous stirring.

Following the electrophoretic run, the power supply was disconnected and the assembly was removed from the tank. After removing the layers of filter paper, the nitrocellulose was peeled off the gel and briefly stained in Ponceau S to determine if the antigen transfer onto the nitrocellulose membrane was successful. This was confirmed by also staining the gel with CBB G250. The molecular weight markers were marked on the nitrocellulose membrane using a pencil and the nitrocellulose was subsequently destained with d.H_2O. If the nitrocellulose membrane was difficult to destain, NaOH (1 M) was added dropwise to the d.H_2O until the protein bands were not visible. The nitrocellulose membrane was subsequently air dried between two pieces of filter paper.

Non-specific binding sites on the nitrocellulose membrane were blocked with 5% (w/v) non-fat milk powder in TBS (overnight, 4ºC), washed (3 x 5 min) in TBS and incubated with the

primary antibody in 0.5% (m/v) BSA-TBS containing 0.3% (v/v) Brij 35 (2 h, RT). The nitrocellulose was washed (3 x 5 min) in TBS, incubated with the secondary antibody at a 1:150 000 dilution in 0.5% (m/v) BSA-TBS containing 0.3% (v/v) Brij 35 (1 h, RT) and washed (3 x 5 min) in TBS. The nitrocellulose membrane was incubated in the alkaline phosphatase substrate (pH 9.5) until bands developed, after which the substrate was replaced with d.H_2O, and dried between two pieces of filter paper to remove excess substrate and preserve the bands before capturing the image using the Versadoc imaging system.

2.9 Enzyme-linked immunosorbant assay (ELISA)

ELISA also uses a detection system similar to western blotting and was first employed by Engvall and Perlmann (1972) to measure immunoglobulin G (IgG). Numerous antibodies and antigen samples can be tested at one time using an ELISA and, therefore, this procedure is ideal for testing cross reactivity (Dennison, 2003). Although not as useful for showing specificity, ELISA is a highly sensitive procedure that is often used in conjunction with western blotting to obtain qualitative and quantitative information about the antibodies (Dennison, 2003).

ELISA has been used for quantifying hormones, serum proteins, including specific antibodies, and measuring toxin levels for the diagnosis of certain infections (Voller *et al*., 1978). For our purposes, an ELISA was suitable for evaluating the progress during immunisation, such as when the highest antibody titres occurred in the chicken (Dennison, 2003). To test optimum antibody production, the egg yolk, which serves as the primary antibody, can be diluted 1:3 in Na-phosphate buffer (Patterson *et al*., 1962). The direct approach of diluting the egg yolk, as opposed to isolating the IgY antibodies first, reduces the risk of antibodies being lost or destroyed during the isolation procedure and, therefore, provides a more accurate representation of the antibodies titres produced by the chicken over time. Before establishing the immune response curve, however, it is important to optimise the antibody and antigen concentrations by varying the concentration of antigen used to coat the ELISA plates and the primary antibody incubated with the antigen. This may be achieved using a checkerboard ELISA.

For establishing the antibody response curve, the indirect ELISA method is usually employed. Here the antigen is passively immobilised on the supporting medium (i.e. microtitre plate). Once bound to the plate, the antigen may be partially denatured. The immobilised antigen is subsequently incubated with the primary antibody in the presence of a blocking protein, i.e. BSA, to prevent non-specific binding of the antibody to the antigen surface. Any unbound antibody is washed away and the secondary antibody-enzyme conjugate binds to the bound primary antibody. Upon addition of a chromogenic substrate, the enzyme conjugate degrades the substrate producing a coloured product (Voller *et al*., 1978). The coloured product is, subsequently, measured colorimetrically. The intensity of the colour product is proportional to the amount of enzyme-linked secondary antibody present, which is an indication of the amount of primary antibody bound to the antigen. (Voller *et al*., 1978). In contrast to western blotting, the substrate used in an ELISA needs to produce a soluble coloured product. HRPO, which breaks down the ABTS substrate, produces this soluble coloured product (Voller *et al*., 1978) and, thus, was the substrate of choice in this study.

2.9.1 Reagents

Phosphate buffered saline (PBS), pH 7.4. NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 800 ml d.H_2O, adjusted to pH 7.4 with HCl and made up to 1 litre.

Blocking agent [5% (m/v) non-fat milk powder in PBS]. Elite non-fat milk powder (5 g) was dissolved in 90 ml PBS and made up to 100 ml.

0.5% (m/v) Bovine serum albumin (BSA) in PBS (BSA-PBS). BSA (0.5 g) was dissolved in 80 ml PBS and made up to 100 ml.

0.1% (v/v) Tween-PBS. Tween 20 (1 ml) was dissolved in 1 litre of PBS.

ABTS substrate [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in 150 mM citrate-phosphate buffer, pH 5.0]. ABTS (15 mg) and H_2O_2 (15 µl) were dissolved in 30 ml citrate phosphate buffer (for two ELISA plates).

Stopping solution [0.1% (w/v) NaN_3 in citrate phosphate buffer]. NaN_3 (0.015 g) was dissolved in citrate-phosphate buffer (15 ml).

2.9.2 Procedure

A checkerboard ELISA was conducted to determine the optimal antigen and antibody concentrations (2 µg-0.25µg) giving the most linear and sensitive (i.e. steepest) response to use in determination of antibody response. The antigen concentrations which gave such a relationship (1 µg/ml) was coated, in PBS, on a 96 well microtitre plate (over night, RT). A duplicate 96 well microtitre plate was also prepared to serve as the control. Non-specific binding sites were blocked with 0.5% (w/v) BSA-PBS (200 µl) (1 h, 37ºC) and the wells were washed (3 x 5 min) with 0.1% (v/v) Tween-PBS. A serial two-fold dilution of a crude dilution of egg yolk (1:3 in PBS), from immunised hen's eggs (1 egg from each week) and non-immunised hens eggs, was prepared on the plate beginning with 1:320 and ending with a 1:5620 dilution. The test and control ELISA plates were incubated in the test and pre-immune antibodies, respectively (2 h, 37ºC), washed (3 x 5 min) with 0.1% (v/v) Tween-PBS and incubated with rabbit anti-chicken-HRPO IgG (1:5000, 120 µl, 37ºC, 1 h). The ABTS substrate was added to the wells (150 µl) and the plate was incubated in the dark (10-15 min) until a distinctive colour reaction developed against the controls. The reaction was subsequently stopped by the addition of citrate-phosphate buffer containing 0.1% (w/v) NaN_3 (50 µl) and the A_{405} read using an ELISA plate reader. Eggs showing the best titre, established by plotting absorption at 405 nm against –log dilution of egg yolk, were selected for antibody (IgY) isolation.

2.10 Cell Culture

The MCF10A and the MCF10A-neoT cell lines were supplied by Dr. Bonnie Sloane (Department of Pharmacology, Wayne State University, Detroit, MI) and cultured as in the source laboratory to make sure the cell phenotype, in the present laboratory, is consistent with the cell phenotype in the source laboratory, as the protocol used in this study differs slightly from that used by other workers. The passage numbers of cells were similarly kept as low as possible. All glassware was washed in 7X-PF detergent and copiously rinsed in tap water and d.H_2O before autoclaving.

2.10.1 Reagents

Hanks' balanced salt solution (HBSS). Powdered HBSS and sodium hydrogen carbonate (1.2 g) were dissolved in double distilled water (dd.H_2O), adjusted to pH 7.3 with NaOH and made up to 1 litre. The HBSS solution was filtered a through 0.22 µm filter into sterile, autoclaved bottles under sterile conditions and stored at 4ºC.

Epidermal growth factor (EGF) (50 µg/ml). EGF (100 µg) was dissolved in d.H_2O (2 ml) and stored at -20ºC.

Insulin (600 µg/ml). Insulin (10.2 mg) was dissolved in HBSS (16.5 ml) and 0.1 M NaOH (500 µl) was added. This solution was prepared just before use.

Hydrocortisone (3.33 mg/ml). Hydrocortisone (10 mg) was dissolved in ethanol (3 ml) and stored at -20ºC.

Fungizone (250 µg/ml). Fungizone (5 mg) was dissolved in d.H_2O (20 ml) and stored at -20ºC.

DMEM: Ham's F-12 complete medium [DMEM: Ham's F-12 cortisone (0.5 µg/ml), insulin (10 µg/ml), EGF (20 ng/ml), horse serum (5% (v/v)), fungizone (0.25 µg/ml), pH 7.3). Powdered medium (0.5 mg) and sodium hydrogen carbonate (1.2 g) were dissolved in 900 ml dd.H_2O, adjusted to pH 7.3 with NaOH and made up to 1 litre. The medium was filtered through a 0.22 µm filter into sterile autoclaved bottles under sterile conditions and horse serum (50 ml), hydrocortisone (150 µl), insulin (17 ml), EGF (400 µl) and fungizone (10 ml) were added aseptically and the medium stored at 4ºC.

2.10.2 Procedure

The MCF10A-neoT cells were cultured at 37ºC in 25 cm^3 flasks in complete medium in a humidified atmosphere containing 5% CO_2, fed every 3-4 days and passaged upon reaching 80% confluence. Cells were washed three times with HBSS, rinsed in trypsin-EDTA so that it just covered the monolayer and incubated at 37ºC until the cells detached. The cells were diluted in complete medium, split into a 1:3 ratio for each passage and seeded into new flasks.

2.11. Fixation and pre-embedding processing of cells

Fixation is used to rapidly preserve cellular structure including cell shape, volume and spatial arrangement of organelles and proteins, such that the cell resembles its living state. Fixation also protects the cells from being severely damaged after exposure to subsequent treatments, such as resin embedding and sectioning. For immunocytochemistry, a fixative

must be able to fix the specimen in position, in an insoluble form and preserve the protein in such a way that it is recognisable by the antibody.

Chemical fixation is the most commonly used method for stopping all cellular activity and preserving cellular constituents. This fixation involves, either precipitation of proteins within the cell (eg. alcohol fixation), or cross linking of proteins via their side groups (e.g. aldehyde fixation) (Brorson, 1998a). Fixation with alcohols (i.e. ethanol and methanol) or ketonic solvents (i.e. acetone) inactivates cellular processes and permeabilises the cell. This allows membrane lipids to be removed, but may also allow partially precipitated proteins to diffuse out of the cell. Reduction in these intracellular proteins may, consequently, weaken the antibody response against specific antigens. Alcohols also destroy cell ultrastructure, which is unsuitable for viewing under the electron microscope (EM). Aldehydes (i.e. glutaraldehyde and paraformaldehyde) and osmium tetroxide are more efficient in fixing tissue than alcohols (Boon *et al*., 1988) as they can rapidly penetrate the cells, forming crosslinks between cellular constituents. This allows surface and intracellular organelles and proteins to be fixed. Aldehydes also minimise cell shrinkage (Ma *et al*., 2002). Recent reports, however, have suggested that crosslinking between cellular constituents may diminish antigen preservation (Benavides *et al*., 2006). Despite this, aldehydes maintain membrane integrity, which prevents the contents from escaping from the cell and makes the antigen in the cellular compartments more available for detection. Aldehydes also provide excellent preservation of the ultrastructure in conjunction with good membrane contrast, such that organelles like the ER, Golgi and mitochondria can be identified (Murk *et al*., 2003).

Osmium tetroxide is not suitable in imunocytochemsitry as it completely denatures antigens, making them unrecognisable by the antibody. Although glutaraldehyde may also cause partial damage to antigens, this fixative is able to penetrate the cell slowly and at the same time fixes the tissue rapidly, which is better for preserving ultrastructure compared to osmium tetroxide. In contrast, paraformaldehyde (PFA) penetrates the cell rapidly, with minimal crosslinking between cellular constituents, which is less destructive to antigens and, thus, maintains optimal reactivity with antibodies (Elliott *et al*., 1995). The degree of

penetration and cross-linking of PFA is affected by concentration and pH conditions. Low PFA concentrations combined with high pH increases penetration and results in rapid cross-linking. High PFA concentrations combined with lower pH (i.e. physiological pH) allows the cross-linking to be more widespread (Elliott *et al*., 1995). Elliott *et al*. (1995) showed that employing both sets of conditions optimises antigen immobilisation. Although PFA is better than glutaraldehyde at preserving antigen structure, it does result in poor ultrastructure preservation compared to glutaraldehyde (Brorson, 1998a; Boon *et al*., 1988). For the best results, therefore, glutaraldehyde and PFA are usually used together to fix the tissue. These fixatives were, consequently, used in in this study in the fixation of the MCF10A-neoT cell line, according to the protocol of Santama *et al*. (1998) with a few minor modifications.

2.11.1 Reagents

800 mM HEPES stock solution, pH 7.3. HEPES (43.68 g) was dissolved in 180 ml d.H_2O, adjusted to pH 7.3 with NaOH and made up to 200 ml. The solution was aliquoted and stored at -20ºC. Prior to use, the solution was diluted to 200 mM and the pH was adjusted if necessary.

16% (m/v) Paraformaldehyde stock solution. Paraformaldehyde (16 g) was dissolved in 90 ml d.H_2O, warmed to a maximum temperature of 60ºC, cleared with a minimum amount of 1 M NaOH, made up to 100 ml and stored at -20ºC.

8% (m/v) Paraformaldehyde in 200 mM HEPES, pH 7.3. Paraformaldehyde stock solution (25 ml) was added to HEPES stock solution (12.5 ml), adjusted to pH 7.3 with 1 M HCl, made up to 50 ml with d.H_2O and stored at -20ºC.

4% (m/v) Paraformaldehyde, 0.05% (v/v) glutaraldehyde in 200 mM HEPES, pH 7.3. Paraformaldehyde stock solution (25 ml) and glutaraldehyde [200 µl of 25% (v/v)] were added to HEPES stock solution (25 ml) and made up to 90 ml with d.H_2O.The pH was adjusted to pH 7.3 with 1 M HCl and the solution was made up to 100 ml and stored at -20ºC.

20 mM Glycine in 200 mM HEPES, pH 7.3. Glycine (0.015 g) was dissolved in 200 mM HEPES and made up to 10 ml.

10% (m/v) Gelatin in 200 mM HEPES, pH7.3. Microbiological grade gelatin (10 g) was added to 200 mM HEPES (100 ml) and dissolved by gentle heating. The solution was made up to 100 ml if necessary with dH_2O, chilled rapidly on ice and stored at 4ºC.

2.11.2 Procedure

MCF10A-neoT cells were fixed with 4% (m/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 200 mM HEPES (30 min, RT). The fixative was removed and replaced with 8% (m/v) paraformaldehyde in 200 mM HEPES. The monolayers were carefully scraped off the bottom of the flasks using a rubber policeman, transferred into eppendorf tubes and stored overnight at 4ºC. The cells were gently pelleted, the excess fixative removed and any remaining free aldehyde groups quenched with 20 mM glycine in 200 mM HEPES (2 x 15 min). The cells were pelleted, excess glycine was removed and the pellet was infiltrated with 10% (m/v) gelatin in 200 mM HEPES (2 h, 37ºC). The cells were pelleted again, excess gelatin removed and the cell pellet chilled rapidly on ice. A thin layer of buffer was placed over the gelatin-infiltrated cell pellet to prevent drying out. The pellet was cut into 1 mm^3 cubes that were dehydrated and embedded in LR White resin for sectioning and immunolabelling for EM.

2.12 LR White embedding of cells

There are two main types of resins used for embedding tissue, the acrylic and epoxy resins. Epoxy resins are more suitable for tissue used in transmission electron microscopy as they are more efficient in preserving ultrastructure and more stable when exposed to the electron beam. In the tissue, however, epoxy resins tend to form covalent bonds with the protein, thereby co-polymerising with the embedded tissue and, as a result, proteins are often cleaved at the plane of sectioning (Brorson, 1998b; Murray, 1992). Acrylic resins, in contrast, do not bind to the embedded tissue, but polymerise around the tissue without forming covalent bonds with tissue proteins, which allows for thinner sections to be obtained and minimal protein loss (Brorson, 1998b; Murray, 1992). For embedding tissue that will be used in immunocytochemical localisation of antigens, London Resin (LR) white acrylic resin is frequently used (Murray, 1992). LR white is a “hydrophilic mixture of acrylic monomers” (Newman *et al*., 1983), which can produce high resolution ultrastructure. The resin has a low viscosity and toxicity and its hydrophilic nature makes it versatile and easy to use but, more importantly, it can fully infiltrate the section without causing damage to tissue antigens and other intracellular structures (Newman *et al*., 1983). Being hydrophilic, LR white also minimises lipid breakdown, which enables easy discrimination between membranes and

organelles. LR white resin does not bind to antibodies or inhibit the antibodies from binding to the protein antigen, which is often the case with hydrophobic resins. For these reasons, LR white resin was used for embedding the MCF10A-neoT cells in this study.

2.12.1 Reagents

25% (v/v) Ethanol. Ethanol (2.5 ml) was made up to 10 ml with d.H_2O.

50% (v/v) Ethanol. Ethanol (5 ml) was made up to 10 ml with d.H_2O.

70% (v/v) Ethanol. Etahnol (7 ml) was made up to 10 ml with d.H_2O.

90% (v/v) Ethanol. Ethanol (9 ml) was made up to 10 ml with d.H_2O.

Resin:ethanol (1:1). LR White (2.5 ml) was mixed with an equal volume of absolute ethanol (2.5 ml).

Resin:ethanol (2:1). LR White (4 ml) was mixed with absolute ethanol (2 ml).

2.12.2 Procedure

The gelatin blocks were dehydrated in a graded ethanol series (25%, 50%, 70%, 90% and 100% ethanol for 15 min, 15 min, 1 h, 30 min and 30 min respectively). Once dehydrated, the blocks were transferred to increasing amounts of LR White resin (1:1 and 2:1 for 30 min each followed by undiluted resin for 30 min, 30 min and 1 h respectively). The gelatin blocks were transferred to fresh undiluted embedding media and allowed to further infiltrate overnight. Fresh resin was dispensed into gelatin capsules and the samples were placed on top of the resin, taking care to minimise resin carryover, and allowed to sink to the bottom of the capsule. The capsules were quickly closed ensuring no air bubbles were present, as oxygen prevents polymerisation, and allowed to polymerise (24 h, 50ºC).

2.13 Coating grids with formvar, preparation of the knife and cutting of sections

Copper grids need to be coated to provide some mechanical strength to the section during immunolabelling and to keep the section stable under the electron beam. Formvar is frequently used to coat the grids.

2.13.1 Reagents

0.25% (m/v) Formvar in chloroform. Formvar (0.25 g) was dissolved in chloroform (100 ml).

2.13.2 Procedure

For the preparation of formvar-coated grids, a dish (20 x 10 x 10 cm) was completely filled with d.H_2O. A clean glass microscope slide was dipped into formvar solution, the excess allowed to drain and the remaining film dried. The dried film was loosened around the edges of the slide with a razor-blade and floated onto the surface of the d.H_2O. Copper grids (hexagonal, 100 mesh) were placed, shiny side up, on the floating film and, using a rectangular piece of wire mesh as a support, the film was recovered using a scooping motion. The grids, adhered to the underside of the film, were allowed to dry. Formvar-coated grids were removed from the wire mesh by carefully perforating the formvar film surrounding individual grids.

For the preparation of the glass knives, a glass strip was cleaned with detergent and water-dried using tissue paper and positioned on a Type 7801B LKB Knife Maker (Morewood *et al*., 1992) with the ridged imperfect edge facing downwards. The glass strip was repeatedly scored and fractured in the middle until square pieces were produced. Each square was placed in the glass holder at a 45º angle, scored quickly through the middle and fractured into two right-angled triangles. Usually, the one triangle has a sharp usable edge while the other has the blunt counter face. A trough to hold d.H_2O, upon which cut sections would be floated out, was made below the sharpest knife-edge using silver adhesive tape. The knives were stored in a dust-free container for later use.

Prior to sectioning, the LR White blocks were trimmed into a trapezoid using a fine hacksaw. The blocks were further trimmed with a glass knife on an RMC MT6000XL ultramicrotome. The trough of the previously prepared knife was filled with sufficient d.H_2O to ensure that the d.H_2O just reaches the knife-edge. Silver-gold ultrathin sections (80-100 nm) were cut and floated onto the water trough for retrieval onto formvar-coated copper grids. Grids were dried and could be stored indefinitely before immunolabelling.

2.14 Immunolabelling of MCF10A-neoT cells for transmission electron microscopy

Immunolabelling of tissue sections involves the incubation of antibodies with the tissue section and the antibody binding is visualised by incubating the tissue section with a labelled secondary antibody system that can be detected by the transmission electron microscope (TEM). TEM uses electron dense molecules to detect immunolabelled biological tissue. Colloidal gold particles are favourable markers for immunolabelling as they are non-toxic, and easy to prepare and their electron-dense nature allows protein antigens to be easily identified (Birrell *et al*., 1987; Mayer and Bendayan, 1999). Due to their unique shape, gold probes also facilitate the discrimination and identification of tissue, that are similar in morphology (Birrell *et al*., 1987; Schofer *et al*., 2004).

Gold particles are usually adsorbed either directly to the antibody (i.e. immunogold probes) or to a protein A molecule (i.e. protein A-gold probes). Protein A is derived from *Staphylococcus aureus* and binds to the Fc portion of many species of rabbit IgG. The rabbit IgG, directed against the primary antibody, binds to it and acts as the linker between the primary antibody and the protein A-gold probe. The antibody-antigen interaction is visualised under the electron microscope with high resolution and sensitivity.

Both immunogold and protein A-gold probes result in a similar degree of sensitivity. Protein A-gold probes, however, are better suited if antigen quantitation is required as only one protein A molecule binds to one primary antibody, whereas more than one immunogold probe can bind to a single primary antibody. In addition, one or more antigens can be detected in different regions of the cell labelled with protein A-gold. This is crucial for determining distribution patterns and behaviour of cellular proteins (Mayhew and Desoye, 2004). Therefore, protein A-gold probes were used to label the MCF10A-neoT cell line in this study.

2.14.1 Reagents

Blocking agent (FBG): 1% (v/v) fish skin gelatin, 0.8% (m/v) BSA, 20 mM gelatin in PBS. Fish skin gelatin (5 ml), BSA (4 g) and glycine (0.75 ml) were combined and made up to 500 ml in PBS. Impurities were sedimented (10 000 x g, 4 h).

Phosphate buffered saline (PBS), pH 7.4. NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 800 ml d.H_2O, adjusted to pH 7.4 with HCl and made up to 1 litre.

1% (m/v) Bovine serum albumin (BSA) in PBS (BSA-PBS). BSA (1 g) was dissolved in 80 ml PBS and made up to 100 ml.

1% (v/v) Glutaraldehyde in PBS. Glutaraldehyde (10 µl) in 1 ml PBS.

2% (m/v) Uranyl acetate. Uranyl acetate (1 g) was dissolved in d.H_2O (50 ml), 95% ethanol (1 ml) was added to assist in solubility and the solution was stored in the dark at 4ºC.

1M NaOH. NaOH (0.4 g) was dissolved and made up to 10 ml in d.H_2O.

Lead citrate. Lead citrate (1.33 g) and trisodium citrate.$2H_2O$ (1.76 g) were added to recently boiled and cooled d.H_2O (40 ml) and shaken occasionally over 30 min. The solution appeared milky and was cleared with 1 M NaOH (8 ml). The solution was made up to 50 ml with additional boiled and cooled d.H_2O and stored in the dark at 4ºC.

2.14.2 Procedure

Ultra-thin sections (100 nm) were cut with a LKB ultramicrotome, collected on a formvar-coated copper grid and allowed to dry for 2-24 h before use. Immunolabelling was conducted by placing the copper grid, section-side down, on a droplet of each reagent on a sheet of parafilm and the grid was transferred to different reagents using a 1 mm nichrome wire loop. Non-specific binding sites on the section were blocked with FBG (10 min) and the section was incubated with the specific primary antibody (40 µg/ml) in 1% BSA-PBS (30 min). The section was washed with PBS (5 x 2 min) and incubated with the rabbit anti-chicken IgG secondary antibody (1:200 dilution in FBG, 30 min), washed with PBS (5 x 2min) and probed with the 10 nm protein A-gold (1:200 dilution in FBG, 30 min). After washing in PBS (6 x 2 min), the section was stabilised with 1% (v/v) glutaralderhyde in PBS (5 min), washed with d.H_2O (4 x 5 min) and allowed to dry before counterstaining. Two controls were run concurrently. The first control involved incubating the section with pre-immune IgY (40 µg/ml) and the second was a method control where the primary antibody step was omitted.

For counterstaining, the section was incubated with uranyl acetate (10 min) and washed three times with d.H_2O, the second wash using a large volume of d.H_2O to ensure excess uranyl acetate was removed. The section was incubated with lead nitrate (5 min) under CO_2-

free conditions (otherwise the lead nitrate precipitates). To obtain these conditions, the section was incubated with the lead nitrate in the presence of NaOH and kept covered. The section was washed twice with $d.H_2O$, again the second wash using a large volume of $d.H_2O$, and dried with filter paper ready for viewing.

2.15 Immunofluorescence labeling of MT1-MMP for confocal microscopy

2.15.1 Reagents

Phosphate buffered saline (PBS), pH 7.4 [8 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$, pH 7.4]. $Na_2HPO_4.2H_2O$ (1.42 g) and KH_2PO_4 (0.20 g) were first dissolved in $d.H_2O$ (200 ml). NaCl (7.99 g), KCl (0.20 g), $CaCl_2.2H_2O$ (0.15 g) and $MgCl_2.6H_2O$ (0.10 g) were added and the solution made up to 1 l with $d.H_2O$. The solution was filtered and stored at 4°C.

16% (m/v) Paraformaldehyde (PFA) stock solution. (see Section 2.11.1 on tissue processing for EM)

3.7% (m/v) Paraformaldehyde in PBS, pH 7.4. 16% PFA stock solution (23.13 ml) was added to PBS, pH 7.4 (76.87 ml). The solution was made up just before use.

1% (m/v) Bovine serum albumin (BSA) in PBS, pH 7.4. BSA (1 g) was dissolved in PBS, pH 7.4 (100 ml). The solution was made up just before use.

0.1% (m/v) Saponin in PBS, pH 7.4. Saponin (0.1 g) was dissolved in PBS (100 ml) and filtered through Whatman No. 1 filter paper. The solution was made up just before use.

Moviol. Moviol (24 g) and glycerol (6 g) were dissolved in $d.H_2O$ (6 ml) and added to 0.2 M Tris (12 ml) pH 8.5-9 and stirred overnight. The solution was centrifuged (500 x g, 15 min) (Ono *et al*., *2001*). Aliquots were stored at -20°C.

2.15.2 Procedure

MCF10A and MCF10A-neoT human breast epithelial cells were seeded onto sterile round coverslips (in Tersaki plates/ 24 well plates) and grown to 70% confluency in DMEM-HAM's F12 medium supplemented with 10% decomplemented horse serum. Cells were washed with HBSS and fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and permeabilised in PBS containing 0.1% (m/v) saponin. After washing with PBS, cells were blocked with 1% (m/v) BSA-PBS. All subsequent antibody and wash solutions contained 0.1% (m/v) saponin. Cells were incubated with specific primary antibodies [i.e. MT1-MMP (PROcat) and MT1-MMP (PEXcat) antibodies] for 1 h and

washed. Cells were incubated for 1 h with Cy3-conjugated goat anti-chicken IgG, (1:1500), followed by six washes in PBS containing 0.1% (m/v) saponin. In controls, the primary antibody was substituted by preimmune chicken serum or PBS. Finally, cells were mounted with Mowiol anti-fade reagent and observed with a Zeiss LSM 510 META confocal laser scanning microscope. Images were saved (in a database) for further image analysis.

Optical sections were obtained at increments of 0.4 μm in the Z-axis and digitised with a scanning mode format of 1024 X 1024 pixels. Selected image planes were exported as RGB images. ImageJ program was used in this study for post acquisition processing. The relative fluorescence intensities were calculated at various points such as leading edge lamellipodia and along the plasma membrane. Exported images were scaled using the magnification bars on each image. Images were separated into red, green and blue (RGB) components. The red image component represented captured Cy3 fluorescence. These images were converted to 256 gray scale images. Pixels with values between 80 and 256 on the gray scale were determined to represent fluorescence. The total area represented by these pixels was calculated and represented the relative amount of fluorescence in that image. The area of each cell analysed was also calculated by outlining the cell and measuring the area.

Chapter 3

Membrane type-1 matrix metalloproteinase (MT1-MMP) domain selection, expression, protein recovery and solubilisation

3.1 Selection of MT1-MMP domain

Recombinant expression of proteins, or protein domains, is a useful way of quickly obtaining, at low cost, a large amount of protein for studies to improve the understanding of structure-function relationships, possible enzymatic activity, and behaviour and role of an enzyme in *in vitro* and *in vivo*. Recombinant proteins/protein domains can also be used as antigens to generate specific antibodies. This may be achieved by immunisation of laboratory animals. In this study, we needed to express MT1-MMP (MMP-14) in order to raise MT1-MMP-specific and potentially immunoinhibitory antibodies. Before this could be attempted, however, identification of target MT1-MMP domains for antibody production was needed. As mentioned in Chapter 1 (Section 1.5), the propeptide, hemopexin-like and catalytic domains of MT1-MMP play a critical role in the enzyme's function and activity. Antibodies that target one or more of these domains potentially may inhibit MT1-MMP and, hence, MT1-MMP-associated invasion and were, therefore, considered as primary targets for immunisation.

Such domains, however, are highly conserved across MMP classes (Figure 1) (Folgueras *et al*., 2004). The primary aim of this study was, therefore, to find suitable target domain(s) for expression and against which to raise antibodies that recognise and inhibit only MT1-MMP. It was, therefore, critical to determine which target domain/domain combinations would be the most suitable (i.e. have the least number of conserved residues across all MMP subtypes and species compared to human MT1-MMP), in order to have the least cross-reactivity. It was also important to establish the best species of laboratory animal in which to raise antibodies (i.e. the species in which human MT1-MMP would be the most different and, therefore, the most immunogenic). Predictions may be made by performing multiple sequence alignments using a program such as that from the Clustal series (Clustal W and Clustal X).

Multiple sequencing alignments, established progressively from a series of pairwise alignments, and construction of phylogenetic trees, may be used to guide the alignment of the multiple protein sequences. Whole protein sequences or domain-specific sequences may be analysed in this manner. Unlike other multiple sequence alignment programs, Clustal W and Clustal X both align the most closely related sequences first. Such programs take into account sequence length and gaps existing between alignments. These include those gaps introduced between conserved sequences during the calculation of the scoring matrix. High scores indicate well conserved amino acids between different proteins and, conversely, low scores suggest the sequences are less conserved (Thompson *et al*., 1997).

The Clustal X program was modified from the original Clustal W program by Thompson *et al*. (1997). This was to allow the multiple sequence alignment, including viewing of the results, alignment editing and improvement, to be conducted in a single window interface. Compared to Clustal W, Clustal X is also faster, more user friendly and easily installed (Chenna *et al.*, 2003). The data is displayed in a coherent and simple-to-follow manner, as the conserved sequences between different proteins are highlighted in the same colour. Misaligned sequences are emphasised, by being highlighted in white with a grey background (Thompson *et al*., 1997). This allows easy and quick assessment of sequence homology and difference and also enables the user to better refine the alignment (Chenna *et al*., 2003; Thompson *et al*., 1997). Clustal X can also read sequences in the Fasta format. The benefit of this format is compatibility with other software programs and portablility between different operating systems, such as the Microsoft windows and Macintosh operating systems (Thompson *et al*., 1997). In this study, Clustal X (Thompson *et al*., 1997) was used to predict the optimal laboratory host species and target domains of MT1-MMP for antibody production.

3.1.1 Overall sequence homology of human MT1-MMP (MMP-14) with that of mouse and chicken

The choice of laboratory animal for raising antibodies was initially determined by aligning the whole human MT1-MMP sequence with that in other animals and birds. This allowed overall sequence homology or potential antigenicity in potential antibody generating host animals to be descerned (Chenna *et al*., 2003).

3.1.1.1 Procedure

In this study, all the relevant MT1-MMP (MMP-14) amino acid sequences were obtained from the NCBI protein data bank and the FASTA amino acid sequences of rabbit, mouse and chicken MT1-MMP were each aligned with that of human MT1-MMP (Sato *et al*., 1994). This was performed using the Thompson *et al*. (1997) modified Clustal X program and the animal showing the least sequence homology with human MT1-MMP, was selected as a target sequence for antibody production.

3.1.1.2 Results

Chicken MT1-MMP (Figure 10 C) showed a 54% overall homology with human MT1-MMP compared to that of the mouse (Figure 10 A) and rabbit (Figure 10 B) MT1-MMP, which were 97% and 93% identical to human MT1-MMP overall, respectively. The chicken was, therefore, selected to raise antibodies against human MT1-MMP, having the overall largest number of differences in the amino acid sequence compared to the human MT1-MMP against which antibodies were required.

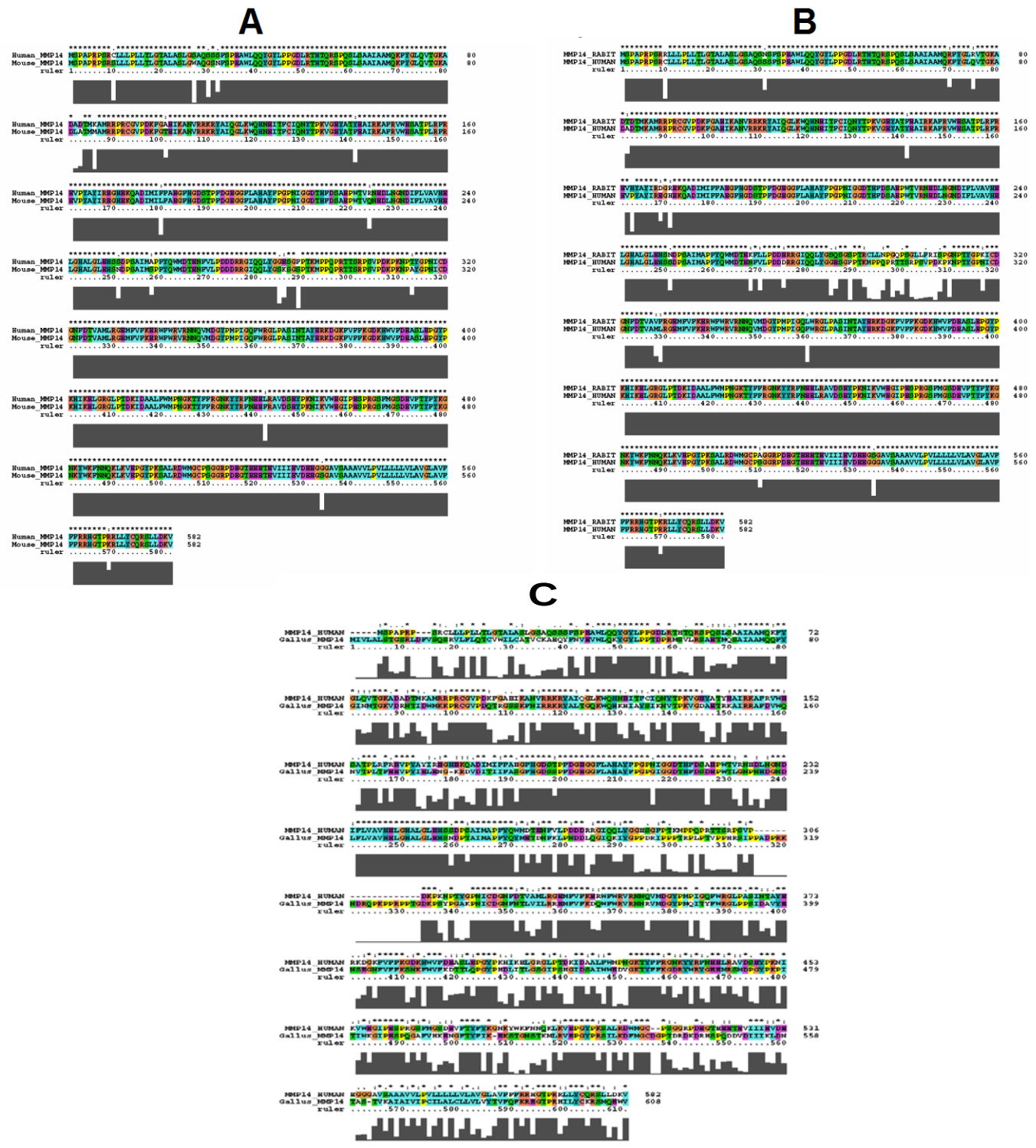

Figure 10. Overall sequence homology between human MT1-MMP (MMP 14) and mouse (A), rabbit (B) and chicken (C) MT1-MMP.

The sequence alignment was conducted using Clustal X (Thompson *et al*., 1997). The "*" represents fully conserved amino acids. The histogram below the ruler represents the degree of similarity between the sequences, with peaks indicating regions of high similarity and valleys indicate regions of low similarity.

3.1.2 Assessment of MT1-MMP domain homology between chicken and human MT1-MMP and other gelatinases

Although chicken MT1-MMP has an overall low sequence homology with human MT1-MMP (Figure 10 C), it was considered important to also check the homology of specific potential target domains, such as the propeptide, catalytic and hemopexin-like domains in the chicken, compared to that of the human. The selectivity and titre of antibodies raised against domains usually depends on the extent of sequence difference, the greater the sequence difference between the chicken and human MT1-MMP sequences, the better the antibody titres. The specificity of the antibodies in distinguishing MT1-MMP from other gelatinases, which have similar domains also depends on the extent of sequence difference or lack of domain homology in the propeptide, hemopexin-like and catalytic domains of other MMPs with domains similar to MT1-MMP e.g. MMP-2 and MMP-9. For this reason, the sequence homologies of MMP-2 and MMP-9, against that of MT1-MMP, were also checked.

3.1.2.1 Procedure

The catalytic, hemopexin-like and propeptide domain sequences of human MT1-MMP (Sato *et al*., 1994) were aligned with their respective domains in chickens, using Clustal X program (Thompson *et al*., 1997). Each domain sequence was downloaded from the NCBI protein data bank in the FASTA format prior to performing the sequence alignment. The propeptide, catalytic and hemopexin-like domains of human MT1-MMP, MMP-2 (Collier *et al*., 1988) and MMP-9 (Huhtala *et al*., 1991) were similarily aligned to check sequence homology between MMP classes (within the gelatinases, MMP-2 and -9).

3.1.2.2 Results

The human catalytic domain appeared to have the highest sequence homology (63%) with the catalytic domain of chicken MT1-MMP (Figure 11 A). The hemopexin-like domain of human MT1-MMP proved to be 57% homologous with that of the chicken (Figure 11 B). The propeptide domain had the lowest sequence homology with that of the chicken (37%). It was, therefore, considered that the human propeptide domain may be the most immunogenic

in chickens (Figure 11 C). Since the catalytic domain appeared to be the least immunogenic, if antibodies against the catalytic domain were required, it was considered possibly better to inject the catalytic domain expressed together with the propeptide domain, as together these domains may also induce a conformational epitope antibody response in chickens, which may differ from that raised to a more linear sequence, improving specificity.

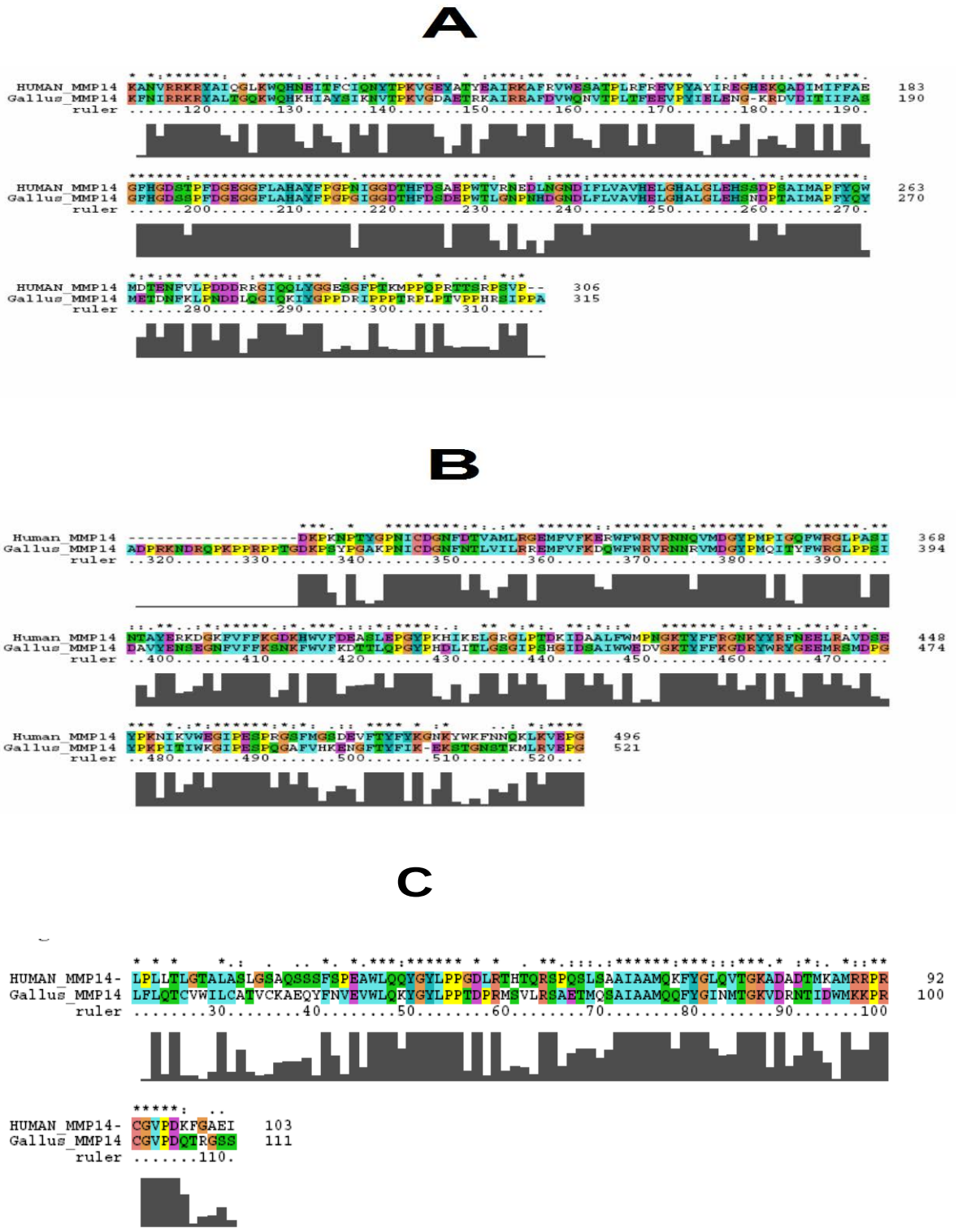

Figure 11. Sequence homology of individual MT1-MMP (MMP 14) domains between humans and chickens.

The catalytic (A), hemopexin-like (B) and propeptide (C) domains of MT1-MMP in chickens and humans were aligned using ClustalX according to Thompson *et al*., (1997). The "*" represents amino acid sequences that are completely conserved. The histogram below the ruler represents the degree of similarity between the sequences, with peaks indicating regions of high similarity and valleys indicate regions of low similarity.

The hemopexin-like domain was observed to be the least conserved across the MMP classes, with an 11% homology observed between MT1-MMP, MMP-2 and MMP-9 (Figure 12 A). On the other hand, the propeptide domains of MT1-MMP, MMP-2 and MMP-9 (Figure 12 C), have a homology of 20% and the highest sequence homology (30%) was observed between the catalytic domains of the three MMPs (Figure 12 B). Antibodies raised against the propeptide and catalytic domains of MT1-MMP were, therefore, considered more likely to cross-react non-specifically and undesireably with MMP-2 and MMP-9 than the antibodies targeting the hemopexin domains. It was, therefore, believed that expression and injection of the catalytic domain linked to the hemopexin-like domain (the larger unit creating more different conformational epitopes) may, therefore, limit antibody cross-reactivity with other MMPs.

Expression and injection of the hemopexin-like domain alone was also thought possibly to be most likely to give rise to MT1-MMP-specific antibodies. The propeptide/catalytic domain combination was thought possibly to be more immunogenic than hemopexin-like/catalytic domain combinations (Figure 11 C). Such strategies were, therefore, considered to be the best approach as, production of chicken antibodies against such domains has not been previously attempted.

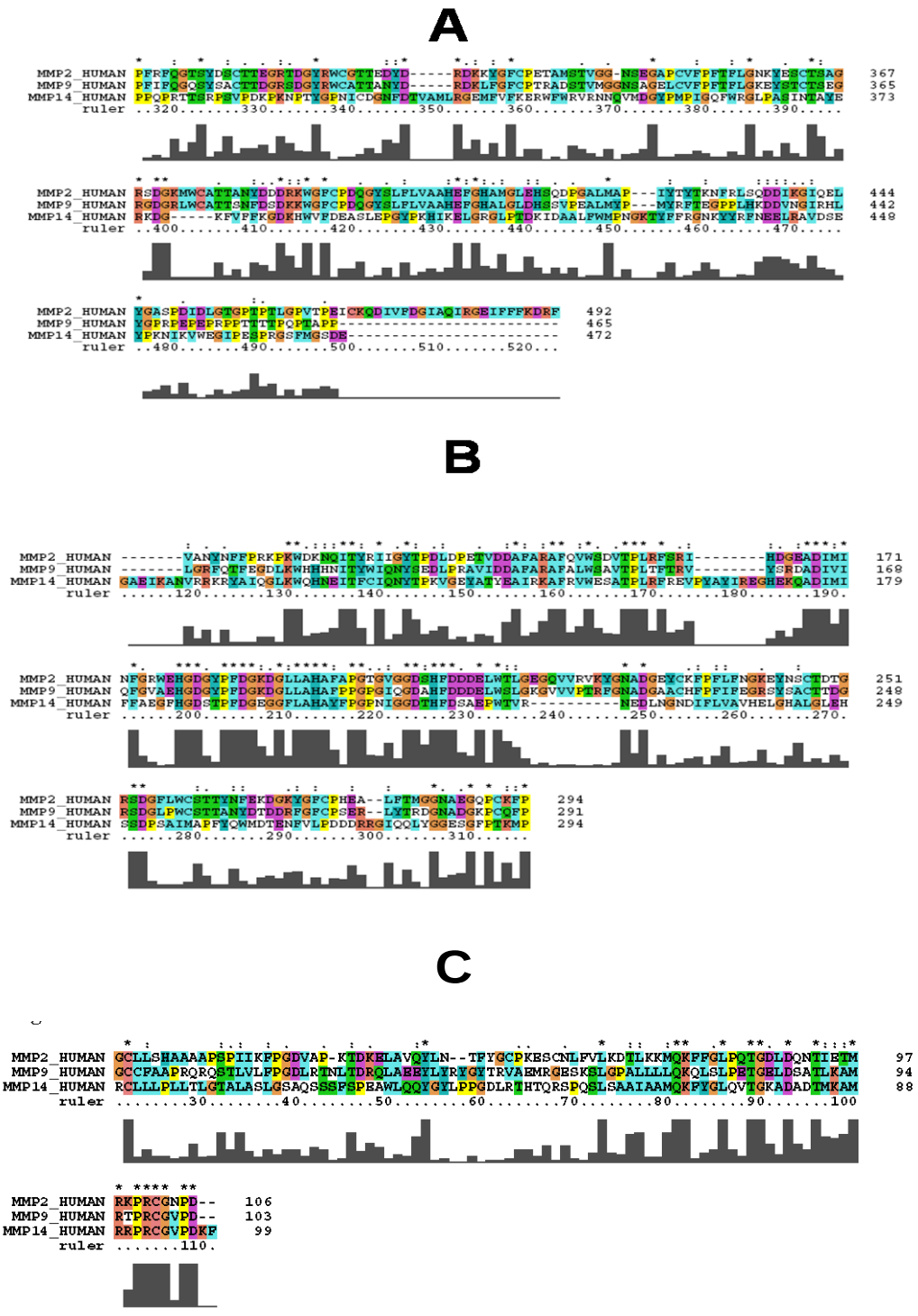

Figure 12. Sequence homology between MMP-2, MMP-9 and MT1-MMP (MMP-14).

The hemopexin domains (A), catalytic domains (B) and propeptide domains (C) were aligned using ClustalX (Thompson *et al.*, 1997). The "*" represents fully conserved amino acids. The histogram below the ruler represents the degree of similarity between the sequences, with peaks indicating regions of high similarity and valleys indicate regions of low similarity.

In summary, the three domain combinations of MT1-MMP chosen for raising antibodies in chickens, were the propeptide and catalytic (PROcat), the hemopexin-like and catalytic (PEXcat) and the hemopexin-like (PEX) domains of human MT1-MMP (Figure 13). The PEXcat expressed protein and the PEX domains of human MT1-MMP had previously been expressed by collaborators and the expressed protein obtained collaboratively. Recombinant 24 kDa MT1-MMP (PEX) peptide (comprised of 201 amino acids, Pro^{316}-Gly^{511}), and 50 kDa MT1-MMP (PEXcat) (Tyr^{112}-Glu^{523}) (Figure 13), both with six histidine residues flanking the C-terminus, were supplied by Prof. Yoshifumi Itoh (Imperial College, London), as a nickle column-purified protein supplied in 6 M urea/50 mM Tris-HCl (pH 8.6)/300 mM imidazole/0/02% (w/v) NaN_3. The 33 kDa PROcat (Ala^{21}-Ile^{316}) target protein (Figure 13) needed to be expressed. This construct was obtained collaboratively from Prof Harald Tschesche, University of Bielefeld. The resultant protein, however, would have no His tag.

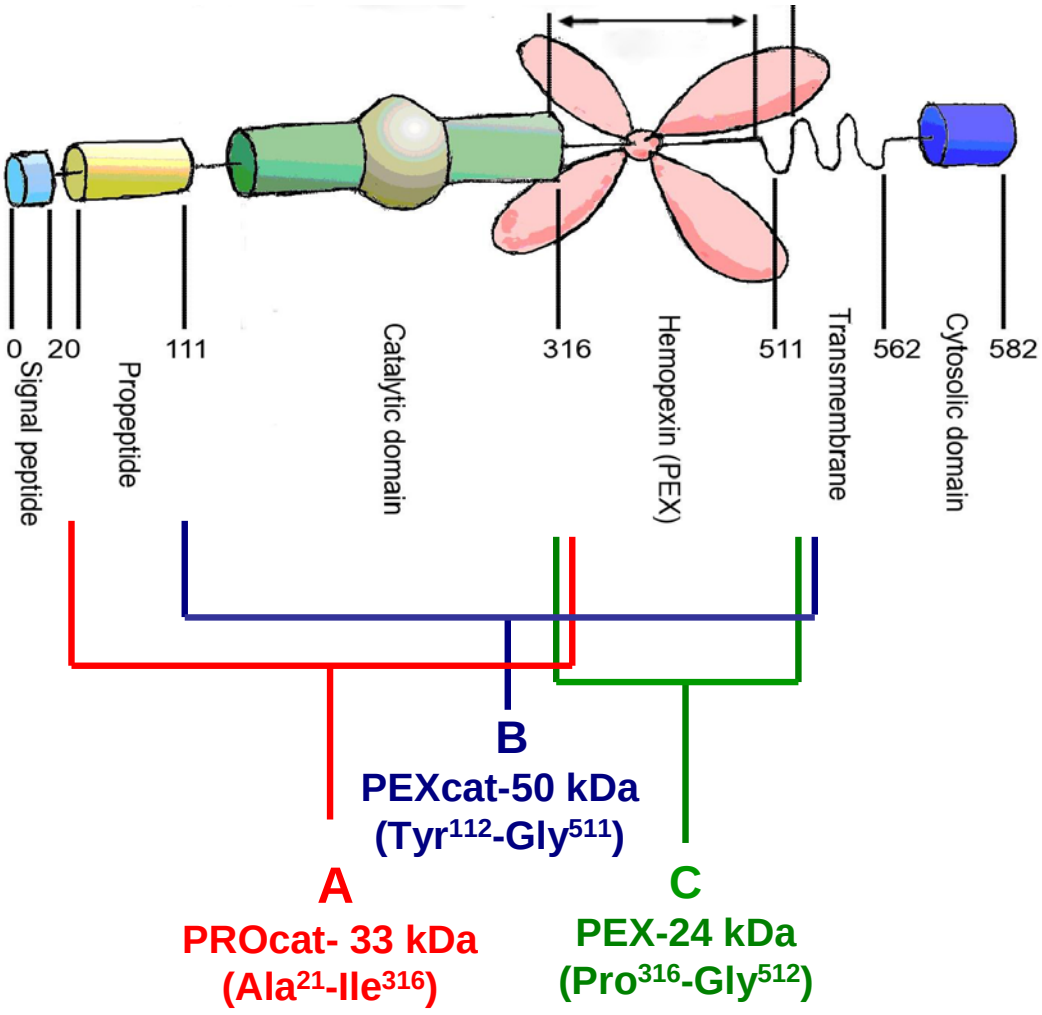

Figure 13. Domains of MT1-MMP used to raise antibodies in chickens.

The first MT1-MMP construct comprised the catalytic and propeptide domains (PROcat) (A), the second construct comprised the hemopexin-like and catalytic domains (PEXcat) and the third construct consisted of the hemopexin-like domain (PEX) (C) of MT1-MMP (Adapted from Folgueras *et al.*, 2004)

3.2 Choice of system for expression of recombinant PROcat MT1-MMP

When choosing an expression system for recombinant protein production, there are important considerations. These include the cost of using a particular system, its growth characteristics, the protein yield in such a system, the protein recovery procedure and whether the expression system processes the protein in its correct conformation (Makrides, 1996). Even upon choosing the host expression system, there are other factors to consider. These include host cell-vector interactions, plasmid stability, toxicity of the protein to the host cell and cellular responses to stress (Ramchuran *et al*., 2005).

Human MT1-MMP has been expressed in a range of host systems, including mammalian cells (Sato *et al*., 1996; Overall, 2002; Lang *et al*., 2004; Mysliwy *et al*., 2006), the Baculovirus system in insect cells (Murphy and Knauper, 1997; Valtanen *et al*., 2000; Maquoi *et al.*, 2003), and as a truncated form in *Pichia pastoris* and *Escherichia coli.* The methylotrophic yeast, *P. pastoris*, has also been used to express the propeptide, catalytic and hemopexin-like domains of human MT1-MMP (Roderfeld *et al*., 2000), whereas *E. coli* has been used to express human MT1-MMP catalytic domain alone (Lehti *et al*, 2000) or in combination with the the propeptide domain and signal sequence (Lichte *et al*., 1996) or the hinge region (Koo *et al*., 2002).

3.2.1 Eukaryotic host expression systems

Mammalian and insect cells are eukaryotic in nature and can, thus, effect post-translational modifications, such as glycosylation. Such systems, therefore, express a protein, such as MT1-MMP, in its native form. Compared to other hosts, cells of mammalian and insect origin are expensive, more difficult to handle and give lower yields. Many mammalian expression systems also contain tissue inhibitors of matrix metalloproteinases (TIMPs), which can be co-expressed with MT1-MMP and can affect the enzyme's activity (Roderfeld *et al*., 2000).

The *P. pastoris* expression vector, a eukaryotic system, also glycosylates and expresses the protein in its correct conformation. As *P. pastoris* cultures can reach very high densities, they also produce high protein yields (Valtanen *et al*., 2000). During expression in yeast, the

recombinant protein may often be secreted into the culture medium, making purification simple as the cells do not need to be lysed to effect harvesting of protein. As an expression system, *P. pastoris* is cheaper and easier to use than mammalian or insect cells, but yeast cells grow more slowly and are more difficult to maintain and manipulate compared to bacterial cells (i.e. *E. coli*).

3.2.2 Prokaryotic host expression systems

Gram negative bacteria, i.e. *E. coli*, have been extensively used to express many proteins, including MT1-MMP (Lichte *et al*., 1996; Pei and Weiss, 1996; Lehti *et al*., 2000; Koo *et al*., 2002; Kridel *et al.*, 2002; Choi *et al*., 2006), giving high yields over a short time period. Compared to other microrganisms, the *E. coli* genome is also well characterised (Baneyx, 1999). Being prokaryotic, *E. coli* do not post-translationally modify eukaryotic proteins and effect disulfide bond formation (Makrides, 1996). The protein of interest is, therefore, expressed as incorrectly folded cytoplasmic protein aggregates, called inclusion bodies (Marston, 1986). Inclusion bodies consist of a mixture of denatured multimers and dimers and are formed when the inner hydrophobic residues of the protein interact with one another causing the expressed protein to precipitate (Vincentelli *et al*., 2004).

As a protective mechanism, *E. coli* proteinases normally degrade foreign proteins. When the foreign protein precipitates and forms insoluble aggregates at a faster rate than it can be degraded, however, inclusion bodies are generated (Prouty *et al*., 1975). It has also been suggested that the formation of inclusion bodies may be initiated by the overexpression of a given protein as even *E. coli* proteins expressed in *E. coli*, using recombinant technology, are often produced as insoluble aggregates.

Although the protein in inclusion bodies is inactive and requires renaturation, the inclusion bodies themselves are easy to isolate. Their dense nature allows them to sediment quickly and easily, even at very low centrifugation speeds, precipitation constituting the first purification step (Parker *et al*., 2000; Kridel *et al*., 2002). Inclusion bodies also, not only protect the protein from proteolytic degradation by the host enzymes, but, particularly for toxic proteins, expression in an inactive form prevents toxicity hindering cell growth.

A major concern, when expressing eukaryotic proteins in *E. coli*, is that different codons are preferentially used during protein translation in prokaryotes and eukaryotes. This may affect the eukaryotic protein expression and yield, as the *E. coli* protein translation machinery may not recognise some eukaryotic codons (Finkelstein *et al*., 2003).

The major reason for choosing *E. coli* as an expression system is that the recombinant protein expression may be tightly controlled by using inducible promotors (Yansura and Henna, 1984). The *E. coli* genome contains a *lac* operon, which comprisies a series of regulatory genes involved in lactose metabolism (Figure 14). Adjacent to these genes is the *lac* promoter, which controls gene transcription (Figure 14). The *lac* operon lies within the promotor region and is bound by a *lac* repressor protein (coded for by the *lac* I gene), which blocks the expression of RNA polymerases (i.e. T7 RNA polymerases) (Yansura and Henna, 1984). When an inducer is present (i.e. lactose), it binds to the *lac* repressor protein, reduces the affinity of the *lac* repressor protein for the *lac* operator and, thus, allows the genes to be transcribed by *E. coli* RNA polymerases (Yansura and Henna, 1984).

Almost all of the *E. coli* biosynthetic capacity may, however, be channeled towards the expression of a target eukaryotic protein, if T7 RNA polymerases, not normally present in the *E. coli* genome, are inserted (Studier *et al*., 1990) (Figure 14). The inserted gene, being denoted by (λDE3) (Figure 14), results in the transcription of eukaruyotic proteins at a rate five times fater than that of *E. coli* RNA polymerases, reducing the effectiveness of *E. coli* RNA polymerases in *E. coli* protein expression (Studier *et al*., 1990), and resulting in the dominent expression of the inserted eukaryotic protein.

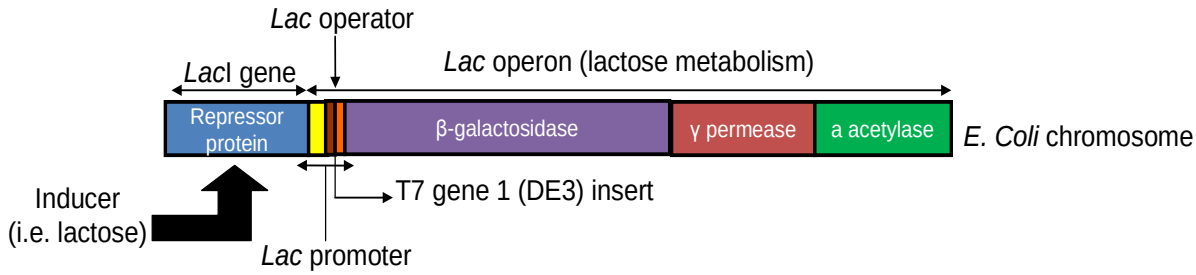

Figure 14. *E. coli* genome.

T7 gene 1 coding for bacteriophage T7 RNA polymerases (denoted by DE3) is inserted into the *lac* operon.

The *E. coli* strain BL21 has been widely used to express MT1-MMP (Lichte *et al*., 1996; Koo *et al*., 2002; Kridel *et al*., 2002) while the JM109 strain has been less frequently used (Sato *et al*., 1996). Both strains of *E. coli* may be transformed with T7 plasmid containing the gene coding for T7 RNA polymerases and be designated λ DE3 derivatives [i.e. BL21(DE3) and JM109 (DE3)] (Honrubia-Marcos *et al*., 2005). The *E. coli* JM109 strain, however, is more easily transformed with plasmid T7 polymerase pET DNA system (Sarkar *et al*., 2002). The *E. coli* JM109 (DE3) strain (containing the T7 polymerase pET system) was, therefore, used in the expression of MT1-MMP (PROcat) domains.

3.2.3 T7 polymerase pET system

As mentioned, the T7 polymerase pET system, first developed by Studier *et al*. (1990), is usually used to overwhelm *E. coli* protein expression. The inserted bacteriophage T7 RNA polymerases, which direct expression of the target gene, inserted into the pET plasmid under the control of the T7 promoter (Figure 15), is *lac*-derived or incorporates the *lac* operator or operon to control expression. In uninduced, transformed *E. coli* cells, the *lac* repressor protein is bound to the *lac* operator, preventing expression of the T7 RNA polymerases. When induced with IPTG, however, the *lac* repressor protein is blocked allowing T7 RNA polymerases to transcribe the inserted gene under the control of the lac UV5 promoter (P_{uv5}) (Figure 15) (Weng *et al*.,2006).

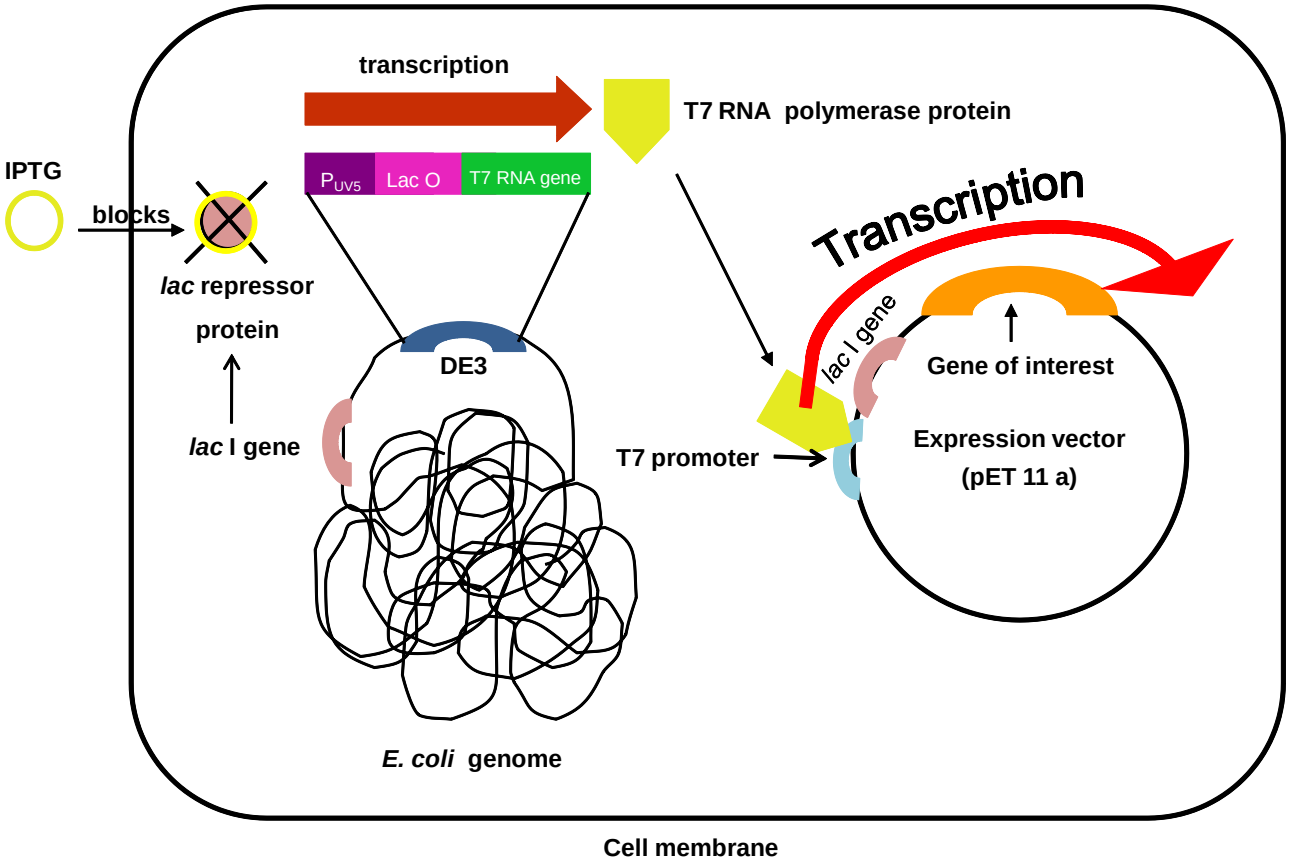

Figure 15. IPTG induced protein expression in *E. coli* using the T7 polymerase pET system.

The DE3 insert, coding for T7 RNA polymerases, is genetically inserted into the *E. coli* genome (dark blue). The *lac* I gene, coding for the *lac* repressor protein (light pink), is present in the *E. coli* genome and the pET 11a plasmid and regulates basal protein expression. When an inducer (IPTG) is present it blocks the *lac* repressor protein from binding to the *lac* operon (dark pink) and allows the T7 RNA polymerases to be transcribed. The T7 RNA polymerases (yellow) bind to the T7 promoter (light blue) and directs transcription of the gene insert (orange). Adapted from the Invitrogen pET system catalogue.

Transcription of the protein of interest using this system is tightly controlled as the T7 RNA polymerases are promoter-specific and will only transcribe DNA under the control of the T7 promoter (Studier *et al.*, 1990). The T7 promoter is also not recognised by *E. coli* RNA polymerases and, therefore, no pET 11a gene inserts may be transcribed in the absence of the promoter for T7 RNA polymerase translation. The *lac*I gene, coding for the *lac* repressor protein, is also present in both the *E. coli* genome and the pET plasmid (i.e. pET 11a plasmid) (Figure 16). This allows regulation at two levels, preventing basal protein expression, and allowing IPTG induction of expression of the inserted protein construct.

This T7 polymerase pET system has frequently been used to express MT1-MMP (Lichte *et al*., 1996; Pei and Weiss, 1996; Lehti *et al*., 2000; Roderfeld *et al*., 2000; Koo *et al*., 2002; Kridel *et al*., 2002; Finkelstein *et al*., 2003; Ou *et al*., 2006) and, for this study, the pET11a-MT1-MMP (PROcat) plasmid construct, as prepared by Lichte *et al*. (1996), was used to express MT1-MMP (PROcat) in JM109 (DE3) *E. coli*.

3.3 Analysis of pET11a-MT1-MMP plasmid DNA

In the pET11a-MT1-MMP (PROcat) plasmid construct, prepared by Lichte *et al*. (1996), the gene of interest, encoding Ala^{21}-Ile^{318} MT1-MMP (PROcat) (Figure 13), is inserted into the polylinker region of the pET11a plasmid between the *Nde*I and *Bam*HI restriction sites, downstream of the T7 promoter (Figure 16). The size of the pET11a plasmid (Novagen) and the MT1-MMP gene insert has previously been shown to be 5.7 Kb and 1 Kb, respectively, and expression of the pET11a-MT1-MMP (PROcat) plasmid DNA in *E. coli* has been shown to result in a 33 kDa protein (Lichte *et al*., 1996). This is the anticipated size of the protein to be expressed in the current study.

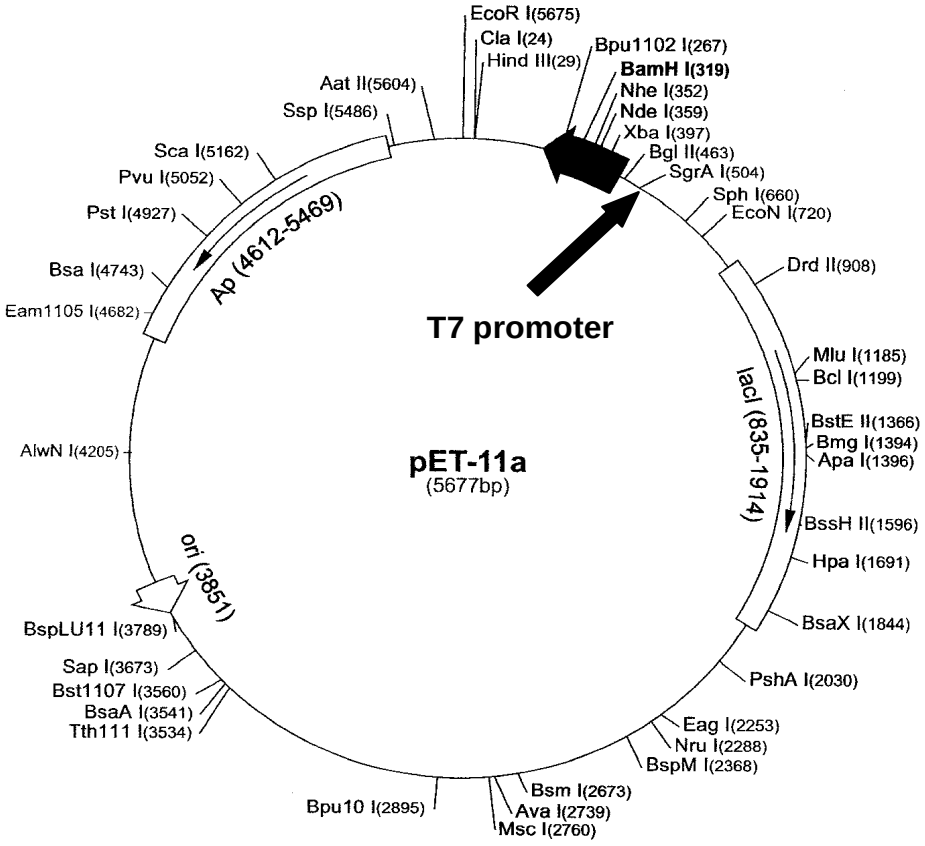

Figure 16. pET11a plasmid used for the expression of MT1-MMP.

The pET11a pasmid (5.7 Kb) allows a 1 kb MT1-MMP DNA insert to be inserted between the *Bam*HI and *Nde*I restriction site (as indicated by arrows), according to Lichte *et al*. (1996). The vector map was obtained from the Merck Biosceinces website (http://www.merckbiosciences.co.uk)

To check that the gene coding for PROcat MT1-MMP (Ala^{21}-Ile^{318}) is still present, restriction endoncleases (i.e. *Nde*I and *Bam*HI) that recognise the *Nde*I and *Bam*HI sites in the pET 11a plasmid (Figure 16) may be used, as described above. A single cleavage with one restriction enzyme may linearise the plasmid without altering its molecular weight. A double digest (with two restriction enzymes), removing the gene insert completely, however, allows the molecular weight of the gene insert and pET 11a plasmid to be determined via agarose gel electrophoresis. This was, therefore, undertaken to check the presence of the PROcat MT1-MMP (Ala^{21}-Ile^{318}) plasmid.

3.3.1 Reagents

10 X TAE buffer [0.4 M Tris-Acetate, 10 mM EDTA]. Tris-Base (48.46 g), anhydrous sodium acetate (4.1 g) and EDTA (3.72 g) were dissolved in 900 ml d.H_2O, adjusted to pH 7.8 with glacial acetic acid and made up to 1 l.

1 X TAE buffer. 10 X TAE buffer (5 ml) diluted to 50 ml with d.H_2O.

1% (m/v) agarose gel. Agarose (0.5 g) was re-suspended in 1 X TAE buffer (50 ml). The solution was boiled until the agarose dissolved. Once lukewarm to the touch, ethidium bromide (2.5 µl) was added and the solution was carefully poured into the gel mould, ensuring no air bubbles were visible, and the gel was allowed to set (30 min, RT).

Ethidium bromide stock solution (10 mg/ml). Ethidium bromide (100 mg) was dissolved in 10 ml $d.H_2O$ and stored in a dark reagent bottle at room temperature.

Buffer D. Commercial buffer (Promega) compatible with *Nde*I and to a lesser extent with *Bam*HI restriction enzymes.

Buffer E. Commercial buffer (Promega) primarily compatible with *Bam*HI restriction enzyme.

Preparation of single digest with either *Nde*I or *Bam*HI. Plasmid DNA (10 µl) combined with buffer D or E (1.5 µl), $d.H_2O$ (2.5 µl) and *Bam*HI or *Nde*I (1 µl).

Preparation of double digest with *Nde*I and *Bam*HI. Plasmid DNA (10 µl) combined with buffer D (1.5 µl), $d.H_2O$ (1 µl) and *Bam*HI (2 µl) and *Nde*I (2 µl).

3.3.2 Procedure

The pET11a-MT1-MMP plasmid, containing the cDNA fragment encoding Ala^{21}-Ile^{318} (PROcat) [pET 11a-MT1-MMP (PROcat) plasmid], provided by Prof. H. Tscheche (Bielefeld, Germany), was cut with *Bam*HI and *Nde*I restriction enzymes (RT, overnight) in the presence of 1% (m/v) bovine serum albumin (BSA). The result of a single or a double digest was analysed by electrophoresis (80 V) on a 1% (m/v) agarose gel containing ethidium bromide (0.5 µg/ml) to assess whether the double digest gave an excision product of the anticipated molecular weight of the plasmid insert (approximately 1 Kb).

3.3.3 Results

In the absence of restriction enzymes, recombinant pET 11a plasmid ran at approximately 10 kb suggesting that the Ala^{21}-Ile^{318} (PROcat) insert remained associated with the circular plasmid (Figure 17, lane 2). In a single digest with *Nde*I (Figure 17, lane 3) or *Bam*HI (Figure 17, lane 4), a 6.7 Kb linearised band was produced. Digestion with both enzymes, resulted in the pET 11a plasmid migrating to 5.7 Kb (as expected) (Figure 17, lane 5) and removal of the Ala^{21}-Ile^{318} (PROcat) insert of 1 Kb, although the insert itself was not readily visible on the gel (Figure 17, lane 5). Based on these results, the MT1-MMP (PROcat) DNA was present in the pET 11a plasmid and, thus, was suitable for use in transforming JM109 (DE3) *E. coli* cells.

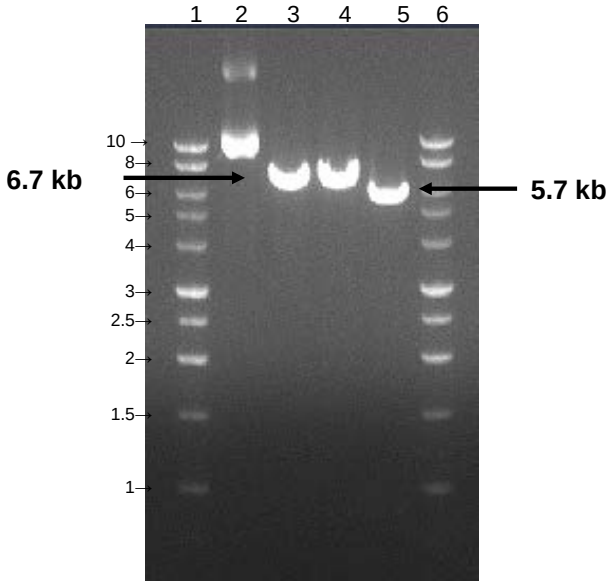

Figure 17. Analysis of the pET11a-MT1-MMP plasmid.

The plasmid construct was restricted with *Nde*I and *Bam*HI restriction endonucleases and analysed on a 1% (m/v) agarose gel and visualised with 0.5 µg/ml ethidium bromide. Lane 1 & 6, 1 Kb DNA molecular weight markers (Promega); lane 2, unrestricted plasmid (6.7 Kb); lane 3, plasmid restricted with *Bam*HI; lane 4, plasmid restricted with *Nde*I; lane 5, double digest with *Nde*I and *Bam*HI

3.4 Transformation of *E. coli* JM109 (DE3) with MT1-MMP (PROcat)-pET 11a and protein expression

E. coli cells may only be transformed with plasmid DNA if the cells are made competent (Bergmans *et al*., 1981). This may be achieved by exposing the cells to a ice cold buffer containing Ca^{2+} and Mg^{2+} ions, followed by heat shock (Sarkar *et al*., 2002). Ca^{2+} and Mg^{2+} ions disrupt the cell membrane leading to membrane permeabilisation. This facilitates the transformation process (Sarkar *et al*., 2002). Transformation is subsequently completed by exposing the Ca^{2+} and Mg^{2+} treated *E. coli* cells to low temperatures (0ºC). This initially causes the plasmid DNA to bind to the *E. coli* cell membrane and results in a partial uptake of bound DNA. Only after heat-shock (42ºC), however, does the *E. coli* cells preferentially take up most of the bound plasmid DNA (Bergmans *et al*., 1981; Sarkar *et al*., 2002). The ability of the *E. coli* cells to take up the plasmid DNA is still highly variable, however (Sarkar *et al*., 2002). Transformation [i.e. of the JM109 (DE3) *E. coli* cells with MT1-MMP (PROcat) plasmid DNA] may be checked by plating the *E. coli* cells on a medium containing an antibiotic (i.e. ampicillin), as the pET11a plasmid, chosen in this study, also contains an ampicillin resistance gene. This allows selection of transformed cells.

E. coli cells may subsequently be bulked up in a nutritious growth medium (i.e. Luria broth or Terrrific broth). Luria broth (LB) is commonly used in the maintenance of bacterial strains, plasmid propagation and purification, as well as protein expression. Terrific broth (TB), however, is more nutritious than LB and results in higher cell densities and, consequently, higher plasmid and protein yields. For protein expression, therefore, TB is preferable, but for bulking up the bacterial cells, LB broth is adequate. For this reason, LB was chosen to bulk up competent *E. coli* cells and TB was used to bulk up transformed *E. coli* cells during IPTG-induced protein expression.

During protein expression, once again, the inclusion of ampicillin in the growth medium ensures only transformed cells will grow and express the desired protein. Ampicillin, however, can easily be inactivated, if the pH in the growth medium drops, due to *E. coli* fermentation, and due to β-lactamase enzyme activity, an enzyme produced by many *E. coli* cells (Studier *et al*., 1990; Frere, 1995). Inactivation of ampicillin may allow untransformed *E. coli* cells, lacking the plasmid, to grow faster than the transformed cells, competing for

nutrients and leading to an overall reduced expressed protein yield. Ampicillin should, therefore, be added frequently to the culture medium during protein expression, consequently maintaining a selection pressure in the transformed cells, to prevent plasmid loss and ensure optimum protein expression.

3.4.1 Reagents

Agar plates. (Bacto) peptone (2 g), (Bacto) yeast extract (1 g), NaCl (2 g), glucose (0.4 g) and agar (4 g) were dissolved in 200 ml d.H_2O and autoclaved (121ºC, 15 min). After cooling the solution <50ºC, ampicillin (100 µg/ml) was added and the medium was poured into sterile Petri dishes (25 ml per Pertri dish) and allowed to cool and solidify (overnight, RT). The plates were stored at 4ºC for up to 4 weeks.

Ampicillin stock solution (1 mg/ml). Ampicillin (0.010 g) was dissolved in 10 ml d. H_2O, filter-sterilised through a 0.2 µm sterile filter and stored in a sterile tube at -20ºC.

Plasmid transforming buffer [0.05 M $MgCl_2$, 0.05 M $CaCl_2$ and 10% (v/v) glycerol]. $MgCl_2$ (0.04 g), $CaCl_2$ (0.074 g) and glycerol (1 ml) were dissolved in 10 ml d.H_2O and autoclaved (121ºC, 15 min).

Luria broth (LB). (Bacto) tryptone (10 g), (Bacto) yeast extract (5 g) and NaCl (10 g) was dissolved in 1 l d.H_2O and autoclaved (121˚C, 15 min).

Terrific broth (TB). (Bacto) tryptone (12 g), (Bacto) yeast extract (24 g) and glycerol (4 ml) were dissolved in 900 ml d.H_2O. A 10 x TB phosphate buffer was made up separately by dissolving KH_2PO_4 (2.31 g) and K_2HPO_4 (12.54 g) in 100 ml d.H_2O. Both solutions were autoclaved (121 ºC, 15 min) and, once cooled, the two solutions were combined.

IPTG stock solution (10 mg/ml). IPTG (0.1 g) was dissolved in 10 ml d.H_2O, filter-sterilised through a 0.2 µm sterile filter and stored in a sterile tube at -20ºC.

3.4.2 Procedure

To make JM109 (DE3) *E. coli* cells competent, JM109 (DE3) *E. coli* cells (Novagen), stored in glycerol (-80ºC), were streaked onto an agar plate and incubated overnight (37ºC). A single colony was selected and used to innoculate LB (10 ml) and bulked up overnight (30 ºC, with shaking). The culture was transferred, as a 1:10 dilution, to fresh sterile LB (90 ml) and incubated (37ºC with shaking) until OD_{600} reached 0.6. The cells were cooled on ice (10 min), centrifuged (6000 rpm, 5 min, 4ºC) and the pellet re-suspended in the plasmid transforming buffer. The solution was subsequently incubated on ice (15 min),

centrifuged a second time (6000 rpm, 10 min) and the pellet re-suspended in plasmid transforming buffer and stored (-80ºC) until further use.

Competent *E. coli* cells were transformed with pET11a-MT1-MMP encoding Ala^{21}-Ile^{318} (PROcat) (Lichte *et al*., 1996) by incubating cells (100 µl) with the recombinant plasmid DNA (2.5 µl, on ice, 30 min), followed by heat shock (42ºC, 90 s) and incubation on ice (2 min). The transformation of *E. coli* cells was checked by plating the cells onto an agar plate containing ampicillin (100 µg/ml, 37ºC, overnight).

A single colony of the surviving bacteria was selected and bulked up overnight in TB (100 ml) containing ampicillin (50 µg/ml, 30°C, with shaking) and transferred, as a 1:10 dilution, to TB (900 ml) containing ampicillin (50 µg/ml). The culture was incubated (37°C, with shaking) until the OD_{600} reached 0.6 and protein expression was induced with IPTG (1 mM, 4 h). Ampicillin (50 µg/ml) was added hourly during the expression period to maintain a selection pressure on the *E. coli* cells to ensure only plasmid-transformed cells survived.

3.5 Recovery and solubilisation of MT1-MMP inclusion bodies from *E. coli*

Various approaches may be used to isolate and recover MT1-MMP protein from the *E. coli* cells in a soluble form. These procedures include *E. coli* cell lysis and MT1-MMP inclusion body recovery and solubilisation.

MT1-MMP inclusion bodies are usually recovered from *E. coli* cells in a lysis buffer containing ethylenediaminetetraacetic acid (EDTA), lysozyme and Triton X-100 (Koo *et al*., 2002). EDTA is a metal chelator that binds to divalent cations, such as iron, magnesium and calcium, on the cell membrane and indirectly affects cellular integrity and function (Kim *et al*., 2006). The lysozyme breaks down the inner peptidoglycan layer in the bacterial cell wall via cleavage of the N-acetylmuramic acid and N-acetyl glucosamine glycosidic bond. Triton X-100, a non-ionic detergent that disrupts the outer membrane of *E. coli* cells releasing cellular components, may also be used. To ensure complete lysis, a homogeniser, such as an Ultra Turrax, has also been used to mechanically disrupt the *E. coli* cell wall (Lichte *et al*., 1996). Treatment with an Ultra Turrax, however, is harsh and there is a fine line between

breaking cell walls and denaturing protein precipitates. Bacterial cells are also small and they may not always be broken up by the Ultra Turrax.

An alternative lysing method may be to subject the bacterial cells, suspended in the lysis buffer, to freezing and thawing, followed by sonication (Koo *et al*., 2002; Weng *et al*., 2006). The freezing and subsequent thawing causes ice crystal damage and the *E. coli* cells to rupture, releasing the inclusion bodies. The sonication also assists in cell lysis, but is primarily used to fragment DNA. Sonication alone has been used in the recovery of MT1-MMP inclusion bodies from *E. coli* (Knauper *et al*., 1996; Koo *et al*., 2002) and freezing and thawing followed by sonication has been used in the recovery of other MMP inclusion bodies, such as MMP-7 (Ou *et al*., 2006), MMP-12 (Parker *et al*., 2000) and MMP-13 (Pathak *et al*., 1998) from *E. coli*. It is important to note that sonication generates heat. This may cause protein degradation and should, therefore, be applied, for only brief periods, preferably on ice.

When the inclusion bodies are extracted, they may be contaminated with other *E. coli* proteins. These can be removed by re-suspending the inclusion bodies in a solution containing a mild detergent (Triton X-100 or Tween 20). This aids in solubilisation of the *E. coli* proteins but not the inclusion bodies. Being dense, the inclusion bodies remain in the pellet with a high degree of purity. Often, however, *E. coli* proteins also sediment with the inclusion bodies, and in order to remove them, additional purification steps need to be employed. These require the inclusion body proteins to be solublilised.

The process of solubilisation involves “disrupting non-covalent hydrogen bonds, ionic or hydrophobic interactions and unfolding the polypeptides” (Marston, 1986). Chaotrophic agents such as 5-8 M guanidium-HCl and 6-8 M urea are commonly used to solubilise protein in inclusion bodies (Lin *et al*., 2004). Urea has been most widely used to solubilise MT1-MMP (Lichte *et al*., 1996; Mucha *et al*., 1998; Koo *et al*., 2002; Kridel *et al*., 2002) and other MMPs, including MMP-3 (Suzuki *et al*., 1998), MMP-9 (Kroger and Tschesche, 1997) and MMP-13 (Pathak *et al*., 1998). The solubilisation is assisted by the presence of a reducing reagent (i.e. β-mercaptoethanol), which cleaves intermolecular disulfide bonds in the inclusion bodies. Guanidinium-HCl has been considered to be a stronger denaturant

compared to urea, however, this may potentially effect restoration of the enzyme into an active form, as shown previously for organophosphorus acid anhydrolase (Ong *et al*., 2004). For this reason, urea is the preferable denaturant to use. For solubilising MT1-MMP, 6 M (Lichte *et al*., 1996; Koo *et al*., 2002) and 8 M (Mucha *et al*., 1998; Kridel *et al*., 2002) urea has been used, however, higher concentrations of urea are preferable (i.e. 8 M), as inclusion bodies solubilise very quickly under these conditions (Arakawa *et al*., 1970). This procedure was, consequently, used in this study. Once solubilised, the protein may be subjected to purification and renaturation.

3.5.1 Reagents

Solution A: Monomer solution [30% (m/v) acrylamide, 2.7% (m/v) bis-acrylamide]. (Section 2.3)

Solution B: 4 x Running gel buffer [1.5 M Tris-HCl, pH 8.8]. (Section 2.3)

Solution C: 4X Stacking buffer [500 mM Tris-HCl, pH 6.8]. (Section 2.3)

Solution D: 10% (m/v) SDS. (Section 2.3)

Solution E: 10% (m/v) Ammonium persulfate. (Section 2.3)

Solution F: Tank buffer [250 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (m/v) SDS]. (Section 2.3)

Reducing treatment buffer [125 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v 2-mercaptoethanol]. (Section 2.3)

Bromophenol blue loading dye (0.1%). (Section 2.3)

Coomassie Blue G250 [0.02% (m/v) CBB-G250, 2% (w/v) phosphoric acid, 5% (m/v) aluminium sulfate, 10% (v/v) ethanol]. (Section 2.4)

Lysis buffer [50 mM Tris-HCl buffer, pH 8.0, 2 mM EDTA]. Tris base (0.6 g) and EDTA (0.07 g) were dissolved in 90 ml d.H_2O, adjusted to pH 8.0 with HCl and made up to 100 ml with d.H_2O.

Lysozyme stock solution (1 mg/ml). Lysozyme (10 mg) was dissolved in 10 ml d.H_2O and stored in 1 ml aliquots (-20^{o}C).

Inclusion body washing solution [1% (v/v) Tween 20]. Tween 20 (2 ml) was dissolved in 200 ml d.H_2O.

Solubilisation buffer [50 mM Tris-HCl buffer, pH 8.5, 30 mM 2-mercaptoethanol, 8 M urea]. Tris Base (0.061 g), 2-mercaptoethanol (26 µl) and urea (4.8 g) were dissolved in 5 ml d.H_2O, adjusted to pH 8.5 with HCl and made up to 10 ml with d.H_2O.

3.5.2 Procedure

E. coli cells were harvested by centrifugation (5000 x g, 10 min, 4°C), re-suspended in a 1/10 culture volume of lysis buffer (100 ml) and frozen at -20ºC overnight. After thawing, lysozyme stock solution (1 mg/ml) was added to a final concentration of 100 µg/ml (10 µl) and 0.1% (v/v) Triton-X 100 (100 µl) were added to the lysis buffer and incubated (30°C, 15 min). The lysate was sonicated (3 x 30 s, on ice) until all viscosity was lost and the inclusion bodies were recovered by centrifugation (12 000 x g, 15 min, 4°C), washed twice with d.H_2O containing 1% (v/v) Tween 20 (100 ml) and twice with d.H_2O (100 ml). After each wash, the pellet was recovered by centrifugation (10 000 x g, 10 min, 4°C) and solubilised in 50 mM Tris-HCl buffer (10 ml, containing 8 M urea and 150 mM 2-mercaptoethanol, pH 8.5) (2 h, stirring, 4ºC). Insoluble material was removed by centrifugation (12 000 x g, 25 min, 4°C) and the urea-solubilised protein (10 ml) was stored at -80ºC until further use. A sample of the supernatant and pellet after each centrifugation was kept for analysis. Electrophoretic analysis was subsequently performed using SDS-PAGE gel analysis on a 12.5% reducing SDS-PAGE gel, as described in Section 2.3, and stained, as described in Section 2.4.

3.5.3 Results:

The MT1-MMP (PROcat) recombinant protein appeared to be expressed in the form of inclusion bodies (5 g/l) to a level of 28% of the total wet weight of the JM109 (DE3) *E. coli* mass (18 g/l). The urea-solubilised MT1-MMP (PROcat) in JM109 (DE3) recovered from inclusion bodies, upon separation on a 12.5% reducing SDS-PAGE gel, revealed a 33 kDa protein (Figure 18, lanes 3, 5, 7, 9, 11 and 13), the molecular weight anticipated for the JM109 (DE3) expressed MT1-MMP protein (Lichte *et al.*, 1996). A protein of similar molecular weight (i.e. 34 kDa) (possibly of *E. coli* origin) was, unfortunately, also observed in the untransformed JM109 (DE3) control, but at much lower concentrations (Figure 18, lane 2). After cells were lysed, however, a large proportion of the soluble *E. coli* proteins

were removed by centrifugation of the cell lysate (Figure 18, lane 4). Proteins were further purified when the inclusion bodies were washed in the presence of 1% (v/v) Tween 20 and/or d.H_2O (Figure 18, lane 6). Due to their insoluble and dense nature, the majority of the inclusion body material remained in the cell pellet (Figure 18, lanes 5, 7, 9 and 11) and was solubilised in the presence of 8 M urea and 30 mM 2-mercaptoethanol (Figure 18, lane 13). There was, however, a small amount of protein loss observed during the isolation and solubilisation steps (Figure 18, lanes, 6, 10, 12 and 13). This may have affected the overall protein yield. The presence of a similar protein band in the *E. coli* control (Figure 18, lane 2) raised a question regarding the identity of the expressed protein and, consequently, the solubilised protein was prepared for N-terminal sequencing (Michele Brillard, University of Torrs, France).

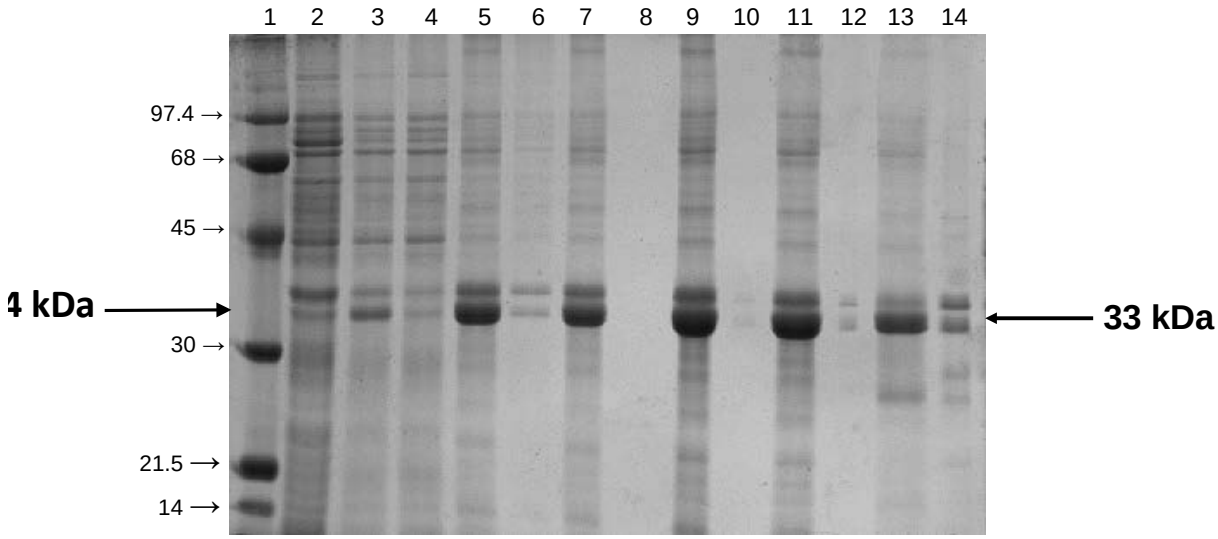

Figure 18. Analysis of MT1-MMP recovered after expression in *E. coli* JM109 (DE3).

The protein expression was induced using 1 mM IPTG and recovered from inclusion bodies using 8 M urea and 150 mM 2-mercaptoethanol. The various steps during cell lysis, MT1-MMP inclusion body recovery and solubilisation were analysed on a 12.5% SDS-PAGE gel under reducing conditions, loading of each sample being standardised to 50 µg/ml. Lane 1, molecular weight marker; lane 2, untransformed JM109 (DE3) (control); lane 3, *E. coli* cell lysate; lane 4, supernatant following centrifugation of cell lysate; lane 5 and 7, pellet resuspended in 1% Tween 20 in d.H_2O; lane 6 and 8, supernatant following washing of pellet; lane 9 and 11, pellet resuspended in d.H_2O; lane 10 and 12, supernatant following washing of pellet in d.H_2O; lane 13, soluble MT1-MMP protein; lane 14, insoluble protein in PBS (pH 7.4)

3.6. Preparation of expressed protein for N-terminal protein sequencing

N-terminal sequencing (also referred to as Edman sequencing) was first introduced by Pehr Edman in 1967 and used to identify proteins by determining their amino acid sequence. The proteins can be sequenced directly by separating and excising a protein band from an SDS-PAGE gel or from proteins blotted directly onto polyvinylidene difluoride (PVDF) membrane. Due to high protein binding capacity, chemical stability and physical strength, it is preferable to sequence western blotted proteins blotted onto a PVDF membrane rather than a nitrocellulose membrane (Kurien and Scofield, 2006). Western blot transfer onto PVDF is also quick, simple and efficient (Kurien and Scofield, 2006). For these reasons, PVDF was used in this study to prepare the solubilised protein sample for N-terminal sequencing.

3.6.1 Reagents

Solution A: Monomer solution [30% (m/v) acrylamide, 2.7% (m/v) bis-acrylamide]. (Section 2.3)

Solution B: 4 x Running gel buffer [1.5 M Tris-HCl, pH 8.8]. (Section 2.3)

Solution C: 4X Stacking buffer [500 mM Tris-HCl, pH 6.8]. (Section n 2.3)

Solution D: 10% (m/v) SDS. (Section 2.3)

Solution E: 10% (m/v) Ammonium persulfate. (Section 2.3)

Solution F: Tank buffer [250 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (m/v) SDS]. (Section 2.3)

Reducing treatment buffer [125 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v 2-mercaptoethanol]. (Section 2.3)

Bromophenol blue loading dye (0.1%). (Section 2.3)

Blotting buffer. Section 2.8

Staining solution [0.025% (m/v) Coomassie Blue R250, 40% (v/v) methanol]. Coomassie blue R250 (0.025 g) was dissolved in methanol (40 ml) and made up to 100 ml with $d.H_2O$.

Destaining solution [50% (v/v) methanol]. Methanol (50 ml) was made up to 100 ml with $d.H_2O$.

3.6.2 Procedure

The solubilised protein sample was separated by 12.5% SDS-PAGE, under reducing conditions, in a buffer containing SDS, as described in Section 2.3. Sufficient protein (50 µg/ml) was loaded on the gel to ensure that a minimum of 1-5 µg/ml protein per band was blotted onto the PVDF membrane (Amersham) and made available for sequencing. The hydrophobic PVDF membrane was pretreated with 100% methanol before equilibrating in the blotting buffer. The protein sample was subsequently electroblotted (170 mA, 90 min, RT). The protein bands were identified, on the basis of their molecular weight, on the PVDF membrane after staining with 0.025% (m/v) Coomassie R250 in 40% (v/v) methanol (no acetic acid) and destaining with 50% (v/v) methanol. The immobilised protein was excised from the membrane and submitted for sequencing on an Applied Biosystems Procise 491 protein sequencer (Courtaboeuf, France), attached to a Model 140 C Micro-Gradient System and a 610 A Data Analysis System.

3.6.3 Results:

A light stain with 0.025% (m/v) Coomassie R250 stain proved suitable for detection of the 33 kDa protein band (Figure 19, lane 2). This was excised from the PVDF membrane and used in Edman sequencing. Nine sequencing cycles were performed on a sequencer, and a sequence, ^{21}ALASLGSAQ^{29} 100% homologous to the propeptide domain of human MT1-MMP was determined proving that the 33 kDa band, presumptively identified as MT1-MMP, is infact MT1-MMP and, hence, suitable for immunisation to raise anti-MT1-MMP antibodies

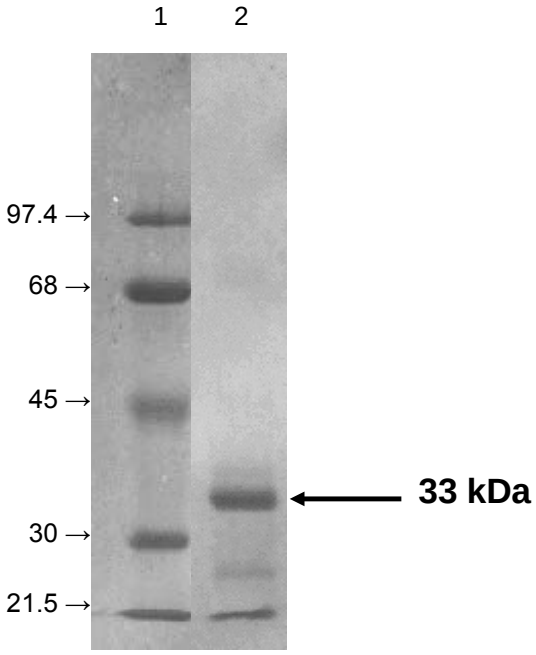

Figure 19. Transfer of MT1-MMP protein onto PVDF for protein sequencing.

The protein was reduced, incubated at 37ºC for 15 min, separated on a 12.5% SDS-PAGE gel (35 mA, 4ºC) and electroblotted onto PVDF (170 mA, 90 min, RT). The transferred protein was visualised with 0.025% Coomassie R250 and subjected to Edman sequencing. Lane 1, molecular weight marker; lane 2, solubilised MT1-MMP in 8 M urea and 30 mM 2-mercaptoethanol.

3.7 Discussion

Recombinant protein expression is a particularly valuable and relatively inexpensive way of obtaining abundant amounts of protein over a short time period, especially when the protein of interest is present in low levels or is difficult to isolate in abundance from its natural source, as is the case with MT1-MMP. Although the catalytic, hemopexin-like and propeptide domains of MT1-MMP are considered to be highly conserved across MMPs (Folgueras *et al*., 2004), PROcat, PEXcat and PEX combinations possibly may be the best target domains against which to raise specific antibodies against MT1-MMP if chickens are used, as these mammalian domains had a very low sequence homology with chicken MT1-MMP. The PROcat domains, although possibly the most immunogenic in chickens, as determined by ClustalX sequencing, may, however, cross react with MMP-2 and MMP-9. So this needed to be assessed after antibodies were raised. Fortunately, only the MT1-MMP (PROcat) domain needed to be expressed as the PEXcat and PEX protein domains could be accessed collaboratively.

Though *E. coli* JM109 (DE3) strain had not been frequently used to express MT1-MMP (Sato *et al*., 1996), we showed here that this strain was successful in expressing MT1-MMP (PROcat) in abundance, as the inclusion body material was observed to be 28% of the total *E. coli* wet weight. This is greater than the 20% level of inclusion bodies previously recovered from BL21 (DE3) cells (Lichte *et al*., 1996). This suggests that a higher inclusion body recovery and yield may have been achieved when MT1-MMP (PROcat) is expressed in JM109 (DE3) *E. coli* compared to the BL21 (DE3) expression system.

The pET11a plasmid, containing the MT1-MMP (PROcat) DNA insert (Lichte *et al*., 1996), analysed using *Bam*HI and *Nde*I restriction enzymes, though not visible on the gel, was determined to be 1 kb due to the 1 kb decrease in weight in the double cleavage with *Bam*HI and *Nde*I restriction enzymes. Amplifying the DNA using the polymerase chain reaction (PCR) could have improved the yield and, hence, visibility of the band, but, since the expression system produced a band at 33 kDa, a molecular weight previously seen for MT1-MMP (Lichte *et al*., 1996), and sequencing confirmed the identitity of the expressed protein as human MT1-MMP, PCR confirmation of the plasmid insert identity did not seem necessary. Though the enzyme was expressed in the form of insoluble inclusion bodies and

required renaturation (Tsumoto *et al*., 2003), previous reports have shown that soluble, active protein is easily recoverable from inclusion bodies (Lichte *et al*., 1996; Koo *et al*., 2002).

Strategies to reduce the formation of inclusion bodies, e.g. the use of a lower temperature (e.g. 23-30ºC) to reduce degradation induced by *E. coli* heat-shock proteins during IPTG-induced protein expression (Schein and Noteborn, 1988; Vasina and Baneyx, 1996), was not attempted in this study as many promoters do not function optimally at lower temperatures. To improve protein yields it is, therefore, perferable to use a cold-shock, inducible promoter, such as *csp*A (Vasina and Baneyx, 1996). But the T7 promoter has the advantage of being, not only durable, but also allows strict control of protein expression, minimising any basal protein expression in uninduced cells. This was evident in this study (Figure 18).

Another approach would have been to express the protein of interest as a fusion protein with a highly soluble protein, such as maltose binding protein (Pryor and Leiting, 1997). The presence of the carrier protein, however, means that additional purification steps would have been required. These would include digestion with site-specific proteases, to cleave the carrier protein. This approach was, thus, not considered in this study.

MT1-MMP inclusion bodies seemed reasonably well recovered from *E. coli* cells in a lysis buffer containing lysozyme, EDTA and Triton X-100, using freezing and thawing and sonication. When cells are lysed, however, DNA may contaminate the lysate and may interfere with the inclusion body recovery as well as protein renaturation (Batas *et al*., 1999). As sonication appears to mechanically shear the DNA into smaller fragments (Sambrook and Russell, 2006), it may facilitate the incusion body recovery procedure as these smaller fragments are more easily removed following centrifugation and, thus, sonication appears to be more critical for DNA shearing than for cell lysis. This certainly seemed to be the case in this study. Inclusion bodies have previously been recovered from *E. coli* cells using a lysis buffer, similar to the one used in this study (i.e. buffer containing EDTA, Triton-X 100 and lysozyme), however, these reporters used an Ultra Turrax, instead of freezing and thawing, to mechanically shear the cells (Lichte *et al*.,1996). As cell lysis using an Ultra Turrax may

destroy intracellular proteins, this treatment may, therefore, have also resulted in the reduced protein yields (20%) previously obtained (Lichte *et al*., 1996), compared to the 28% yield achieved in this study. This, therefore, suggests that the inclusion body recovery procedure, employed in this study, may have improved the recovery of inclusion bodies.

Apart from DNA, there are also many other impurites present when the cell are lysed. These include *E. coli* proteins and they also need to be removed. This may be achieved using detergents (Batas *et al*., 1999). In this study majority of the soluble *E. coli* proteins seemed to be removed with Tween 20 and d.H_2O and the protein in inclusion bodies, being insoluble and dense, seemed to remain in the cell pellet, as indicated by SDS-PAGE gel analysis.

SDS-PAGE analysis also showed that most of the protein in inclusion bodies seemed to be solubilised with urea and β-mercaptoethanol. Urea is not always the ideal denaturant to use as, in aqueous solutions, the urea breaks down resulting in ammonium and cyanate ions accumulating in the solution (Tsumoto *et al*., 2003). Cyanate, in particular, reacts with the amino terminus of the protein producing carbamyl derivatives, potentially altering protein/peptide conformation and charge, rendering the protein inactive (Lin *et al*., 2004). Guanidinium-HCl has also been used (Kim and Lee, 2000; Golubkov *et al*., 2007) and does not usually result in carbamylation of proteins. In this study, however, urea was chosen as it seemed the most widely used in solubilising MT1-MMP inclusion bodies and does not seem to hinder enzyme renaturation (Lichte *et al*., 1996; Koo *et al*., 2002; Kridel *et al*., 2002).

Carbamylation of proteins can be avoided if the solubilised protein is stored at temperatures below 30ºC and in a low pH buffer i.e. citrate or phosphate buffer below pH 6.0 (Lin *et al*., 2004). In this study, the solubilised protein was stored at 4ºC and in a Tris buffer at pH 8.0. Although Tris buffers have been considered to be poor at controlling carbamylation of proteins at high pH's (Lin *et al*., 2004), MT1-MMP is more stable at neutral to basic pH's (Cotrim *et al*., 2002; Chintala *et al*., 2005) and so it was considered better to solubilise the protein from inclusion bodies in a high pH buffer. In addition, previous reports have demonstrated successful solubilisation and renaturation of MT1-MMP protein from inclusion bodies under these conditions (Lichte *et al*., 1996; Koo *et al*., 2002; Kridel *et al*.,

2002). To attempt to minimise the effects of carbmaylation on the protein, the solubilisation was, therefore, performed at 4°C.

The cyanate ions produced during carbamylation have been reported to bind to the amino terminus of the protein. This can hinder N-terminal sequencing (Lin *et al*., 2004). In this study, a protein band, of similar molecular weight to the expressed protein (i.e. 34 kDa), was observed in the untransformed *E. coli* cells and so the identity of the expressed protein needed to be confirmed via N-terminal sequencing. The urea-solubilised sample fortunately appeared to have had no dentrimental effect on the sequencing in this study, however.

In conclusion, MT1-MMP was successfully expressed in JM109 (DE3) *E coli* using the T7 polymerase-pET system and inclusion body recovery (using sonication and freezing and thawing) and urea-solubilisation (at high pH) seems to have been more successful than previous isolation procedures, yielding a 28% here, instead of a 20% recovery of inclusion bodies from *E. coli* cells previously (Lichte *et al*., 1996). For renaturation, the MT1-MMP protein needed to be re-purified as there were a few remaining minor *E. coli* proteins still present. These contaminating *E. coli* proteins were removed using chromatography and this process and renaturation are discussed in Chapter 4.

Chapter 4

Purification and renaturation of MT1-MMP (PROcat)

4.1 Principle approaches in MT1-MMP purification and renaturation

Although proteins in inclusion bodies are partially purified during the isolation procedure (i.e. washing with detergents and solubilisation in urea), as outlined in Chapter 3 (Section 3.5), it is necessary to remove the remaining bacterial proteins and DNA, as these may hinder renaturation of the protein by stabilising it in an unfolded form (Huang *et al*., 1996; Tsumoto *et al*., 2003), and for the production of specific antibodies, it is essential that the antigen (i.e. protein) is in its purest form (Harlow and Lane, 1988). MT1-MMP inclusion body proteins have previously been purified using gel filtration (Lichte *et al*., 1996), ion exchange chromatography (IEC) (Mucha *et al*., 1998; Kridel *et al*., 2002) or affinity chromatography on a nickel nitrilotriacetic acid (Ni-NTA) column (Koo *et al*., 2002). In addition, hydroxamic acid affinity chromatography has been used to purifiy native MT1-MMP (Roderfeld *et al*., 2000).

Gel filtration, also referred to as size exclusion chromatography, is usually one of the first methods used to purify MT1-MMP inclusion bodies (Lichte *et al*., 1996). This technique separates proteins according to their size and has been shown to achieve a purity from approximately 80% (Valtanen *et al*., 2000) to 95% (Lichte *et al*., 1996). Gel filtration is a relatively simple procedure. Since the purity achieved depends on the distribution of protein size, however, similar sized proteins are not easily separated and often gel filtration needs to be accompanied by other chromatographic procedures, such as affinity chromatography (Kroger and Tschesche, 1997). Lichte *et al*. (1996) used gel filtration to purifiy MT1-MMP (PROcat) inclusion bodies. This procedure was not considered in this study, however, due to the similar size of contaminating *E. coli* proteins to the expressed MT1-MMP protein (Chapter 3, Section 3.5.3).

Affinity chromatography, on the other hand, uses the relationship between a protein and its complementary ligand to specifically select out a protein of interest from a crude extract (Dennison, 2003), and, thus, a higher purity can generally be achieved using this procedure

compared to gel filtration chromatography. Two types of affinity chromatography have been previously used to successfully purify MT1-MMP. The first, hydroxamic acid affinity chromatography, has been used to purify correctly folded MT1-MMP and has been shown to result in a purity >90% (Roderfeld *et al*., 2000). In this procedure, Pro-Leu-Gly-NHOH moiety is coupled to agarose and mimics a substrate sequence recognised by MT1-MMP (Moore and Spilburg, 1986). The active site cleft of the active enzyme binds to the substrate and the hydroxamic acid (NHOH) specifically binds to the zinc ion in the active site cleft (Moore and Spilburg, 1986). This procedure requires the protein to be in its native conformation as even partial denaturation can affect the binding of the hydroxamate substrate to the active site cleft (Moore and Spilburg, 1986; Roderfeld *et al.,* 2000). The expressed MT1-MMP in this study was urea-denatured, which means the active site cleft would not be correctly folded. As the PROcat domains of MT1-MMP was expressed, even if the protein had been correctly folded, the active site cleft may have been blocked by the propeptide domain, hence, may not easily bind. Hydroxamic acid affinity chromatography, therefore, could not be employed. This procedure could, however, possibly be used after renaturation of the protein, if necessary. The second procedure, Ni-NTA affinity chromatography, has been used to purify insoluble and denatured MT1-MMP catalytic domain with a purity of 90% (Koo *et al*., 2002). This procedure, however, relies on the binding of the protein, via a tag of 6 histidines, to the metal ions on the resin to select out the protein. Although it can be used to purify denatured proteins (Koo *et al*., 2002), unlike hydroxamic affinity acid chromatography, this procedure, does require that the protein of interest is His-tagged. The pET11a-MT1-MMP plasmid construct used in this study lacked a His-tag (Lichte *et al*., 1996) and so, this purification procedure also could not be used.

Another way of selecting out the desired protein is to couple anti-MT1-MMP antibodies to a resin (e.g. Sepharose 4B). The protein, to which the antibodies selectively bind, may subsequently be eluted by changing the buffer pH or ionic strength. This procedure has not frequently been used to purify MT1-MMP and often requires the protein to be in its native conformation as the antibodies may not recognise denatured protein. For this study, anti-MT1-MMP antibodies were not readily available and so it was, therefore, decided that, for the purposes of this study, affinity chromatography was not the most suitable procedure to

employ to initially purify the unfolded protein. Ion exchange chromatography, however, was an option.

Anion exchange chromatography (AEC) has been used to purify the denatured catalytic domain of MT1-MMP (Kridel *et al*., 2002), whereas cation exchange chromatography (CEC) has been used to purify soluble and active recombinant MT1-MMP comprising the hemopexin-like, catalytic and propeptide domains (Valtanen *et al*., 2000). Both procedures have resulted in a purity >90%. It was, therefore, decided to test both procedures in this study for the reasons described in Section 4.1.1. Once purified on the ion exchange column, the MT1-MMP (PROcat) protein fractions, showing the highest purity, could be subjected to renaturation.

The renaturation of an enzyme, such as MT1-MMP depends largely on its structure, stability, complexity, purity and concentration. Other conditions, which influence protein renaturation include pH, ionic strength and temperature, all of which need to be optimised (Kim and Lee, 2000; Roy *et al*., 2004; Ou *et al*., 2006).

As mentioned previously, the structure of MT1-MMP's catalytic domain is rich in β-pleated sheets (Fernandez-Catalan *et al*., 1998) and since this domain is responsible for the enzyme's activity, this suggests these sheets play a role in maintaining the enzyme in an active form (Section 1.2.2). It was, therefore, considered critical to use a renaturation technique that allows the catalytic domain to twist into its β-pleated sheet conformation. MT1-MMP also contains a zinc and calcium ion in its active site cleft, both of which function to stabilise the structure of the β-pleated sheets (Lichte *et al*., 1996; Kroger and Tscheche, 1997). Exposing the unfolded protein to these stabilising factors was considered to facilitate renaturation. Usually the presence of disulfide bonds makes protein renaturation more difficult and is considered the rate limiting step in any renaturation procedure (Middleberg, 2002). This, however, is not a concern in this study as the catalytic domain of MT1-MMP lacks disulfide bonds (Fernandez-Catalan *et al*., 1998).

The concentration of the unrenatured enzyme used in the renaturation procedure determines the yield of active enzyme obtained. Lower protein concentrations (<100 μg/ml) allow

intramolecular interactions to form more frequently than intermolecular interactions and subsequently prevent the formation of insoluble protein aggregates, thereby allowing the protein to renature into an active enzyme (Marston, 1986; Kroger and Tschesche, 1997). Hence, it is preferable to use lower protein concentrations during protein renaturation.

Several important features of isolation techniques for MT1-MMP are now known. The pH of the renaturation buffer has been shown to influence the activity and stability of an enzyme and for MT1-MMP, should be maintained at pH 7.5 (Lichte *et al*., 1996). To prevent autodegradation of the protein it is also best to conduct the renaturation procedure at low temperatures (Ou *et al*., 2006), such as 4°C for MT1-MMP (Lichte *et al*., 1996). At lower temperatures, carbamylation of proteins in the presence of urea is also reduced, as previously discussed (Chapter 3, Section 3.7). With these criteria met, the renaturation of MT1-MMP into the active enzyme has been reported to be achievable.

Techniques frequently used to renature proteins involve solubilising using a strong denaturant (urea, guanidinium-HCl) or detergent (SDS), followed by dialysis against a buffer with decreasing concentrations of a denaturant/detergent. Detergents are not commonly used to solubilise and renature inclusion bodies and have never been used in the case of MT1-MMP. Urea was previously considered preferable, as it has been used more frequently than guanidinium-HCl to renature MT1-MMP, and the PROcat domain of MT1-MMP, in particular, has been shown to be successfully renatured into its native conformation in the presence of urea (Lichte *et al*., 1996). Urea has also been successful in renaturing the catalytic domain alone (Kridel *et al*., 2002) and in combination with the hinge region (Koo *et al*., 2002).

In the solubilised state, the protein adopts a random coil conformation and as a result it becomes flexible and highly disordered (Creighton, 1990; Tsumoto *et al*., 2003). As the denaturant (i.e. urea) is gradually removed, the protein becomes more compact, loses flexibility, thereby avoiding aggregation and the protein renatures into its correctly folded form. MT1-MMP, comprising the catalytic domain and hinge region, has been successfully renatured using a stepwise gradient dialysis in the presence of $ZnCl_2$ and $CaCl_2$ (Koo *et al*., 2002). The urea-solubilised PROcat domain of MT1-MMP has been renatured using a one

step dialysis in the presence of $ZnCl_2$ to quickly remove the denaturant (Lichte *et al*., 1996). Lichte *et al*. (1996) demonstrated that, once renatured with this one-step dialysis procedure, the catalytic domain of MT1-MMP achieves optimum conformation and can remain in its native conformation, maintaining activity for up to several days at room temperature. Rapid removal of the dentaurant, however, may result in protein aggregation and precipitation (Tsumoto *et al*., 2003). For this reason, stepwise gradient dialysis was chosen for the current study. First, however, MT1-MMP (PROcat) inclusion bodies needed to be purified and this was achieved using AEC and CEC.

4.1.1 Purification of MT1-MMP (PROcat) using anion and cation exchange chromatography

Ion exchange chromatography (IEC) involves the separation of proteins according to their surface charges. For most proteins, their net charge can be manipulated by varying the pH of the buffer. The isoelectric point (pI) of MT1-MMP (PROcat) (Ala^{21}-Ile^{318}) is 6.5, as determined from the Expasy proteomics server (http://au.expasy.org/tools/protparam.html). Increasing the pH of the buffer to exceed the pI of MT1-MMP, results in an overall negative charge on the protein, whereas, reducing the buffer pH to below the protein's pI, results in a positively charged protein. Using an oppositely charged resin, therefore, the protein may be bound to the column and eluted by increasing ionic strength. AEC is usually effected by the binding of a negatively charged protein (pH>pI) to a positively charged resin (e.g. Q-Sepharose), whereas, CEC requires a positively charged protein (pH<pI) to bind to a negatively charged resin (e.g. S-Sepharose). Both procedures are simple to use, have a very high binding capacity and concentrate proteins so samples that are very dilute or contain very little protein may be applied and concentrated (Machold *et al*., 2005).

Purifying MT1-MMP inclusion bodies via AEC and CEC was considered easily achievable, as the pI of MT1-MMP (PROcat) was already known (Expasy proteomics server), and, by using different pH buffers, the overall charge of MT1-MMP could be easily manipulated. These procedures were, thus, considered the most suitable procedures to employ to purify MT1-MMP inclusion bodies. Both AEC and CEC were adopted, to see which would result in a higher purity. The protocol of Valtanen *et al*. (2000) excluded urea and a reducing agent

(i.e. β-mercaptoethanol) in the elution buffer, a process which may affect protein solubility. The method of Kridel *et al*. (2002), however, used an elution buffer containing urea and β-mercaptoethanol similar to the protein solubilisation buffer (i.e. Section 3.5.1) and, consequently, it was thought that the protein would remain more soluble and stable using this procedure.

4.1.1.1 Reagents

S-Sepharose equillibrating buffer [15 mM Na-Phosphate buffer, pH 6.0]. $NaH_2PO_4.H_2O$ (0.21 g) was dissolved in 90 ml $d.H_2O$, adjusted to pH 6.0 with NaOH. NaN_3 (0.02 g) was added and the volume made up to 100 ml.

Q-Sepharose equillibrating buffer [50 mM Tris-HCl buffer, pH 8.5, 6 M urea, 30 mM 2-mercaptoethanol]. Tris Base (0.6 g), urea (36 g) and 2-mercaptoethanol (260 µl) were dissolved in 90 ml $d.H_2O$, adjusted to pH 8.5 with HCl and made up to 100 ml.

4.1.1.2 Procedure

The purification procedure was conducted at 4ºC. The MT1-MMP inclusion bodies, dissolved in 8 M urea and 30 mM 2-mercaptoethanol (5 mg/ml) were purified on an S-Sepharose (cationic) column [15 cm x 1 cm, 4 ml bed volume, equilibrated with 3 column volumes of S-Sepharose equillibrating buffer (12 ml), pH 6.0] or on a Q-Sepharose (anionic) column [15 cm x 1 cm, 6 ml bed volume, equilibrated with 3 column volumes of Q-Sepharose equillibrating buffer (18 ml) containing 6 M urea and 30 mM 2-mercaptoethanol, pH 8.5]. Before applying to the S-Sepharose column, the urea-solubilised MT1-MMP inclusion bodies (1 ml) were initially diluted in S-Sepharose equillibration buffer (3 ml), to decrease the pH of the protein sample to below 6.5. When applied to the Q-Sepharose column, however, the urea-solubilised inclusion bodies did not require dilution and were, thus, applied directly in the solubilisation buffer (1 ml) (Section 3.5.1). Unbound protein was eluted [10 column volumes of the equilibrating buffer] and the bound protein was eluted from both columns with a 0-1 M NaCl gradient [15 column volumes] (1 ml/min flow rate). Fractions (0.5 ml) were collected and the A_{280} of the fractions was measured to detect where the MT1-MMP protein was eluted. The presence of MT1-MMP was confirmed by analysis on a reducing 12.5% SDS-PAGE gel and compared to the crude sample (control) (all samples loaded at a final concentration of 50 µg/ml). Those fractions containing the 33 kDa

expressed protein were pooled and the concentration of the pooled samples determined using the extinction coefficient for MT1-MMP i.e. $A_{280}^{1.407}$ = 1 g/l (Expasy proteomics server).

4.1.1.3 Results

Compared to the crude MT1-MMP inclusion body extract (Figure 20, lane 6), the S-Sepharose (Figure 20, lane 3) eluate showed successful removal of a large majority of the *E. coli* proteins, including those at approximately 27 kDa and 36 kDa. Some 33 kDa expressed protein also appeared to be washed out with the *E. coli* contaminants (Figure 20, lane 2) possibly due to overloading of the column. This was not the case with the Q-Sepharose column (Figure 20, lane 4). Most of the MT1-MMP protein appeared to be bound to both the S-Sepharose and Q-Sepharose resins and eluted after the salt gradient was applied (Figure 16, lanes 3 and 5, respectively). In the Q-Sepharose column eluate, however, the 33 kDa expressed protein and a 36 kDa possible *E. coli* contaminating protein seemed present (Figure 20, lane 2), as previously evident in the untransformed *E. coli* homogenate (Figure 18, lane 2) and , therefore did not seem to be removed by this step, as no unbound protein bands could be identified in the unbound eluate (Figure 20, lane 4). Despite this, following the application of a NaCl gradient, the eluted protein appeared to be significantly purer (Figure 20, lane 5) compared to the crude sample (Figure 20, lane 6). Although the Q-Sepharose-purified fraction still contained the 36 kDa *E. coli* protein, it lost the 27 kDa and other *E. coli* proteins, previously evident in the *E. coli* homogenate (Figure 18, lane 2). This suggests that majority of the *E. coli* proteins were removed during the purification procedure or were present at too low a concentration to be visible on the SDS-PAGE gel (Figure 20, lanes 4, 5 and 6).

Comparing the two purification procedures, the S-Sepharose column appeared to purify the protein more efficiently (Figure 20, lane 3 versus lane 5). Some of the expressed protein was lost during the washing step on the S-Sepharose column, compared to the Q-Sepharose column (Figure 20, lane 2 verusus lane 4), however. This would effect protein yield. In addition, a large portion of the purified protein on the S-Sepharose column appeared to precipitate out of solution, whereas the protein purified on the Q-Sepharose column remained in solution (as anticipated). The total protein yield obtained from each purification

procedure was 256 mg per litre culture from the Q-Sepharose column and 218 mg per litre culture from the S-Sepharose column. This equates to approximately 5% of the total amount of protein loaded on the column. Since the Q-Sepharose purified MT1-MMP (PROcat) sample appeared to be the most soluble, it was considered more suitable for use in renaturation of the PROcat MT1-MMP domain.

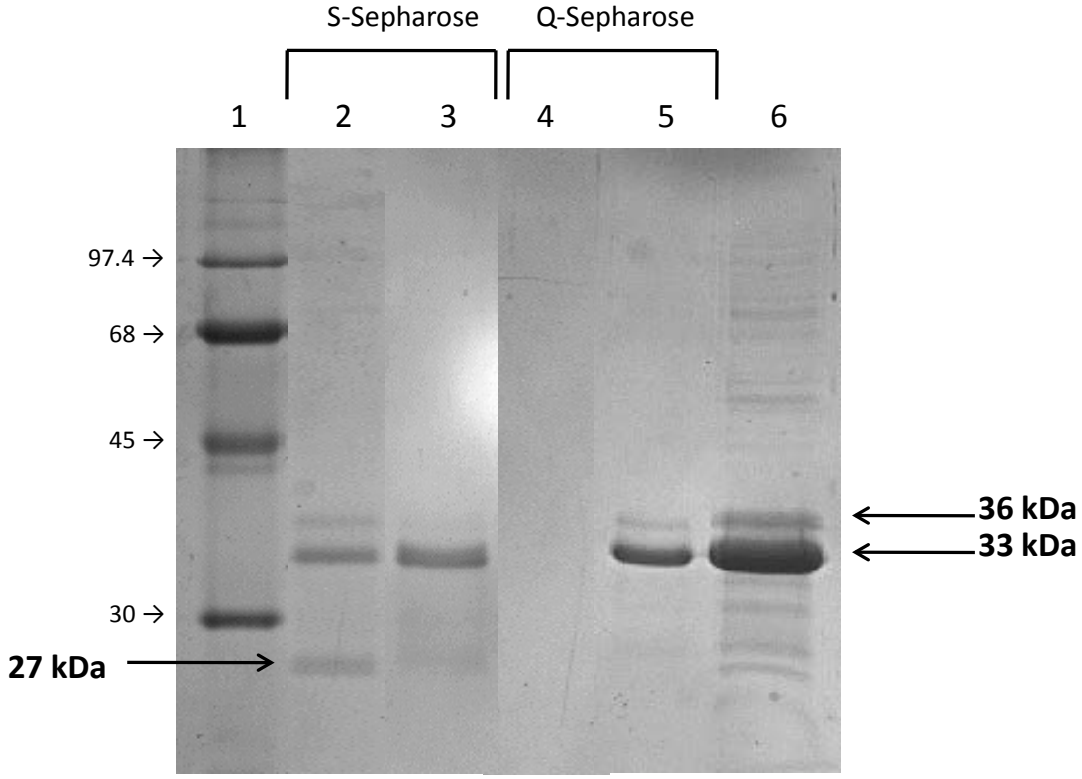

Figure 20. Analysis of MT1-MMP purification after ion exchange eluted with a 0-1M NaCl gradient.

The protein samples were run on a 12.5% SDS-PAGE gel under reducing conditions and visualised with Coomassie G250 dye. Lane 1, protein molecular weight markers; lane 2, unbound protein fraction on S-Sepharose column; lane 3, protein eluted from a S-Sepharose column; lane 4, unbound protein fraction from Q-Sepharose column; lane 5, protein eluted from a Q-Sepharose column; lane 6, crude solubilised expressed protein extract.

4.1.2 MT1-MMP (PROcat) renaturation using gradient dialysis

During gradient renaturation of the PROcat (Ala^{21}-Ile^{318}) domain of MT1-MMP, the NH^{4+} group of Ile^{94} needs to connect via a salt bridge with one of the three conserved aspartate amino acids in the catalytic domain, in order to result in an active enzyme (Lichte *et al.*,

1996). The protein also frequently adopts an unstable intermediate conformation during renaturation. Since the native state is slightly more stable than the unfolded state, the protein usually twists back into its more stable native conformation, which for MT1-MMP, has been shown to involve consecutive autoproteolytic cleavage events (Koo *et al*., 2002). Autocatalytic processing of MT1-MMP catalytic domain into the native form also requires hydrophobic interactions and the formation of β-pleated sheets (Fernandez-Catalan *et al*., 1998). These hydrophobic interactions keep the protein very stable (Creighton, 1990). Lichte *et al*. (1996) showed that during renaturation, recombinant human MT1-MMP (PROcat) proenzyme may be autocatalytically processed from 33 kDa to a 24, 5 kDa form and finally to the active 23 kDa form.

4.1.2.1 Reagents

Renaturation buffer stock [50 mM Tris-HCl buffer, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2.2H_2O$, 0.5 mM $ZnCl_2$l. Tris Base (6.06 g) and NaCl (11.69 g) was dissolved in 900 ml d.H_2O, adjusted to pH 7.5 with HCl and $CaCl_2.2H_2O$ 0(0.74 g) and $ZnCl_2$ (0.068 g) added and made up to 1 l.

Renaturation buffer containing 6 M urea and 150 mM 2-mercaptoethanol. Urea (180.18 g) and 2-mercaptoethanol (5.5 ml) was added to renaturation buffer (500 ml) and pH adjusted to 7.5 if necessary.

Renaturation buffer containing 4 M urea and 100 mM 2-mercaptoethanol. Urea (120.12 g) and 2-mercaptoethanol (3.5 ml) was added to renaturation buffer (500 ml) and pH adjusted to 7.5 if necessary.

Renaturation buffer containing 2 M urea and 50 mM 2-mercaptoethanol. Urea (60.06 g) and 2-mercaptoethanol (1.74 ml) was added to renaturation buffer (500 ml) and pH adjusted to 7.5 if necessary.

1% (m/v) Gelatin stock solution. (Section 2.5)

Non-reducing treatment buffer [125 mM Tris-HCl buffer, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol]. (Section 2.5)

Triton X-100 buffer. (Section 2.5)

Digestion buffer 50 mM Tris-HCl buffer, pH 7.6, 200 mM NaCl, 5 mM $CaCl_2.2H_2O$. (Section 2.5)

Staining solution [0.1% amido black in 30% (v/v) methanol and 10% (v/v) acetic acid]. (Section 2.5)

Destaining solution [30% (v/v) methanol, 10% (v/v) glacial acetic acid]. (Section 2.5)

4.1.2.2. Procedure

The Q-Sepharose-purified human PROcat MT1-MMP domain (100 µg/ml) was dialysed against the renaturation buffer according to the protocol of Koo *et al*. (2002). Here the protein was gradually dialysed using a four-step gradient containing varying amounts of urea (6 M, 4 M, 2 M and 0 M) and β-mercaptoethanol (150 mM, 100 mM, 50 mM and 0 mM) (8 h per step), using fresh renaturation buffer at each of the four steps. This procedure was followed by additional final dialysis against the renaturation buffer (containing 0.05% (v/v) Brij 35) (8 h), all steps being conducted at 4°C. The concentration of the renatured expressed and purified protein was determined using the extinction coefficient of MT1-MMP catalytic domain at $A_{280}^{1.570}$= 1 g/l (Expasy proteomics server).

Activity of the renatured PROcat domain of MT1-MMP (5 µg/ml) was zymographically analysed on a 12.5% (v/v) SDS-PAGE gel containing 0.1% (m/v) gelatin under non-reducing conditions (Section 2.5) In addition, the crude (5 µg/ml), the Q-Sepharose (5 µg/ml) and S-Sepharose (5 µg/ml) purified MT1-MMP samples were analysed and, to ensure that the observed activity was not associated with an *E.coli* enzyme, transformed and untranformed *E. coli* samples were also anaylsed (5 µg/ml). To check gelatinolytic activity was due to a metalloproteinase, all the samples were run on a duplicate gel and the duplicate gel was incubated in digestion buffer containing 1 % (m/v) EDTA-Na_2, a metal chelator and metalloproteinase inhibitor. A human blood sample, obtained from a finger prick (1:1000 dilution in non-reducing treatment buffer), was used as a positive control (Markowski and Ramsby, 1996), MMP-9 and MMP-2 activity usually being observed at 92 kDa and 72 kDa, respectively.

4.1.2.3 Results

Renaturation of the expressed Q-Sepharose-purified (PROcat) protein resulted in gelatinolytic activity at about 25/23 kDa (Figure 21 A, lane 8), as previously described for the proteolytically active, renatured catalytic domain of MT1-MMP (PROcat) (Lichte *et al*., 1996). There was also unanticipated activity, which cannot be easily explained, observed at

42 kDa for this gradient renatured MT1-MMP (Figure 21 A, lane 8). The crude- (Figure 21 A, lane 5) and purified- (Figure 21 A, lanes 6 and 7) PROcat domains of MT1-MMP showed activity mainly at 29 kDa, suggesting these samples underwent partial renaturation prior to the gradient dialysis procedure. In the presence of EDTA-Na_2, enzyme activity at 25/23 kDa in the gradient renatured sample (Figure 21 B, lane 8) and at 29 kDa in the crude, denatured and S-Sepharose and Q-Sepharose purified MT1-MMP PROcat isolates (Figure 21 B, lanes 5, 6 and 7), was extinguished, indicating the presence of possible metalloproteinase enzymes (Figure 21 B, lanes 5-8).

The Q-Sepharose column seemed to be most successful for binding unrenatured PROcat MT1-MMP (33 kDa) and renatured MT1-MMP (29 kDa) (Figure 21 A, lane 7). No activity was observed in the untransformed and transformed *E. coli* samples (Figure 21 A, lanes 3 and 4), indicating gelatinolytic activity was most possibly due to the expressed, renatured PROcat MT1-MMP domain and not an *E. coli* enzyme. The activity observed in the human blood sample possibly represented proMMP-9 (92 kDa) and proMMP-2 (72 kDa) (Figure 21 A, lane 2), as previously reported (Markowski and Ramsby, 1996). Gelatinolytic activity of these MMPs was also extinguished in the presence of EDTA-Na_2 (Figure 21 B, lane 2) indicating that they are also gelatinolytic metalloproteinases. The total amount of renatured PROcat MT1-MMP protein recovered after stepwise gradient dialysis was 213 µg from 246 µg of Q-Sepharose-purified MT1-MMP PROcat protein, as determined using the extinction coefficient for MT1-MMP i.e. $A_{280}{}^{1.570}$= 1 g/l (Expasy proteomics server).

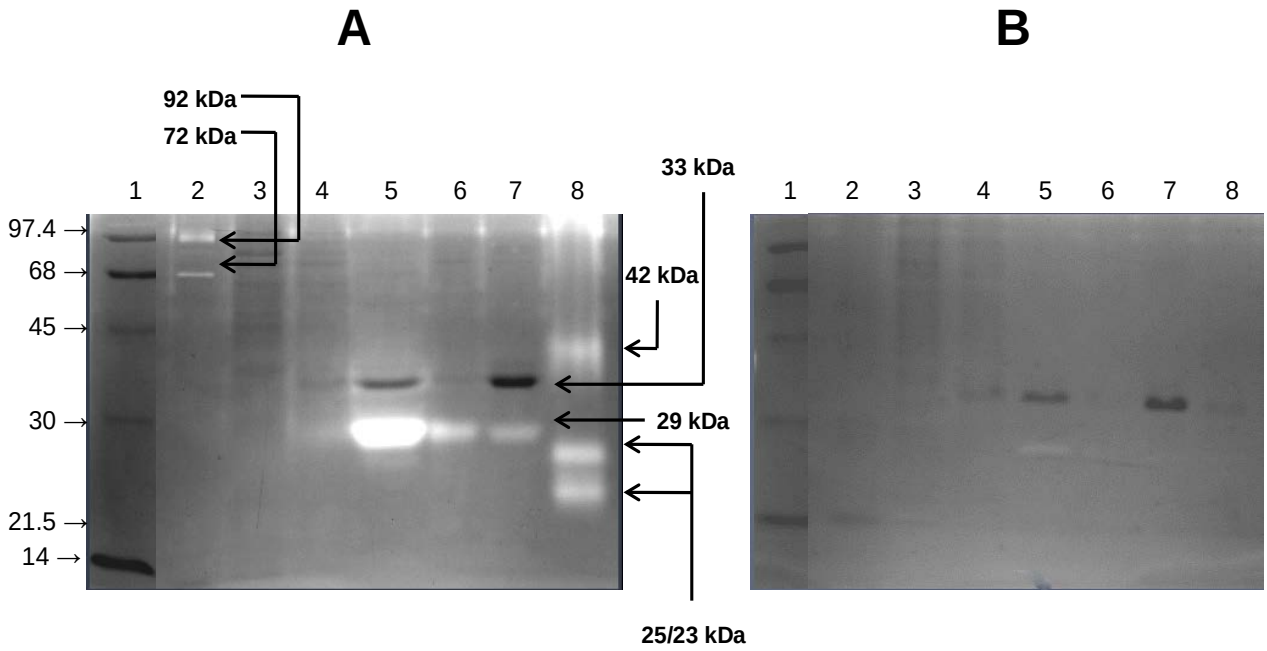

Figure 21. Gelatinolytic activity of renatured PROcat MT1-MMP.

Solubilised MT1-MMP was exposed to 5 mM $ZnCl_2$ and subjected to dialysis in the presence of a four-step gradient against urea and 2-mercaptoethanol**.** The samples were analysed on a 12.5% SDS-PAGE gel containing 0.1% (m/v) gelatin and were prepared without reduction or boiling. Panel A. gelatin gel incubated in digestion buffer (18 h, 37°C). B. gelatin gel incubated in digestion buffer containing EDTA-Na_2 (18 h, 37°C). Lane 1, molecular weight marker; lane 2, human blood sample; lane 3, untransformed *E. coli* cells; lane 4, tranformed *E. coli* cells; lane 5, unrenatured crude MT1-MMP; lane 6, S-Sepharose purified MT1-MMP; lane 7, Q-Sepharose purified MT1-MMP; lane 8, gradient renatured MT1-MMP

4.1.3 Measuring enzyme activity of renatured MT1-MMP (PROcat) using a synthetic substrate

MT1-MMP activity can be measured using the universal MMP fluorogenic substrate (7-methylcoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH_2 cleavage occuring specifically at the Leu-Gly bond (Lichte *et al*., 1996; Roderfeld *et al*., 2000; Koo *et al*., 2002; Oku *et al*., 2003). This fluorogenic substrate comprises a short peptide sequence conjugated to a highly fluorescent 7-amino-4-methyl coumarin (AMC) fluorophore and a quenching group, 2, 4-dinitrophenyl (DNP). As the peptide substrate is cleaved, the quenching is weakened and the AMC group is released, emitting a fluorescence detected by the spectrophotometer at an excitation wavelength of 328 nm and an emission wavelength of 393 nm (Lichte *et al*., 1996). Over time, the intensity

of fluorescence increases as the substrate is hydrolysed and the rate of change of fluorescence is directly proportional to the concentration of active enzyme, provided the substrate is not limiting (Koo *et al*., 2002). The enzyme activity can be calculated from the initial plot of fluorescence versus time. This allows the initial velocity of the enzyme (V_0) to be read from the slope of the linear section of a hyperbolic plot and the determination of K_m by plotting V_0 against substrate concentration [S], where K_m equals ½ V_{max}. It is important to establish K_m, as at least twice the K_m concentration of substrate should be used to ensure substrate is not limiting in stopped time (non-continuous) enzyme assays. Although a Lineweaver-Burk plot has frequently been used to establish K_m (Lineweaver and Burk, 1934), it is a double recipricol plot, which may overemphasise experimental error at lower substrate concentrations, thereby resulting in inaccurate estimates of K_m and V_{max} (Palmer, 1981). This plot is, therefore less desirable to use in the analysis of enzyme kinetics. A Hanes plot (Hanes, 1932) is comparatively better at linearising the Michaelis-Menton equation and, therefore, is a more reliable plot to determine K_m and V_{max} (Palmer, 1981).

A AMC standard can be used to convert enzyme fluorescence into enzyme activity in units per ml. The fluorescence obtained from 0.5 µM standard MEC solution is equal to 1000 arbitrary enzyme fluorescence units, which is equal to 0.1 mU of enzyme activity (Barrett and Kirschke, 1981). The enzyme activity can be calculated using the following equation:

$$\text{mUnits activity} = \frac{\text{Fluorescence of sample x 0.1 mU}}{\text{Fluorescence of 0.5 µM MEC standard}}$$

If such activity is defined in terms of time this activity may also be represented by V, representing velocity (i.e. activity per time).

Fluorogenic peptide substrates are useful in determining enzyme kinetics, stability and substrate specificity. The establishment of latter parameters may contribute towards the development of therapeutic inhibitors and assist in discriminating between different enzymes (Mucha *et al*., 1998). The fluorogenic peptide substrate, mentioned above, was used to assess MT1-MMP enzyme activity to establish whether the PROcat domain of MT1-MMP was successfully renatured.

4.1.3.1 Reagents

Aminomethyl coumarin (AMC) standard (1 mM). 7-amino-4-methyl coumarin (1.8 mg) was dissolved in DMSO (10 ml) and stored at 4°C. A 0.5 µM standard was prepared by diluting stock solution (5 µl) with assay buffer (10 ml).

MMP assay buffer [20 mM Tris-HCl, pH 7.3, 100 mM NaCl, 5 mM $CaCl_2.2H_2O$, 100 µM $ZnCl_2$, 0.02% (m/v) NaN_3]. Tris (1.21 g) and NaCl (2.92 g) were dissolved in 450 ml $d.H_2O$, adjusted to pH 7.3 with HCl, $CaCl_2.2H_2O$ (0.37 g), $ZnCl_2$ (0.007 g) and NaN_3 (0.1 g) were added and the volume made up to 500 ml.

AMC-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2.AcOH stock solution (915 µM). Substrate (1 mg) was dissolved in 1 ml DMSO and stored at -20ºC.

Substrate working solution (50 µM). Substrate stock solution (273 µl) was made up to 5 ml with $d.H_2O$ and stored at 4ºC.

4.1.3.2 Procedure

To determine units of activity of renatured PROcat MT1-MMP, the standard AMC fluorescence (i.e. control), 0.5 µM 7-amino-4-methyl coumarin (200 µl) was added to each of eight wells of a FluoroNunc MaxiSorp microtitre plate and incubated (10 min, 37°C). The fluorescence was measured using a Fluostar Optima fluorescent reader with excitation at 320 nm and emission at 420 nm. The fluorescence values were averaged and substituted in the equation in Section 4.1.3 for calculation of renatured PROcat MT1-MMP activity.

The enzyme kinetics of renatured PROcat MT1-MMP was subsequently established using the protocol of Lichte *et al*. (1996) with minor modifications. The renatured PROcat MT1-MMP sample was diluted in MMP assay buffer (14 µl enzyme in 10 ml buffer, to give a concentration of 330 ng) and added (150 µl) to each well of a 96 well FluoroNunc MaxiSorp microtitre plate and incubated (37˚C, 2 min). The K_m of the enzyme was established by measuring fluorescence over a range of substrate concentrations. Substrate working solution was diluted to five different concentrations using two-fold dilutions in $d.H_2O$ (50 µM to 3.125 µM), substrate (50 µl) was added to each well in triplicate and the fluorescence continuously measured, as described above (10 min, 37°C) using a Fluostar Optima fluorescent reader. To calculate enzyme activity (V_o), the slope of the curve, plotting time versus fluorescence, for each substrate concentration ([S]) needed to be established. This was achieved by substituting the change in fluorescence (i.e. final fluorescence - initial

fluorescence) for each substrate concentration into the equation in Section 4.1.3. The enzyme activity (V_0) was plotted against [S] using the hyperbolic regression plot and the program “hyper” (Studnicka, 1987). The K_m was subsequently confirmed using a Hanes plot:

$$[S]/V=1/(V_{max})[S]+K_m/V_{max}$$

where

x-intercept=$-K_m$

y-intercept = K_m/V_{max}

slope= $1/V_{max}$

4.1.3.3 Result

The plot of fluorescence against time showed a classical plot (data not shown). The rate of hydrolysis of substrate (V_0) showed a relatively linear relationship with increasing substrate concentration until V_{max} was reached, as anticipated (Figure 22 A). This allowed K_m to be estimated as [S] at 1/2 V_{max} and gave a K_m of 16.16 µM (+- 15.47 µM). The K_m was subsequently checked using a Hanes plot (Figure 22 B) and was established to be 19 µM.

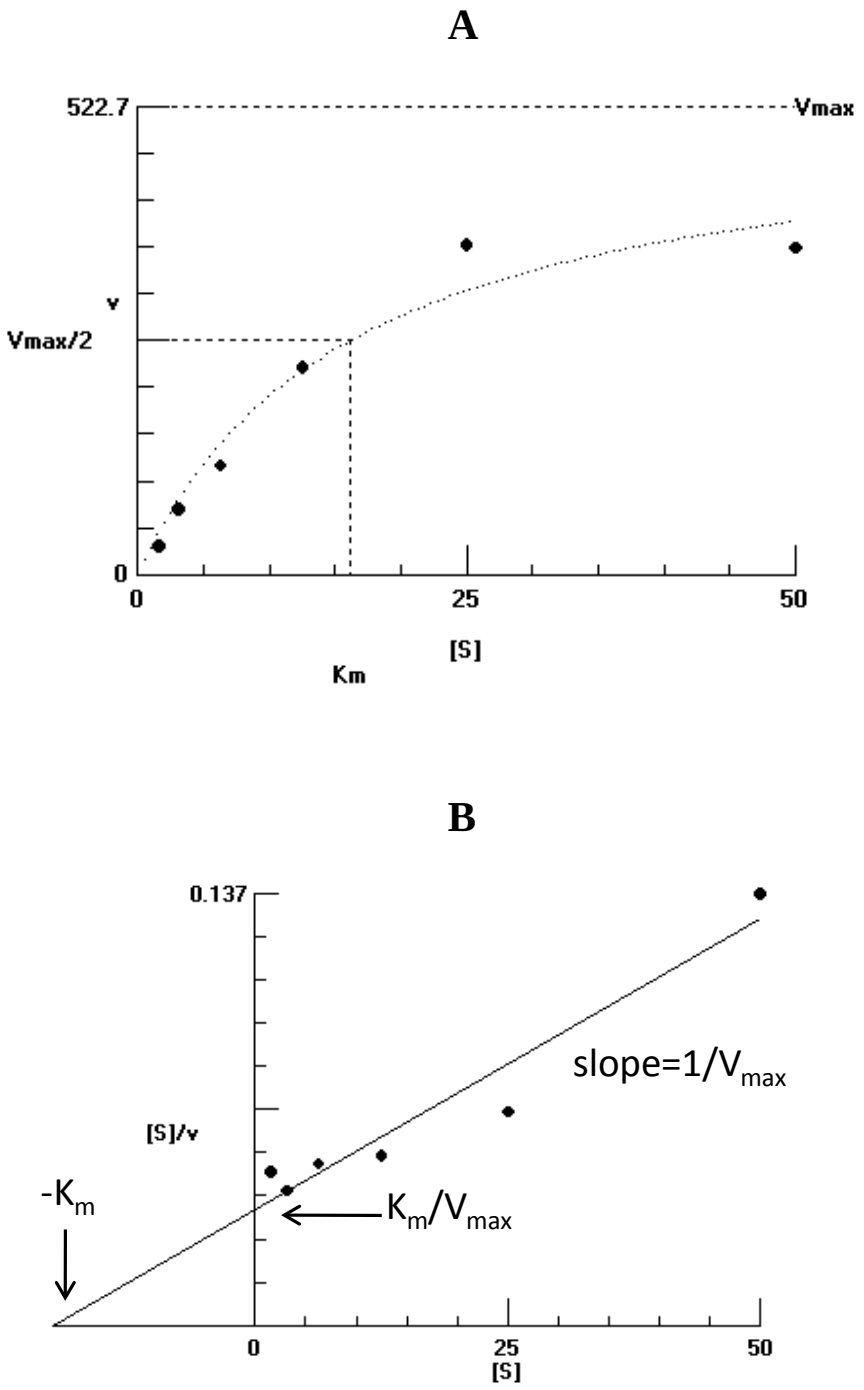

Figure 22. Activity of renatured PROcat MT1-MMP determined using a quenched fluorescent substrate AMC-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2.

The activity was measured over five different substrate concentrations (50 µM - 3.125 µM) and plotted to determine the trend (Panel A). The data was also used in a Hanes plot to calculate K_m (Panel B). The fluorescence was measured continuously at 320 nm excitation and 420 nm emission wavelengths.

4.2 Discussion

In the current study, inclusion body solubilisation with detergents and urea (Chapter 3, Section 3.5) was followed by additional purification procedures using Q-Sepharose (Kridel *et al*., 2002) and S-Sepharose (Valtanen *et al*., 2000). These methods were both used to determine which procedure was more efficient in purifying the PROcat proenzyme domain, expressed essentially according to the procedure of Lichte *et al*. (1996), although Lichte *et al*. (1996) had only used gel filtration to purify the MT1-MMP protein. As antibody generation was required and minor *E. coli* contaminants of similar molecular weight to the PROcat protein were present, both IEC methods (Valtanen *et al*., 2000; Kridel *et al*. 2002), were employed, though these have not been previously used before to isolate the MT1-MMP PROcat domain. This is also the first time the PROcat domain of MT1-MMP has been solubilised using urea (8 M) and β-mercaptoethanol (30 mM) (Chapter 3, Section 3.5) and it was evident from the zymogram that significant amounts of PROcat enzyme was already partially renatured by the process of solubilisation.

S-Sepharose seemed more efficient in removing the *E. coli* proteins compared to Q-Sepharose as there was a 36 kDa *E. coli* protein still present in the eluate from the Q-Sepharose column (Figure 20, lane 5). The Q-Sepharose purified protein, however, appeared to be soluble, whereas a large portion of the S-Sepharose purified protein precipitated out of solution. Q-Sepharose also seemed more effective in isolating the unrenatured (33 kDa) PROcat form of the MT1-MMP expressed protein. Whereas the elution buffer used with the S-Sepharose method was a sodium phosphate buffer, the elution buffer used in the Q-Sepharose purification was a tris buffer and contained urea and 2-mercaptoethanol. These additives (i.e. urea and mercaptoethanol) were possibly responsible for keeping the expressed protein soluble.

As the purity of the protein is more important than its solubility for antibody production (Harlow and Lane, 1988), the S-Sepharose-purified protein was used for antibody production. For the ELISA and renaturation procedures, however, solublility was essential and so the Q-Sepharose protein was used. Both chromatography techniques were, however, unsuccessful in completely eliminating *E. coli* contaminants, though the Q-Sepharose was more successful of the two purification procedures. As ion exchange chromatography

fractionates on the basis of net charge, and net charge depends on the number and type of ionisable amino acid side groups, the presence of *E. coli* contaminants following both cation and anion exchange chromatography (at different pH's) suggests that contaminating *E. coli* proteins may have a similar pI to that of the PROcat domain of MT1-MMP.

The catalytic domain of MT1-MMP, composed primarily of β-pleated sheets, containing a catalytic zinc ion in the active site cleft, which is crucial for enzyme activity (Bode *et al*., 1999; Fernandez-Catalan *et al*., 1998), was relatively easily renatured by exposure to $ZnCl_2$ with decreasing concentrations of a denaturant and reducing agent. As the concentration of the denaturant (urea) and the reducing agent (β-mercaptoethanol) is decreased, the protein is reported to become more compact and stable (Tsumoto *et al*., 2003), and the size of the renatured enzyme produced in this study (25/23 kDa) was of similar molecular weight to that reported previously for human PROcat MT1-MMP (Lichte *et al*., 1996). Reduction of activity in the presence of EDTA-Na_2 seemed to confirm the enzyme is a metalloproteinase, as previously reported (Lichte *et al*., 1996). Prior to renaturation, a 29 kDa gelatinolytic band was evident. As this too was extinguished by EDTA, suggests this was possibly an intermediate form of MT1-MMP. As enzymes are usually not very stable in an intermediate form (Creighton, 1990) renaturation possibly gave rise to autocatalytic processing when exposed to the stepwise renaturation procedure (Lichte *et al*., 1996; Koo *et al*., 2002). The observed apparent activity of a 29 kDa proenzyme in the SDS-PAGE gel is interesting as it suggests that the presence of urea, β-mercaptoethanol and possibly SDS may facilitate renaturation of the unrenatured protein. It must, however, be remembered that zymography is sensitive and the gelatinolytic activity, observed at 29 kDa in the SDS-PAGE gel, may be due to *in situ* activation of the unrenatured enzyme in the gel rather than the enzyme being active due to prior extraction procedures. The band intensity of the S-Sepharose (Figure 21, lane 6) and Q-Sepharose (Figure 21, lane 7) purified samples compared to the crude sample (Figure 21, lane 5) was reduced, which suggests that there was significant loss of enzyme during the purification procedures, as the same total amount of protein was loaded in each case.

The presence of a 42 kDa band on the renatured gel is interesting as it suggests that dimerisation of the renatured PROcat MT1-MMP domain had occurred. As mentioned previously (Section 4.1) the concentration of protein is important in preventing intermolecular cross-linking during renaturation procedures in the presence of a reducing agent (Kroger and Tschesche, 1997). A prescribed concentration of <100 µg/ml for such procedure is suggested. Here 100 µg/ml of PROcat protein was used and perhaps this protein concentration should have been reduced to below 100 µg/ml to avoid exceeding the protein concentration limit, as protein concentrations greater than 100 µg/ml have been reported to facilitate intramolecular dimerisation (Kroger and Tschesche, 1997). Such an event is the only way to explain the appearance of the active but high molecular weight form of presumptive PROcat enzyme seen in the gel post renaturation. Activity at 42 kDa was extinguished in the presence of EDTA (i.e. control) and further suggests this activity was due to a metalloproteinase.

The K_m value of a naturally occuring or a renatured enzyme refers to the affinity of the naturally occuring/renatured enzyme for its substrate, the substrate being inversely proportional to the K_m (i.e. higher the K_m the lower the affinity of the enzyme for the substrate). In the current study, PROcat MT1-MMP enzyme renaturation showed that the Q-Sepharose-purified PROcat domain had a K_m of 16.16 µM (as determined from the hyperbolic plot) and 19 µM (as determined from the Hanes plot). The difference in the K_m values obtained from the Hanes and the Hyperbolic plots, may be associated with differences in accuracy, the Hanes plot being more accurate than the hyperbolic plot (Palmer, 1981). The K_m values in the current study were much higher than 5.5 µM, previously reported for PROcat MT1-MMP by Lichte *et al*. (1996), even though an identical plasmid construct was used. As the same substrate was used, the results here suggest that the renatured enzyme in the current study had a lower affinity for the substrate than the previous renatured enzymes (Lichte *et al*., 1996). This is difficult to explain. The overall discrepency between all K_m results recorded could partially be explained by the plot used to calculate K_m and partly the renaturation procedure used.

Lichte *et al*. (1996) used the Lineweaver-Burk plot for calculation of the K_m for the PROcat construct. As previously explained, this plot has been found to be less accurate than the Hanes plot (Palmer, 1981). Other reports, not using the Lineweaver-Burk plot, but using the hyperbolic plot, have shown similar higher K_m values (10 µM) for renatured catalytic domain of MT1-MMP (Toth *et al*., 2002) to those obtained in this study using this hyperbolic plot. This implies that the Lineweaver-Burk plot gives lower K_m results. The K_m is independent of enzyme concentration (i.e. the amount of enzyme renatured) (Palmer, 1981). Here it is argued, that the only way to explain a higher K_m is in terms of K_m dependance on the level of success of renaturation of the enzyme. This, inturn, may affect the affinity of the enzyme for the substrate and influence K_m. The discrepency in the reported K_m values may, therefore, be related to the choice of renaturation procedure (i.e. success of renaturation process) and the plot chosen to determine K_m. In the current study, PROcat MT1-MMP was renatured using a stepwise gradient dialysis (Koo *et al*., 2002). Previously the catalytic domain of MT1-MMP (Toth *et al*., 2002) and the catalytic domain in conjunction with the propeptide domain (PROcat) of MT1-MMP (Lichte *et al*., 1996) were renatured using a one-step dialysis. This suggests that the gradient dialysis procedure was less successful in renaturation of the protein into its fully native conformation than the one-step dialysis procedure (Lichte *et al*., 1996; Toth *et al*., 2002) (i.e. a rapid removal of denaturants is preferable to a gradual removal of denaturants), the latter giving a higher K_m (i.e. less avidly-binding renatured enzyme).

Although the PROcat domain of MT1-MMP appeared to be partially renatured into the native conformation, the choice of rather using the unrenatured MT1-MMP (PROcat) domains for antibody production was made, as this fraction, from the S-Sepharose column, seemed the most purified and it was thought possibly preferable to inject an inactive enzyme, as active enzymes are often rapidly removed by enzyme inhibitors, such as α-2 macroglobulin.

The unrenatured, partially soluble S-Sepharose PROcat antigen was, therefore, used to raise antibodies against the human PROcat domain, while the MT1-MMP (PEXcat) and MT1-MMP (PEX) un-renatured, urea-solubilised enzymes were also chosen for antibody

production against the PEXcat and PEX domains. The semi-soluble, unrenatured antigens were used as a precipitated or semi-soluble enzyme in adjuvant as these possibly give a better antibody response, being precipitated at the site of injection and, hence, being less easily removed. This potentially gives rise to a depot effect or granulomas at the site of injection, to extend exposure to the antigen. Other strategies for raising antibodies were also attempted (e.g. protein band excision from acrylamide gel and injection) (see Chapter 5), while the renatured protein was to be used to test the specificity and immunoinhibitory activity of the antibodies subsequently raised.

Chapter 5

Production of PROcat, PEXcat and PEX MT1-MMP antibodies and antibody characterisation

5.1 Introduction

Highly specific antibodies are extremely useful reagents not only for recognising proteins separated electrophoretically and western blotted, but also for imaging the protein of interest, in the cell, in order to establish the distribution, trafficking or movement through specific structures, to focus at specific regions or in specific vesicles in cells. This is achieved using immunolabelling and electron or fluorecent microscopy with such antibodies, to establish possible roles of proteins in the cell. By initially working out interacting ligands or enzyme activity using classical biochemical approaches and subsequently establishing associations and possible activity, a distribution similar to the known substrate or ligand presumptive functions *in vivo* may be established. For this investigation, specific anti-MT1-MMP antibodies could be used to investigate the role and distribution of MT1-MMP in tumour cell invasion, a study critical for the development of therapeutic drugs and inhibitors.

For successful antibody production, the immune system has to recognise the substance (antigen) as being foreign. Antigen presenting cells, such as monocytes and dendritic cells, which form part of the adaptive immune system, respond by binding to and internalising the foreign material. This is subsequently processed for presentation by the major histocompatability molecules (MHC class I or II) (Harlow and Lane, 1988; Hau and Hendrikson, 2005). If the presented antigen is viral in nature, MHC class I presentation takes place and a cell mediated immune response will be provoked. This mostly involves a cytotoxic T cell response. On the other hand, a protein antigen presented via a MHC Class II molecule may stimulate uncommitted B cells and T cells to become committed B and T helper cells. On second encounter with the target antigen, these T cells release various cytokines (e.g. interleukin-4) to promote committed B cells to proliferate and become committed to differentiate into specific antibody producing plasma cells (Harlow and Lane, 1988; Hau and Hendrikson, 2005).

In order to raise antibodies, the antigen not only has to be foreign, but usually has to be introduced in a medium, which will facilitate slow release, but will also stimultate the immune response. Such a medium is known as an adjuvant. As the MMPs are homologous in primary sequence, it was decided to use biotechnological expression of specific domains. Those that showed the greatest variation in primary sequence across species and across different MMPs needed to be selected. This exercise (Chapter 3) helped to identify three different target domains or domain combinations, the propeptide and catalytic domains (PROcat), the hemopexin-like and catalytic domains (PEXcat) and the hemopexin-like domain (PEX), and the most optimal species for antibodiy production, i.e. the laboratory animal in which MT1-MMP-like enzymes showed the greatest primary sequence difference to human MT1-MMP, in this case the chicken.

A second reason why it was fortuitous that the chicken should prove to be the most advantageous species for antibody production, arises from the fact that antibodies produced by the innoculated laying hen are packaged into the egg. Should these eggs be fertile and be incubated for 9 days to become vascularised, these eggs constitute an ideal vascularised model system in which cancer cell invasion may be tested (Kim *et al*., 1998). Should the antibodies, produced by the laying hen, prove to be immunoinhibitory to an enzyme primarily involved in cancer cell invasion, and be passed directly into the vascular system of the embryo, such a system should allow the testing of whether it is possible to prevent cancer cell invasion by priming the immune system against such an enzyme (in this case MT1-MMP). Such an investigation would be a unique approach.

5.2 Producing antibodies in chickens versus mammals

A wide range of laboratory animals may be used to produce polyclonal antibodies of which rabbits and chickens are the most common (Harlow and Lane, 1988) and have a number of advantages over other laboratory animals. Smaller animals, such as guinea-pigs, mice, rats and hamsters are not usually convenient for raising polyclonal antibodies, as only small volumes of sera can be obtained. Mice are, instead, used more frequently for producing monoclonal antibodies. Rabbits are a good choice for raising polyclonal antibodies as they are easy to maintain, can be repeatedly bled and the antibodies they produce are easily

characterised and purified (Harlow and Lane, 1988). Rabbits are, however, mammals and mammalian antigens are often not very immunogenic in these animals. Chickens, in contrast, are phylogenetically different from mammals and are more likely to recognise the mammalian antigen as foreign (Ceuninck *et al*. 2001; Narat, 2003). Harvesting antibodies from chickens may also be a non-invasive procedure as the chickens transfer their antibodies directly from the serum to the egg yolk and, therefore, bleeding of the chicken is not necessary if the yolk antibodies is adequate. This would not be the case for rabbits. Compared to other laboratory animals, chickens produce considerably higher amounts of antibodies (up to 100 mg IgY per yolk) and as a result, fewer animals are required for a particular study. This makes the process of antibody production in chickens more cost-effective and ethically acceptable (Narat, 2003; Hau and Hendriksen, 2005).

The IgY antibodies produced in chickens have an overall structure similar to that of IgG found in mammals, and both immunoglobulins carry out a similar function. Compared to IgG, IgY has a greater half-life, being stable at room temperature for up to 6 months (Narat, 2003). Chicken IgY also does not bind to protein A and G or to the mammalian complement system, features which could otherwise give false-positive results (Hau and Hendriksen, 2005). For these reasons, chickens are frequently considered as a first choice host for antibody production. Fortunately, sequence alignments of the target antigens with that of the chicken showed a low sequence homology with the antigen of interest, making the chicken a highly suitable host.

5.3 Use of adjuvants

In order to produce large quantities of good quality antibodies, the protein antigen needs to be administered with a non-specific stimulator of the immune system, also known as an adjuvant (Harlow and Lane, 1988). Adjuvants store the antigen at the injection site creating a depot effect, which allows a slow release of small quantities of the antigen to continually stimulate the immune system to produce antibodies. By storing the antigen in this manner, the adjuvant protects the antigen against cellular removal. Some adjuvants also act as delivery vehicles directing antigens towards the lymph nodes and/or spleen where the antigen is trapped by dendritic cells and where majority of the antibody-secreting cells

(plasma cells) are produced. At some point in time, the antigen does break down completely, after which a second boost of the antigen in adjuvant is required (Harlow and Lane, 1988).

Freund's complete adjuvant (FCA) is one of the oldest and most commonly used adjuvants used to boost immune responses in animals. It consists of a mixture of non-metabolisable oil (mineral oil), a surfactant and mycobacterial cell walls (*M. tuberculosis* or *M. butyricum*) (Harlow and Lane, 1988). The adjuvant is usually administered as a water-in-oil emulsion with the antigen solution at a 1:1 ratio. FCA usually induces a high, long-lasting antibody immune response. The adjuvant, however, can also lead to unrelenting, aggressive granulomas at the site of injection (Harlow and Lane, 1988). Studies have also shown that FCA can reduce the number of eggs laid per chicken, ultimately affecting the antibody yield and suggests that this adjuvant compromises the animal's health (Hau and Hendriksen, 2005). Freund's incomplete adjuvant (FIA) consists of the same mixture of oil and surfactant except without the microbacterial components and so its effect is milder. FIA can be administered as the primary injection, but is less potent than FCA and is commonly used instead to boost immune responses following an initial injection with FCA (Harlow and Lane, 1988). Both these adjuvants are not acceptabe for use in humans due to their high toxicity and should not be ethical for use in animals either and their use has been discontinued.

Adsorbing the antigen onto an aluminium salt, such as aluminium hydroxide or aluminium phosphate, is frequently used as an alternative adjuvant. Murine studies have shown that aluminium adjuvants are associated with T-helper 2 immune responses, stimulating specifically CD4+ T-cells, which activate B lymphocytes to produce antigen-specific IgE (Yamanishi *et al*., 2003; Lindblad, 2004). Aluminium phosphate induces a cyotoxic T-cell and humoral response against DNA and the protein antigens it encodes, whereas aluminium hydroxide induces an immune response against the protein antigen and not the DNA. A possible reason for this is that the DNA suppresses the immune system due to the high binding affinity of the DNA to the hydroxide groups of the adjuvant inhibiting protein transcription (Kwissa *et al*., 2003). Therefore, aluminium phosphate is more useful in inducing a specific immune response and, thus, is the preferable adjuvant to use.

Aluminium based adjuvants cause no serious side effects and are accepted by the World Health Organisation for use in humans, provided the recommended procedures are followed (Harlow and Lane, 1988; Kwissa *et al*., 2003). In human and veterinary vaccine studies, aluminium adjuvants have been used more frequently than others as they are safe and produce high antibody titres, coupled with a long-term immune response (Lindbald, 2004). Aluminium phosphate, i.e. alum, has also been widely used in producing vaccines (Kreuter and Haenzel, 1978). For these reasons aluminium phosphate was chosen in this study to raise anti-MT1-MMP antibodies in chickens.

Intravenous inoculation of the protein antigen in an adjuvant (i.e. alum) can result in a rapid immune response, but can lead to pulmonary embolisms and/ or anaphylactic shock, both of which are potentially lethal, particularly in very sensitive animals (Harlow and Lane, 1988). Bacterial endotoxins contaminating the antigen, may also cause dangerous side effects if injected into the bloodstream. MT1-MMP was expressed in *E. coli* and, although the enzyme was purified, there could still be bacterial components present. For these reasons, intravenous administration of protein antigens was not used in the present study.

Hau and Hendriksen (2005) proposed that oral administration of the antigen is a much "friendlier" way of delivering the antigen to the animal. This procedure, however, is less reliable, as one cannot be sure that the animal has successfully ingested the entire antigen and the antigen may be degraded in the gastrointestinal tract, by stomach acid and hydrolytic enzymes, before entering the blood stream. Oral administration of the protein antigen was, therefore, not considered in this study.

Delivering the protein antigen intramuscularly (im) or intra-dermally into the laboratory animal is preferable. If the antigen is injected into the muscle, it is released slowly from the muscle tissue and eventually reaches the lymph nodes, which stimulates the immune system to produce antibodies. In addition, large antigen volumes (up to 500 μL) can be injected into the muscle tissue. Injecting intra-dermally also releases the antigen slowly, but skill is required to do this and is most effective if the antigen is injected at multiple sites, as only small volumes can be injected into the skin at one time (Harlow & Lane, 1988). The antigen can be administered at one or two sites into the muscle tissue, which makes this procedure

comparably less painful for the animal and, thus, is more ethically acceptable. Therefore, administering the antigen intramuscularly appeared to be the best approach.

5.4 Production of anti-MT1-MMP antibodies using alum or bands from polyacrylamide gels

As mentioned, when using alum as an adjuvant, the MT1-MMP protein antigen is either directly adsorbed to the aluminium salt or trapped in the salt during precipitation, with the injected precipitate establishing a depot effect releasing the antigen gradually into the animal (Harlow and Lane, 1988). According to Yamanishi *et al*. (2003) the minimum effective concentration of antigen required to induce a suitable immune response in BALB/c mice, when administered with alum hydroxide adjuvant, is 1-10 μg/ml. Since chickens were the host of choice in this study, a minimum concentration of 50 μg/ml antigen was used to ensure successful antibody production. Milligrams of the MT1-MMP antigen were expressed (Chapter 3) and so sufficient antigen was available for such an inoculation dosage.

It is preferable that the protein antigen, against which specific antibodies need to be raised, is pure (Harlow and Lane, 1988). Due to the presence of presumptive *E. coli* contaminants following purification of urea-solubilised PROcat MT1-MMP isolated on a Q-Sepharose and S-Sepharose column (Chapter 4, Section 4.1.1), a further approach was tested in an attempt to obtain a more purified MT1-MMP PROcat protein sample for antibody production. This reqiured the antigen to be further purified by SDS-PAGE and excision of the final protein from the SDS-PAGE gel and administration of the protein, in the SDS-PAGE gel, directly into the laboratory animal (Harlow and Lane, 1988). The antigen, prepared in this manner, has frequently been used to raise antibodies in rabbits without the presence of an adjuvant (Weintraub and Raymond, 1963; Platchter *et al*., 1992). This implies that the acrylamide also induces a depot effect. If the Laemmli (1970) reducing SDS-PAGE separation method is used to separate the antigen, antibodies produced against the denatured and linearised protein may not, however, recognise the native protein. Instead, these antibodies may be more suited for studying the presence/absence and distribution of the protein antigen in fixation-denatured tissue, using immunocytochemistry, and SDS-denatured and blotted

proteins in western blots. Purifying proteins from a gel is quick, easy and avoids the complications involved in purifying proteins using other methods (e.g. chromatography).

Harlow and Lane (1988) describe a range of procedures for identifying proteins on gels for excision and injection or analysis. The side-strip method involves staining a side strip of the gel and aligning it with the unstained part of the gel to determine which region of the unstained gel to excise. This avoids fixation of the protein and is suitable if the protein of interest is abundant and well separated from surrounding proteins and if the gels do not shrink during staining. Following MT1-MMP expression and recovery, a few *E. coli* proteins were found to be in close proximity to the expressed protein (Section 3.5.3, Figure 18) and, since high accuracy is required in aligning the strips exactly, it was decided that it would be difficult to ensure the *E. coli* proteins have been excluded during the excision.

Another procedure for locating proteins on the gel is to lightly stain the gel with Coomassie R250 (Harlow and Lane, 1988). Compared to the side-strip method, staining with Coomassie R250 dye is easier and, if the staining duration is short and acetic acid is excluded from the stain, the risk of fixing the protein is reduced. The shortened staining time also means that, to be detected, the protein needs to be present in abundance. In this study, milligrams of MT1-MMP were expressed and so this procedure was judged to be the most convenient. Other commonly used stains for locating proteins on the gel include copper chloride and sodium acetate (Harlow and Lane, 1988). Like Coomassie blue R250, these stains are easy to prepare and the staining time is short and the also reveal protein bands with a high degree of sensitivity. Unlike Coomassie R250, however, both these stain the gel negatively. This tends to result in less contrast compared to the positive stain methods, making band distinction difficult. In light of this, it was decided that staining the gel with Coomassie R250 would be the most suitable and this method was chosen for the current study.

Besides excision of the protein from an SDS-PAGE gel and administration of the protein (antigen) in this form, the electrophoretically separated protein can be excised from a nitrocellulose membrane and administered to the laboratory animal instead (Larsson and Nilsson, 1988). Proteins blotted onto nitrocellulose membrane are fixed, immobilised on the membrane in a particular configuration for a longer duration. This is reported to help to

stimulate a good immune response (Larsson and Nilsson, 1988). Nevertheless, the protein needs to be electroblotted first. Since the protein is transferred from one medium to another, there is also little certainty that all the protein has transferred onto the membrane, and, therefore, the amount of protein loaded onto the gel may not necessarily be the same as that bound to the nitrocellulose (Desai *et al*., 2001). Therefore, for antibody production, excision of the 33 kDa MT1-MMP PROcat protein band from a reducing SDS-PAGE gel and injection of the crushed SDS-PAGE-separated MT1-MMP protein was employed instead, as an additional strategy for raising antibodies.

In larger laboratory animals, such as rabbits, pigs and chickens, such a strategy is possible and more convenient. For easy administration, the gel slice may be broken up into smaller fragments, by passing the gel slice repeatedly between two syringes in a buffer. This procedure is difficult to perform, however, and can result in significant protein loss. An alternative is to subject the gel slice to freeze-drying or conventional freezing (-80ºC) and mechanically breaking it up into smaller pieces using a pestle or mortar. In the present study, conventional freezing was employed.

This approach was used, in an attempt to achieve greater purity of MT1-MMP PROcat from inclusion bodies for use in antibody production, compared to what was achieved using chromatography.

5.4.1 Reagents

Phosphate buffered saline (PBS), pH 7.4. (Section 2.9.1)

Solution A: Monomer solution [30% (m/v) acrylamide, 2.7% (m/v) bis-acrylamide]. (Section 2.3.1)

Solution B: 4 x Running gel buffer [1.5 M Tris-HCl, pH 8.8]. (Section 2.3.1)

Solution C: 4X Stacking buffer [500 mM Tris-HCl, pH 6.8]. (Section 2.3.1)

Solution D: 10% (m/v) SDS. (Section 2.3.1)

Solution E: 10% (m/v) Ammonium persulfate. (Section 2.3.1)

Solution F: Tank buffer [250 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (m/v) SDS]. (Section 2.3.1)

Reducing treatment buffer [125 mM Tris-HCl, pH 6.8, 4% (m/v) SDS, 20% (v/v) glycerol, 10% (v/v 2-mercaptoethanol]. (Section 2.3.1)

Bromophenol blue loading dye (0.1%). (Section 2.3.1)

5.4.2 Procedure

For antibody production against PEXcat (50 µg/ml) and PEX (50 µg/ml) domains of MT1-MMP, SDS was added to a final concentration of 0.5% (w/v) and the proteins were dialysed against PBS and injected in an unrenatured form. The urea-solubilised and S-Sepharose-purified PROcat domain of MT1-MMP was diluted in PBS to a final concentration of 50 µg/ml to be administered to the chicken, also in an unrenatured form.

The crude, urea-solubilised PROcat MT1-MMP extract from *E. coli* inclusion bodies, as described in Chapter 3 (Section 3.5), was prepared in reducing treatment buffer (Section 2.3) (1 mg/ml) and separated on a 10% SDS-PAGE gel and visualised by staining with 0.05% (m/v) Coomassie R250 (10 min, RT). The gel was washed several times with d.H_2O and the desired band was excised from the gel using a scalpel and frozen at -80ºC. The excised protein band was crushed into tiny fragments using a clean glass rod and re-suspended in ultra pure d.H_2O (1 ml) to facilitate administration to the laboratory animal. To lower toxicity, a maximum of two protein bands were used per chicken, each at a concentration between 40-56 µg/ml protein per band.

Chickens were injected intramuscularly (in the large breast muscle) with the antigen, either in polyacrylamide (50-100 µg/ml per chicken) or as a 1:1 (v/v) dilution with alum adjuvant (50 µg/ml per chicken). The antigen (500 µl) was administered at two sites with a maximum of 250 µl of the antigen solution injected at each site. Booster inoculations were administered in the same manner, 2, 4, 6 and 8 weeks after the initial immunisation. Eggs were collected daily and the IgY antibodies isolated from an egg every week (Section 2.7). One egg per week for each chicken was also reserved for an ELISA to determine the antibody titres (Section 2.9). Since two chickens were used per antigen, antibodies from the chicken, which produced the best immune response, were used in subsequent analysis.

5.4.3 Results

After lightly staining the gel with Coomassie R250, the MT1-MMP PROcat protein band was easily identified at the 33 kDa region (Figure 23) and was excised from the gel. The Coomassie R250 stain also revealed an *E. coli* protein positioned just above MT1-MMP at 36 kDa (Figure 23, lanes 2-5). Great care was, therefore, taken to avoid including this protein when the MT1-MMP band was excised. The short incubation time in the Coomassie R250 stain was also sensitive enough to pick up most of the remaining *E. coli* proteins present in the crude MT1-MMP PROcat samples (Figure 23, lanes 2-5) as well as the molecular weight marker (Figure 23, lane 1).

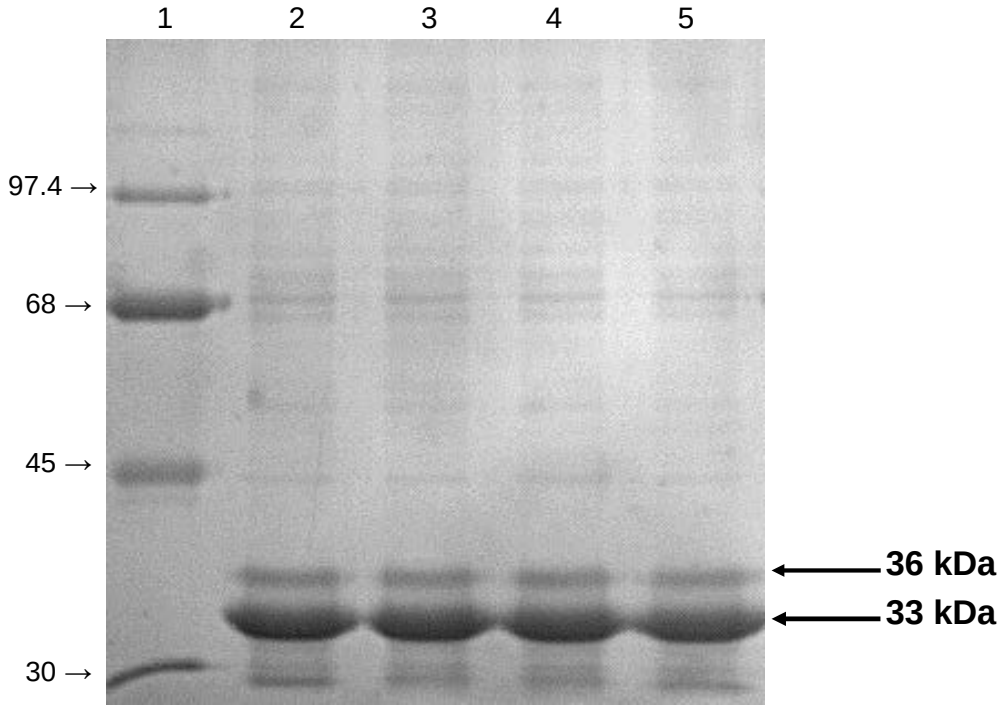

Figure 23. MT1-MMP PROcat separated from *E. coli* proteins on a reducing 10% (v/v) SDS-PAGE gel

Solubilised MT1-MMP PROcat inclusion bodies, prepared and separated as described in Section 5.4.2, were visualised with 0.05% (m/v) Coomassie R250 and the 33 kDa protein bands, each consisting of approximately 50 μg/ml protein, were excised from the gel and stored at -80˚C. Lane 1, molecular weight marker; lanes 2-4, crude urea-solubilised MT1-MMP PROcat domain.

5.5 ELISA assay of immune response of chickens

ELISA is a useful procedure to test recognition and allows selection of eggs with maximum titres to be selected for IgY isolation and further characterisation of these antibodies for specificity and immunoinhibition tests.

5.5.1 Reagents

Phosphate buffered saline (PBS), pH 7.4. (Section 2.9.1)

Blocking agent [5% (m/v) non-fat milk powder in PBS]. (Section 2.9.1)

0.5% (m/v) Bovine serum albumin (BSA) in PBS (BSA-PBS). (Section 2.9.1)

0.1% (v/v) Tween-PBS. (Section 2.9.1)

ABTS substrate [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in 150 mM citrate-phosphate buffer, pH 5.0]. (Section 2.9.1)

Stopping solution [0.1% (w/v) NaN_3 in citrate phosphate buffer]. (Section 2.9.1)

5.5.2 Procedure

The antibody response to the PROcat MT1-MMP antigen in the polyacrylamide gel, and alum adjuvant and the PEXcat and PEX antigens in alum adjuvant, was determined using an ELISA, as previously described (Section 2.9). IgY from eggs showing the highest titres were selected and IgY isolated from these eggs as well as from eggs isolated before inoculation (pre-immune), as described in Section 2.7.

5.5.3 Results

The chicken appeared to have a poor immune response against the PROcat MT1-MMP antigen in acrylamide (Figure 24 A). In contrast, when the PROcat MT1-MMP antigen was adminstered in alum, the chicken produced a potent immune response with the highest antibody titres observed 8 weeks after initial immunisation (Figure 24 B). When comparing the immune response to the PEXcat and PEX domains administered in alum (Figure 24 C and D), to that to the anti-PROcat IgY (Figure 24 B) the highest antibody response was induced by the PROcat domain and the lowest antibody response was seen against the PEX antigen (Figure 24 D). This suggests that the propeptide domain is possibly

more immunogenic or less homologous than the hemopexin-like domain, in agreement with the sequence alignment data (Section 3.1.2). Eggs showing the highest titres for each MT1-MMP antigen, were subsequently selected for IgY isolation (Section 2.7) and further characterisation for specificity (Section 5.6) and immunoinhibitory characteristics (Section 5.7).

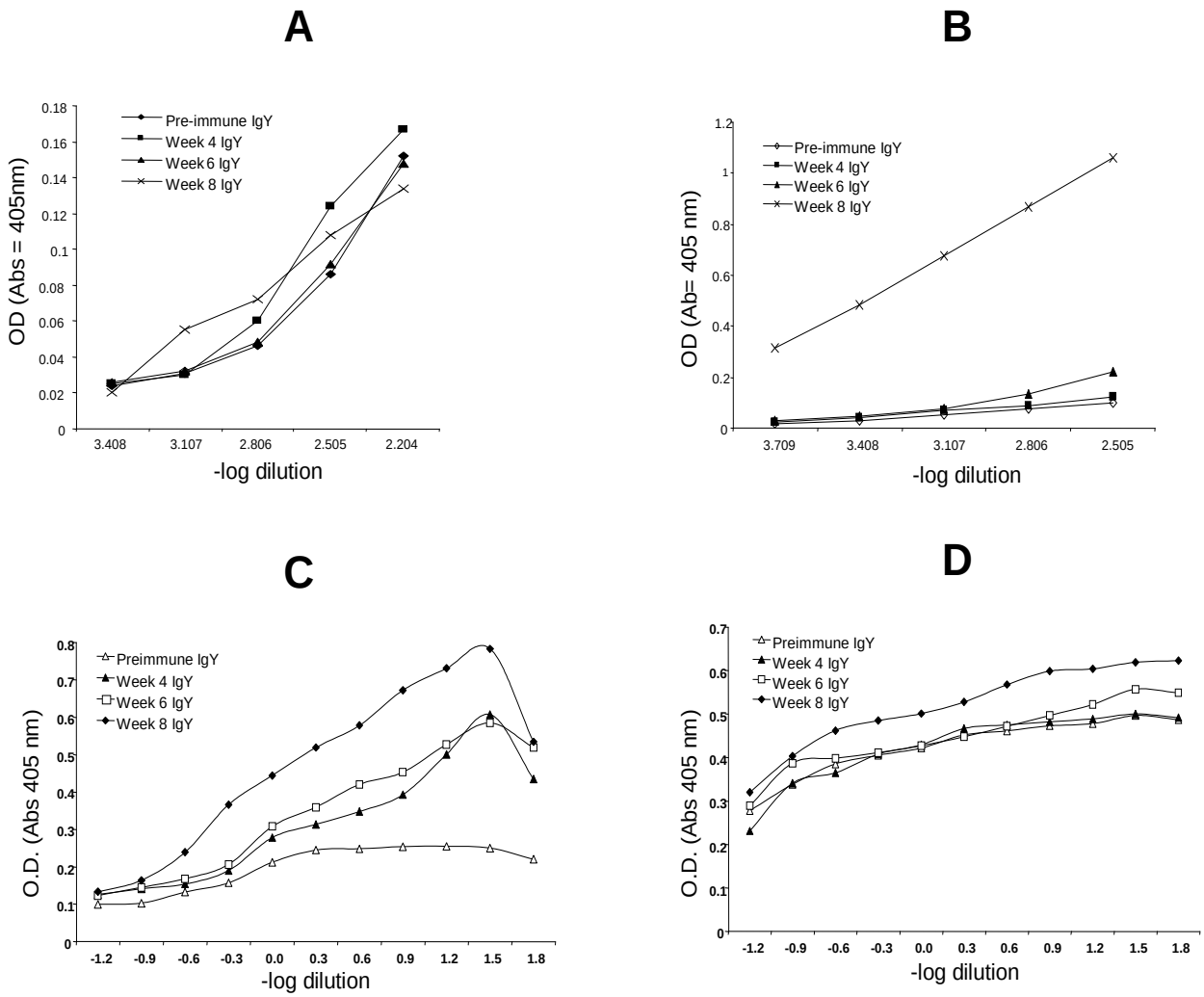

Figure 24. Immune response following immunisation of chickens.

The protein antigens used to inject chickens were PROcat in acrylamide (A), S-Sepharose purified PROcat in alum adjuvant (B), PEXcat in alum adjuvant (C) and PEX in alum adjuvant (D). Each MT1-MMP antigen was probed with antibodies produced 0, 4, 6 and 8 weeks following initial immunisation

5.6 Characterisation of anti-PROcat, PEXcat and PEX antibodies using western blotting

Western blotting was useful in checking the specificity of the antibodies raised against the PROcat antigen in acrylamide and alum adjuvant and the PEXcat and PEX domains in alum adjuvant. Although PROcat, in acrylamide, induced a weak immune response in the chicken, it was considered useful to test these antibodies further to see if, even at low titres, they recognised the MT1-MMP antigen more specifically than those raised using an alum adjuvant. As a first step, therefore, the recognition of these four antibodies was compared [antiPROcat (alum), anti-PROcat (acrylamide), anti-PEXcat (alum) and anti-PEX (alum)].

Besides recognising the PROcat, PEXcat and PEX domains specifically, for therapeutic immunoinhibition studies on human cancer cell lines, the antibodies raised against the three truncated MT1-MMP forms need to be able to detect the native human enzyme *in vivo*. To test possible recognition of this, the antibodies were used to check the recognition of MT1-MMP in the MCF10A cell line and its *ras*-transfected counterpart (MCF10A-neoT), cell lines which may be used in future invasion studies and are reported to be rich in MT1-MMP (Gilles *et al*., 2001).

To check cross reactivity with other MMPs, the antibodies were also used to probe sources rich in human MMPs, such as human blood, which primarily contains MMP-2 and MMP-9 (Wysocki *et al*., 1993; Freitas *et al*., 1999). The J774 mouse macrophage cell line also produces a variety of MMPs, including MMP-2, MMP-9, MMP-12, MMP-13 and MT1-MMP (Gibbs *et al*., 1999; Shapiro, 1999). Blotting of homogenates and probing with the various antibodies may, therefore, also be used to check cross reactivity with MMPs within and across species (using mouse antigens). The antibodies were raised against three different regions of MT1-MMP, two of which have the catalytic domain in common (i.e. PROcat and PEXcat) and so it was considered interesting to see if there is cross reactivity of the antibodies with the different MT1-MMP antigen domains as well. Such characterisation of antibodies is essential to establish the specificity and lack of cross-reactivity of the raised antibodies.

5.6.1 Reagents

Blotting buffer. (Section 2.8.1)

Ponceau S stain [0.1% (m/v) Ponceau S in 1% (v/v) acetic acid]. (Section 2.8.1)

Tris buffered saline (TBS), pH 7.4. (Section 2.8.1)

Blocking agent [5% (m/v) non-fat milk powder in TBS]. (Section 2.8.1)

0.5% (m/v) Bovine serum albumin (BSA) in PBS (BSA-TBS). (Section 2.8.1)

Alkaline phosphatase buffer (50 mM Tris-HCl buffer, 5 mM $MgCl_2$, pH 9.5. (Section 2.8.1)

Bromo-chloro-indolyl phosphate (BCIP) substrate solution [50 mM bromo-chloro-indolyl phosphate]. (Section 2.8.1)

Nitro-blue tetrazolium (NBT) substrate solution [100 mM nitro-blue tetrazolium]. (Section 2.8.1)

BCIP-NBT substrate solution. (Section 2.8.1)

5.6.2 Procedure

Since the highest antibody titres were observed 8 weeks after the inital immunisation with the PROcat antigen in alum, these antibodies were used in western blotting. Although the highest immune response could not be determined for PROcat administered in acrylamide, the week 8 antibodies were also used so that the results were comparable. The crude PROcat (25 µg/ml), S-Sepharose purified PROcat (25 µg/ml) and untransformed *E. coli* cells (25 µg/ml) were probed with the IgY antibodies (10 µg/ml). The pre-immune IgY (10 µg/ml) was used as a negative control. After western blotting was performed, as outlined in Section 2.8, and the results compared, anti-PROcat antibodies were selected for further study and comparisons were made with other antibodies against various MT1-MMP domains.

The anti-PROcat (10 µg/ml), anti-PEXcat (20 µg/ml) and anti-PEX (20 µg/ml) antibodies with the highest titres (i.e. week antibodies) were subsequently used to probe human blood (1:1000), mouse macrophage, untransformed *E. coli* cells (25 µg/ml), unrenatured PROcat and PEXcat recombinant antigens (25 µg/ml), the renatured PROcat MT1-MMP (35 µg/ml) and homogenates of the MCF10A and MCF10A-neoT human cell lines for MT1-MMP recognition.

Mouse macrophage and the human cell line homogenates, of unknown concentration, were prepared by diluting the pellets in reducing treatment buffer (120 µl). Pre-immune IgY (20 µg/ml) was used as a negative control. To compare cross reactivity of crude and purified IgY, the same protein antigens were also probed with crude anti-PROcat IgY, anti-PEXcat and PEX IgY, each antibody at a 1:100 dilution. Western blotting procedure was conducted, as described in Section 2.9. In order to check whether any of the bands detected with the anti-PROcat, PEXcat and PEX antibodies are not MMP-2 and MMP-9 processed forms, and how similar domains in sucn MMPs are compared to those in the expressed PROcat, PEXcat and PEX proteins, we checked the cross reactivity of previously raised anti-MMP-2 and MMP-9 IgY antibodies with the target MT1-MMP domains against which antibodies were raised in this study.

5.9.2 Results

In the case of antibodies raised against the PROcat antigen, using the alum adjuvant, the antibody was shown to recognise crude and purified 33 kDa form (Lichte *et al*., 1996) of the PROcat MT1-MMP (Figure 25 A, alum, lanes 1 and 2), only weakly cross-reacting with proteins from *E. coli* (Figure 25 A, alum, lane 3). The pre-immune IgY from the same chicken, recognised a band at about 34 kDa in the crude PROcat and untransformed *E. coli* samples (Figure 25 B, alum, lane 1 and 3, respectively), but not in the S-Sepharose purified sample (Figure 25 B, alum, lane 2), possibly indicating that the S-Sepharose fraction is free of detectable levels of *E. coli* proteins. IgY antibodies to *E. coli* proteins, therefore, seem to be present before inoculation with the PROcat antigen in alum. Previous data had also shown the presence of a 34 kDa protein in the untransformed *E. coli* cells (Section 3.5, Figure 18, lane 2) and, hence, agrees with this data. Therefore, the antibodies raised against the PROcat MT1-MMP domain using alum adjuvant successfully detected the antigen but, being polyclonal, also cross-reacted with *E. coli* antigens (to a minor degree) (Figure 25 A, alum).

Antibodies raised against PROcat in acrylamide, on the other hand, recognised a 34 kDa protein in the crude PROcat sample (Figure 25 A, acrylamide, lane 1) and many bands in the untransformed *E. coli* cells (Figure 25 A, acrylamide, lane 3), but not in the S-Sepharose

purified protein sample (Figure 25 A, acrylamide, lane 2). This confirms the interpretation that the S-Sepharose fraction contained undetectable amounts of contaminating *E. coli* protein. A similar detection pattern was observed for the pre-immune IgY (Figure 25 B, acrylamide). Therefore, these chickens completely failed to produce an antibody response against the MT1-MMP (PROcat) antigen in acrylamide. Only the alum antibodies were, therefore, used in subsequent studies.

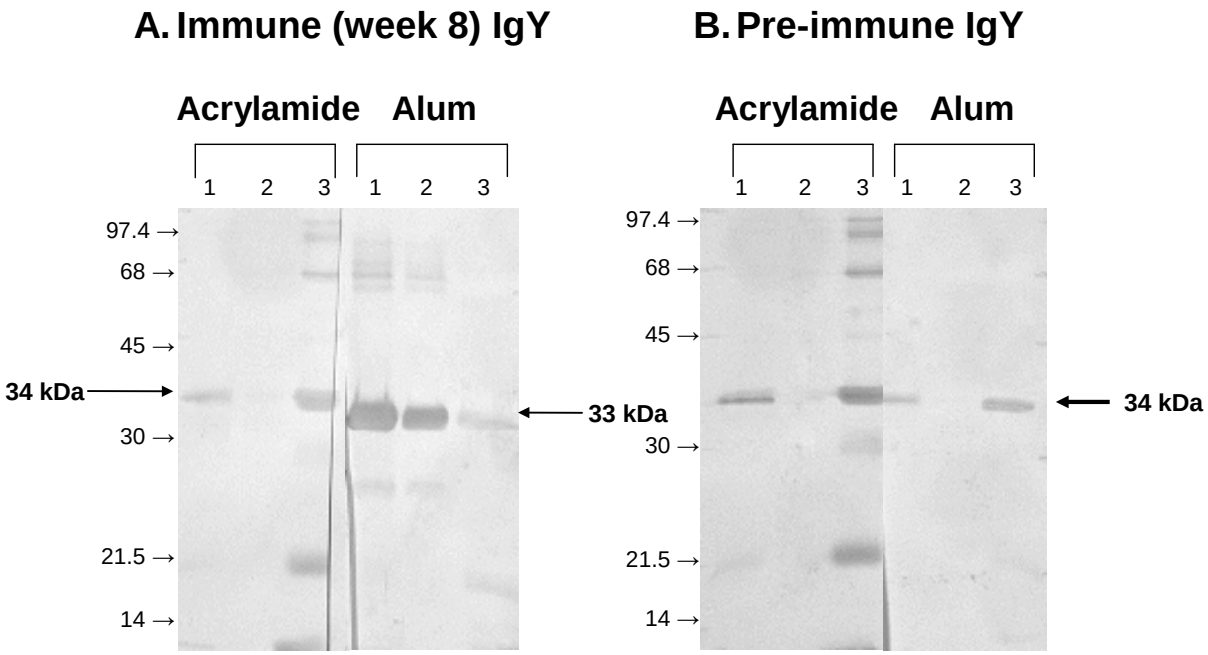

Figure 25. Western blot analysis of the anti-PROcat IgY (week 8) raised in chickens in acrylamide or in alum adjuvant**.**

The immune (week 8) IgY (panel A) and the pre-immune IgY (panel B) were probed against crude MT1-MMP (lane 1), MT1-MMP purified on an S-Sepharose column (lane 2) and against untransformed *E. coli* cells (lane 3). The protein samples (25 µg/ml) were separated on a 12.5% SDS-PAGE gel with reduction, electroblotted onto nitrocellulose (200 mA, 16 h), probed with the primary IgY antibodies (10 µg/ml) (2h, RT) and again probed with the rabbit anti-chicken IgG-alkaline phosphatase secondary antibody (1:150 000 dilution) (1 h, RT). A colour reaction was developed with a BCIP-NBTS substrate catalysed by alkaline phosphatase.

When antibodies raised using alum adjuvant were tested simultaneously, under the same conditions to allow better comparison, the anti-PROcat IgY was shown to detect a 33 kDa protein in the crude (Figure 26 A, lane 4) and S-Sepharose purified samples (Figure 26 A, lane 5), as seen previously for the MT1-MMP PROcat expressed by Lichte *et al*. (1996). Similar results were shown previously (Figure 25, alum, lanes 1 and 2). Slight differences in the intensity of cross reactivity with various *E. coli* proteins, between the two blots, may be due to differences in transfer efficiency from blot to blot, as the same concentration of antigen was used in both cases (Figures 25 and 26, lane 3). The anti-PEXcat IgY and the anti-PEX IgY recognised a 34 kDa protein in untransformed *E. coli* cells and the crude PROcat extract (Figure 26 B and C, lanes 3 and 4). Since the PEX pre-immune antibodies recognised a 34 kDa protein present also in untransformed *E. coli* cells (Figure 26 C, lane 3), similar molecular weight to the expressed 33 kDa PROcat MT1-MMP, and gave the same pattern, suggests that only the anti-PROcat was successful in detecting the 33 kDa expressed PROcat (Figure 26 A, lanes 4 and 5). All three antibodies failed to recognise renatured PROcat MT1-MMP, with an expected molecular weight of 23 kDa (Figure 26 A, B and C, lane 6) (Lichte *et al*., 1996), previously seen during zymographic analysis (Figure 21, lane 8) [perhaps as these are present at low levels on the nitrocellulose membrane].

The crude anti-PROcat IgY, however, detected a 29 kDa protein (Figure 27), as previously seen on the zymogram (Figure 21 A, lanes 5-7), which was not detected by the purified IgY. This protein possibly resembled an intermediate form produced during protein renaturation of PROcat construct into the active enzyme (Lichte *et al*., 1996), but could also be a contaminating *E. coli* protein. The crude, pre-immune IgY showed no such bands (Data not shown). In contrast, the crude anti-PEXcat and anti-PEX IgY (results not shown) showed a similar banding pattern to the purified IgY (Figure 26 B and C).

The anti-PROcat and the anti-PEX IgY recognised a 50 kDa PEXcat antigen (Figure 26 A and C, lane 7), which was not detected by the anti-PEXcat IgY (Figure 26 B, lane 7), despite the latter antibody being raised against this protein antigen. The anti-PROcat possibly recognised the catalytic domain, while the anti-PEX IgY possibly recognised the hemopexin-like domain (Figure 26 A and C, respectively).

Although the anti-PROcat and the anti-PEX IgY detected various recombinant MT1-MMP domains, these antibodies did not bind native MT1-MMP in either the J774 mouse macrophage (Figure 26 A and C, lane 2) or the MCF 10A and MCF10A-neo T human cell lines (Figure 26 A and C, lanes 8-11). In contrast, the anti-PEXcat IgY, which failed to detect recombinant forms of MT1-MMP, recognised a 45 kDa protein in the mouse macrophage (Figure 26 A, lane 2) and a 43 kDa protein in the MCF10A and MCF10A-neoT cell lines (Figure 26 A, lanes 9 and 11, respectively), both of which possibly resembled the 43 kDa processed form of MT1-MMP produced following the activation of proMMP-2 *in vivo* (Lehti *et al*., 1998). The difference in molecular weight observed between the mouse and human species for processed MT1-MMP may be due to differences in glycosylation.

There did not seem to be any cross reactivity between any of the anti-MT1-MMP antibodies and proMMP-2 (72 kDa) and proMMP-9 (92 kDa) in human blood (Figure 26 A, B and C, lane 1). The anti-MT1-MMP (PROcat) IgY, however, did recognise a 62 kDa protein, though extremely weakly (Figure 26 A, lane 1). Binding to a protein of this molecular weight may indicate binding to latent, soluble MT1-MMP (Table 1) (Kazes *et al*., 2000). Alternatively, the catalytic domain is conserved across the MMPs classes (Section 3.1.2) and the catalytic domain recognised may be that of mature MMP-2 (i.e. 62 kDa) (Table 1) (Visse and Nagase, 2003). This data also agrees with the sequence alignment data which suggested that the antibodies targeting the PRO and catalytic domains of MT1-MMP were likely to cross react with other MMPs (Section 3.1.2). The pre-immune IgY from the chicken inoculated with PEXcat antigen also cross reacted with a 62 kDa protein in human blood (Figure 26 B), however, also possibly mature MMP-2 or latent MT1-MMP. This cross reactivity is, therefore, harder to explain.

Of all three antibodies, the anti-MT1-MMP (PEX) IgY appeared to be of poor quality and was subsequently not used in immunocytochemistry studies.

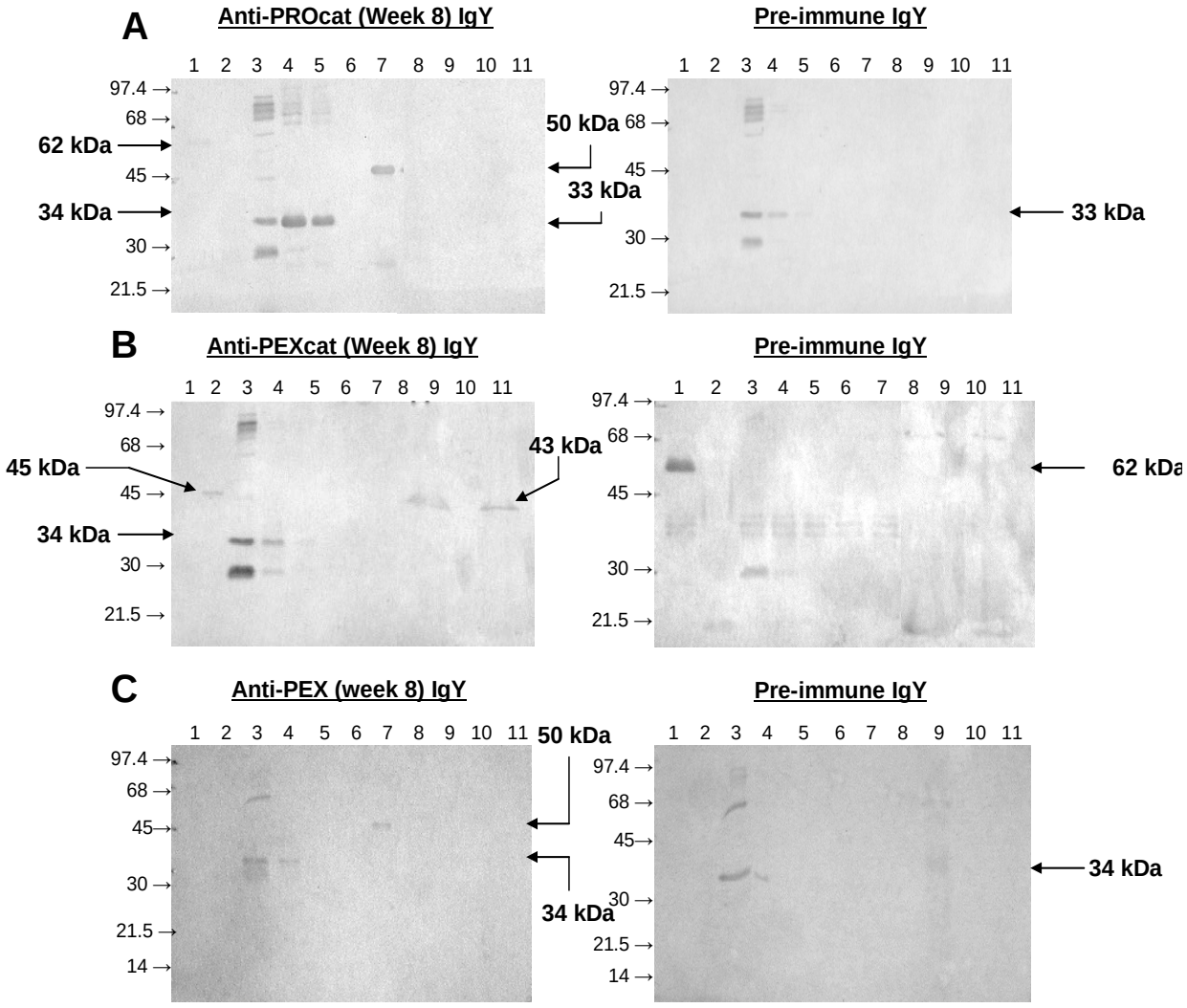

Figure 26. Characterisation of anti-PROcat, anti-PEXcat and anti-PEX MT1-MMP antibodies raised in chickens in alum adjuvant**.**

A. The antigens were probed with 10 µg/ml of the antibody in 0.5% (m/v) BSA-TBS (2 h) B and C. The antigens were probed with 20 µg/ml of the antibodies in 0.5% (m/v) BSA-TBS (2 h). Following incubation with the primary antibody, the protein antigens were also probed with the rabbit anti-chicken IgG-alkaline phosphatase at a 1:150 000 dilution (1 h) and a colour reaction was developed with the NBT-BCIP substrate, catalysed by alkaline phosphatase. Lane 1, human blood sample; lane 2, LPS activated J774 mouse macrophage; lane 3, untransformed *E. coli* cells; lane 4, crude MT1-MMP (PROcat); lane 5, S-Sepharose purified MT1-MMP (PROcat); lane 6, renatured MT1-MMP (PROcat); lane 7, Ni-NTA-purified and unrenatured MT1-MMP (PEXcat); lane 8, MCF 10A-neoT pellet; lane 9, MCF 10A-neoT supernatant; lane 10, MCF 10A pellet; lane 11, MCF 10A supernatant

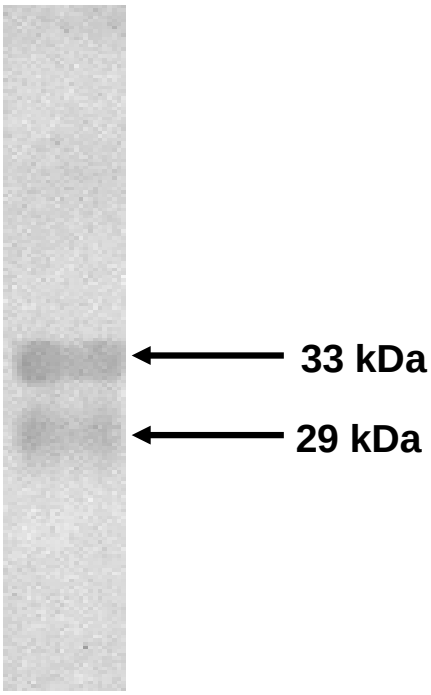

Figure 27. Detection of an intermediate form of renatured PROcat MT1-MP by crude anti-PROcat IgY.

The crude antibody dilutions were prepared by diluting the egg yolk 1:3 in 100 mM Na-phosphate buffer and then preparing a final antibody dilution of 1:100 in 0.5% (m/v) BSA-TBS. Following incubation with the primary antibody, the renatured MT1-MMP (PROcat) antigen was also probed with the rabbit anti-chicken IgG-alkaline phosphatase at a 1:150 000 dilution and a colour reaction was developed with the NBT-BCIP substrate catalysed by alkaline phosphatase.

Both the anti-MMP-9 IgY, raised against recombinant human proMMP-9 (whole protein), and anti-MMP-2 IgG mouse monoclonal antibodies cross reacted with an MMP-2-like 72 kDa protein in the human blood sample, resembling proMMP-2 (Figure 28 A and B, lane 1). This suggests that these antibodies are species-specific as they did not recognise proMMP-2 in the mouse. Neither antibodies detected mature MMP-2. The anti-MMP-9 antibodies, on the other hand, failed to recognise MMP-9 (92 kDa) in human blood or mouse homogenates, however, they did cross-react with *E. coli* proteins (Figure 28 B, lane 3) and MCF10A cell homogenate (Figure 28 B, lane 9). Both anti-MMP-2 and MMP-9 antibodies did not recognise the PROcat, PEXcat and PEX MT1-MMP antigens (Figure 28 A and B) suggesting that these domains are immunologically not that similar to those of MMP-2 and MMP-9.

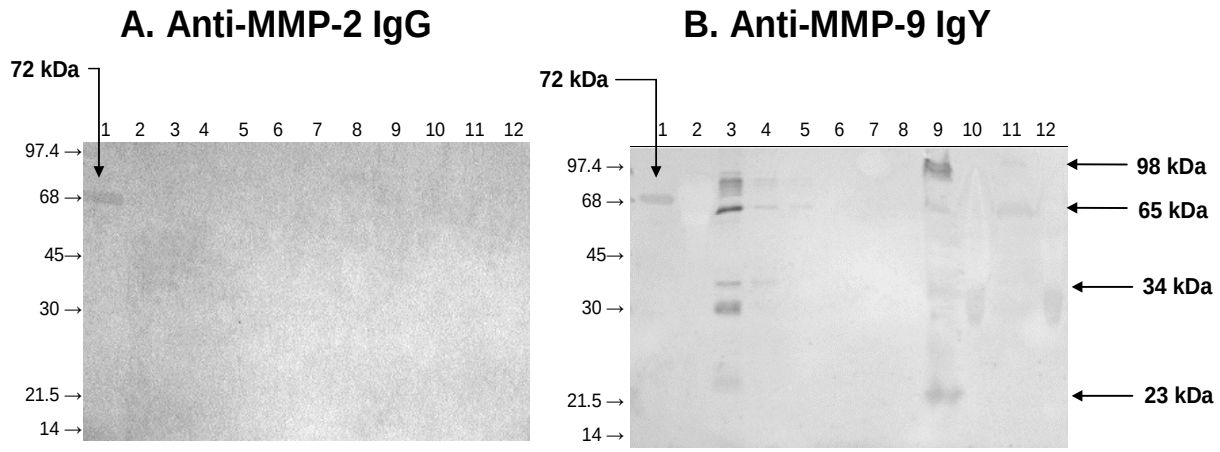

Figure 28. Cross reactivity of MMP-2 IgG and MMP-9 IgY with PROcat, PEXcat and PEX antigens used for raising antibodies.

A. The antigens were probed with anti-MMP-2 monoclonal IgG at a 1:100 dilution in 0.5% (m/v) BSA-TBS (2h). B. The antigens were probed with anti-MMP-9 polyclonal IgY at a concentration of 20 µg/ml in 0.5% (m/v) BSA-TBS (2h). Following incubation with the primary antibody, the protein antigens were also probed with the rabbit anti-chicken IgG-alkaline phosphatase at a 1:150 000 dilution and a colour reaction was developed with the NBT-BCIP substrate catalysed by alkaline phosphatase. Lane 1, human blood sample; lane 2, LPS activated J774 mouse macrophage; lane 3, untransformed *E. coli* cells; lane 4, crude MT1-MMP (PROcat); lane 5, S-Sepharose purified MT1-MMP (PROcat); lane 6, renatured MT1-MMP (PROcat); lane 7, purified MT1-MMP (PEXcat); lane 8, chicken CAM, 9 MFC10A-neoT pellet; lane 10, MCF10A-neoT supernatant; lane 11, MCF 10A pellet; lane 12, MCF 10A supernatant.

5.10 Immunoinhibition of renatured PROcat MT1-MMP

Before the antibodies were applied to immunolabelling of cells and labelling was used to establish the distribution of MT1-MMP on the surface of MCF10A-neoT cells and, hence, a potential target for immunoinhibition (i.e. accessible to antibodies), it was important to assess the inhibitory properties of the antibodies. Immunoinhibition experiments with the anti-PROcat, anti-PEXcat and anti-PEX IgY were, therefore, performed over a range of antibody concentrations (the anti-PEX IgY being used as a control antibody as it should not inhibit the renatured PROcat enzyme as it targets a domain not present in the PROcat construct). Since the crude anti-PROcat IgY appeared to possibly recognise an intermediate form of renatured PROcat MT1-MMP (Lichte *et al*., 1996) (Section 5.9.2) (Figure 27), while purified IgY, failed to detect this protein, the inhibitory potential of the crude IgY was also examined.

5.10.1 Reagents

MMP assay buffer [20 mM Tris-HCl, pH 7.3, 100 mM NaCl, 5 mM $CaCl_2.2H_2O$, 100 µM $ZnCl_2$, 0.02% (m/v) NaN_3]. Tris (1.21 g) and NaCl (2.92 g) were dissolved in 450 ml $d.H_2O$, adjusted to pH 7.3 with HCl, $CaCl_2.2H_2O$ (0.37 g), $ZnCl_2$ (0.007 g) and NaN_3 (0.1 g) were added the volume made up to 500 ml.

AMC-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2.ACOH stock solution (915 µM). Substrate (1 mg) was dissolved in 1 ml DMSO and stored at -20ºC.

Substrate working solution (50 µM). Substrate stock solution (273 µl) was made up to 5 ml with $d.H_2O$ and stored at 4ºC.

Antibody diluent [400 mM sodium phosphate, 0.02% NaN_3 , 0.1% (m/v) BSA, pH 7.6]. $NaH_2PO_4.H_2O$ (13.8 g) and BSA (0.25 g) were dissolvd in 200 ml $d.H_2O$, adjusted to pH 6.0 with NaOH, NaN_3 (0.05 g) was added and the solution made up to 250 ml.

5.10.2 Procedure

Immunoinhibition of renatured PROcat MT1-MMP activity was measured on a continuous basis using the protocol of Coetzer *et al*. (1991), with minor modifications. Anti-PROcat, anti-PEXcat and anti-PEX (2 mg/ml) purified IgY were made up in the antibody diluent, serially diluted (50 µl, 2 mg/ml - 0.0156 mg/ml) in a FluorNunc microtitre plate and pre-incubated with MT1-MMP in MMP assay buffer (330 ng, 50 µl, 15 min, 30˚C). Similarly, crude IgY was also serially diluted, but since this was a crude preparation of egg yolk (Section 2.9) of unknown antibody concentration, it was prepared as a dilution in the antibody diluent (1:10-1:1280 dilution). Additional MMP assay buffer was added to each well and incubated (50 µl, 2 min, 30ºC) to keep the enzyme stable and to initiate MT1-MMP activity. The substrate working solution was added (25 µM, 50 µl) and the fluorescence was measured (excitation of 320 nm, emission of 420 nm, 10 min). The enzyme activity was calculated, as described in Section 4.1.3, and the antibody concentration was plotted against time. The percentage immunoinhibition was subsequently determined by expressing the difference in enzyme activity of the pre-immune IgY and immune IgY, as a percentage of the enzyme activity of the pre-immune IgY, for each antibody concentration, as follows:

% immunoinhibition = [(pre-immune IgY – immune IgY) / pre-immune IgY] x 100

and plotting the log of the concentration of antibody (in µg/ml) or –log of the antibody dilution against % immunoinhibition.

5.10.3 Result

Both the purified and crude antibodies showed inhibition of PROcat MT1-MMP enzyme activity (Figure 29 A and B, respectively). The purified IgY (Figure 29 A), overall, appeared to be more effective than the crude IgY (Figure 29 B), particularly for the anti-PEXcat IgY (Figure 29 A). Of the purified antibodies (Figure 29 A), the anti-PEXcat IgY showed the highest inhibition against the PROcat MT1-MMP enzyme (88%) at high and low antibody concentrations (i.e. at approximately 0.125 mg and 1 mg of antibodies). The second highest inhibition was seen with the anti-PROcat IgY (68%), but only at high antibody concentrations (i.e. 1 mg of antibody) (Figure 29 A), and only giving approximately 10% inhibition at the same lower antibody concentration (i.e. 0.125 mg) as that giving 88% with the PEXcat IgY (Figure 29 A). At lower dilutions (i.e. 1/10) (higher antibody concentration), the crude anti-PROcat IgY showed the highest inhibitory effect (60%), whereas the anti-PEXcat IgY inhibited more effectively (55%) at higher dilutions (i.e. 1/80) (lower antibody concentration) (Figure 31 B). For both crude anti-PROcat and anti-PEXcat IgY preparations (Figure 29 B), however, the inhibitory response was comparably lower than the purified IgY (Figure 29 A). The anti-PEX IgY, overall, appeared to give the lowest inhibition (Figure 29 A and B) with maximum inihibition of 35% induced by approximately 500 µg of purified IgY (Figure 29 A). This was expected as these antibodies were anticipated would not recognise the PROcat enzyme, due to the lack of the hemopexin-like domain. While the inhibitory effect of the purified anti-PROcat IgY generally increased with increasing antibody concentration, the inhibitory effect of the purified anti-PEXcat IgY was more or less consistent from the lowest (16 µg) to the highest (approximately 2 mg) antibody concentrations (Figure 29 A and B). This was not the case with the crude IgY (as mentioned). The anti-PEXcat IgY, overall, appeared to be more inhibitory compared to the anti-PROcat IgY (Figure 29 A and B). In light of these findings, the PEXcat antigen was considered to be most suitable for invasion studies in which antibodies are passed into the fertile eggs to test whether these are able to protect the embryo against invading cancer cells.

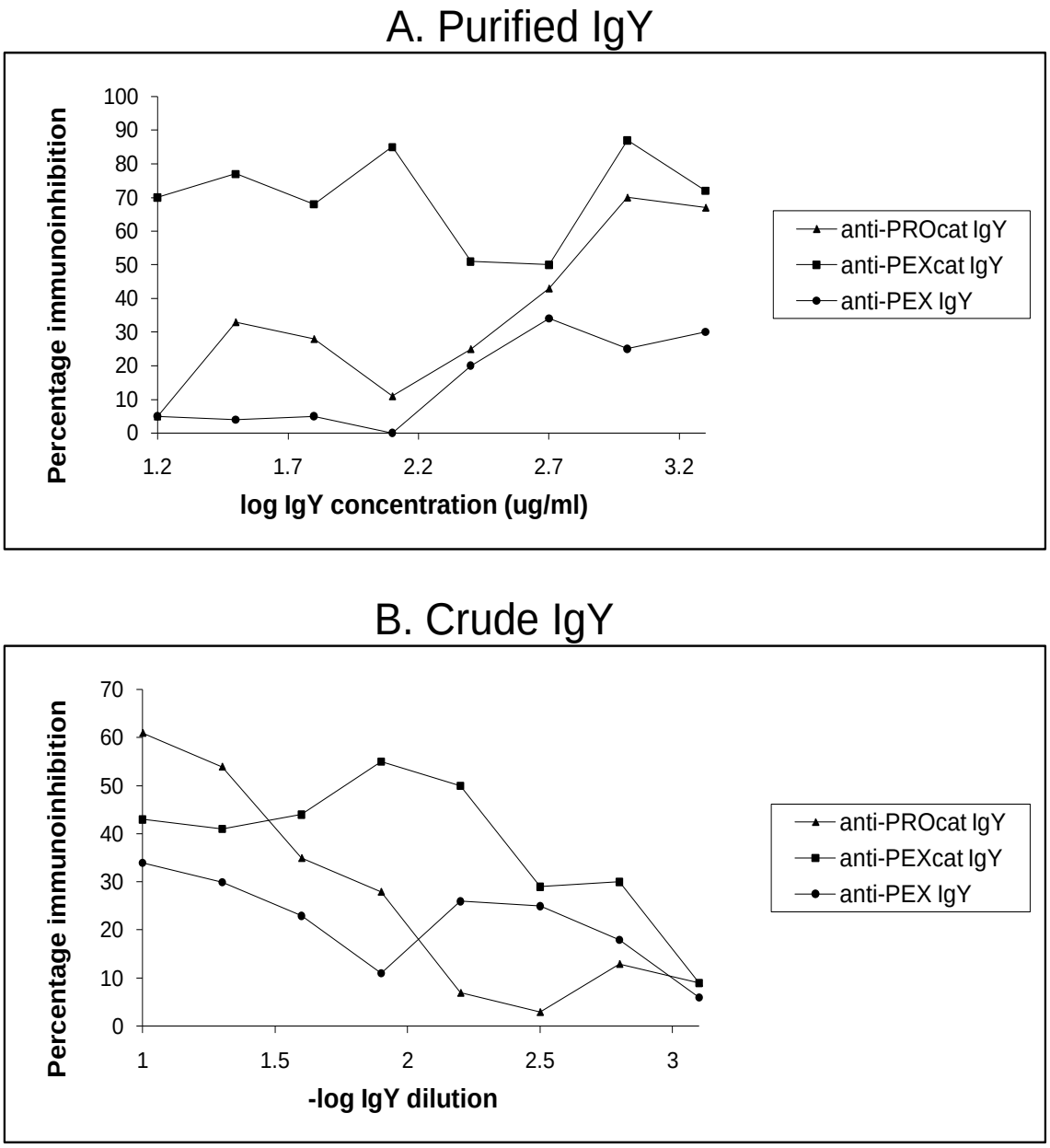

Figure 29. Percentage immunoinhibition of renatured PROcat MT1-MMP in the presence of PEG-purified (A) and crude (B) anti-PROcat, anti-PEXcat and anti-PEX IgY.

5.11 MT1-MMP distribution in the normal (MCF10A) and MCF10A-neoT cells

Since the anti-PEX IgY exhibited such a poor inhibitory response against PROcat MT1-MMP activity (Section 5.10), and weakly labelled MT1-MMP in western blotting (Section 5.9), only the anti-PROcat and anti-PEXcat IgY were considered for imunolablleing of human cell lines. Since MT1-MMP is up-regulated in MCF10A-neoT cells (Kim *et al*., 2007), this human cell line was used to identify the distribution pattern of MT1-MMP in invasive cells. Being a membrane-type MMP, MT1-MMP is reported to be carried in vesicles towards the plasma membrane, directed by its cytoplasmic tail, and to aggregate at the leading edge of the cell, to promote invasion (Yana and Seiki, 2002; Remacle *et al*., 2003). The primary localisation of MT1-MMP in invading cells, such as MCF10A-neoT, it was, therefore, anticipated should be on the cell surface and in invading lamellipodia.

The distribution of MT1-MMP was also determined for the MCF10A and MCF10A-neoT cell lines using fluorescence immunolabelling and confocal microscopy. An overview of labelling distribution is more easily gained using fluorescence microscopy and confocal microscopy than electron microscopy (EM) and it has the additional benefit of being able to "optically section" i.e. assess labelling within an optical slice of defined thickness. This is important as it allows apical versus basal labelling to be assessed and may be used to "guide" EM studies and sectioning.

5.11.1 Reagents

Phosphate buffered saline (PBS), pH 7.4 [8 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2.2H_2O$ and 0.5 mM $MgCl_2.6H_2O$, pH 7.4]. (Section 2.15)

16% (m/v) Paraformaldehyde (PFA) stock solution. (Section 2.15)

3.7% (m/v) Paraformaldehyde in PBS, pH 7.4. (Section 2.15)

1% (m/v) Bovine serum albumin (BSA) in PBS, pH 7.4. (Section 2.15)

0.1% (m/v) Saponin in PBS, pH 7.4. (Section 2.15)

Moviol. (Section 2.15)

5.11.2 Procedure

The MCF10A and the MCF10A-neoT cell lines were labelled with the anti-PROcat (40 µg/ml) and anti-PEXcat (40 µg/ml) IgY and image analysis was performed, as described in Section 2.15 on multiple respresentative cells.

5.11.3 Results

The anti-PROcat IgY (Figure 30), overall, appeared to label the MCF10A and MCF10A-neoT cell lines more intensely compared to the anti-PEXcat IgY (Figure 31). When MCF10A cells were labelled with anti-PROcat IgY, MT1-MMP was observed to be concentrated in podocytes (i.e. lamellipodia) with reduced labelling observed on the plasma membrane (Figure 30 A). In the transfected cells (MCF10A-neoT), however, anti-PROcat IgY labelling was not as intense and was more evenly spread across the invading lamellipodia and the plasma membrane (Figure 30 B). These observations were confirmed with image analysis (using imageJ) to determine the MT1-MMP distribution patterns (Figure 30 C). Image J analysis tool measures the relative fluorescence at a particular region in the cell and it showed here that the enzyme was largely present in lamellipodia in the MCF10A cell line, whereas in the MCF10A-neoT cell line, MT1-MMP was observed to be more or less evenly distributed between the lamellipodia and the plasma membrane (Figure 30 C).

The anti-PEXcat IgY labelled the MCF10A-neoT cells more intensely than the MCF10A cells and labelling intensity was observed to be more or less evenly distributed over the plasma membrane and in lamellipodia in both cell lines (Figure 31 A and B). This was confirmed when analysis was performed using the ImageJ analysis tool (Figure 31 C). Whereas the anti-PROcat IgY gave a greater amount of labelling of the MCF10A and the MCF10A-neoT cells overall, the anti-PEXcat IgY seemed to label the MCF10A-neoT transfected cell line more intensely than the MCF10A cells.

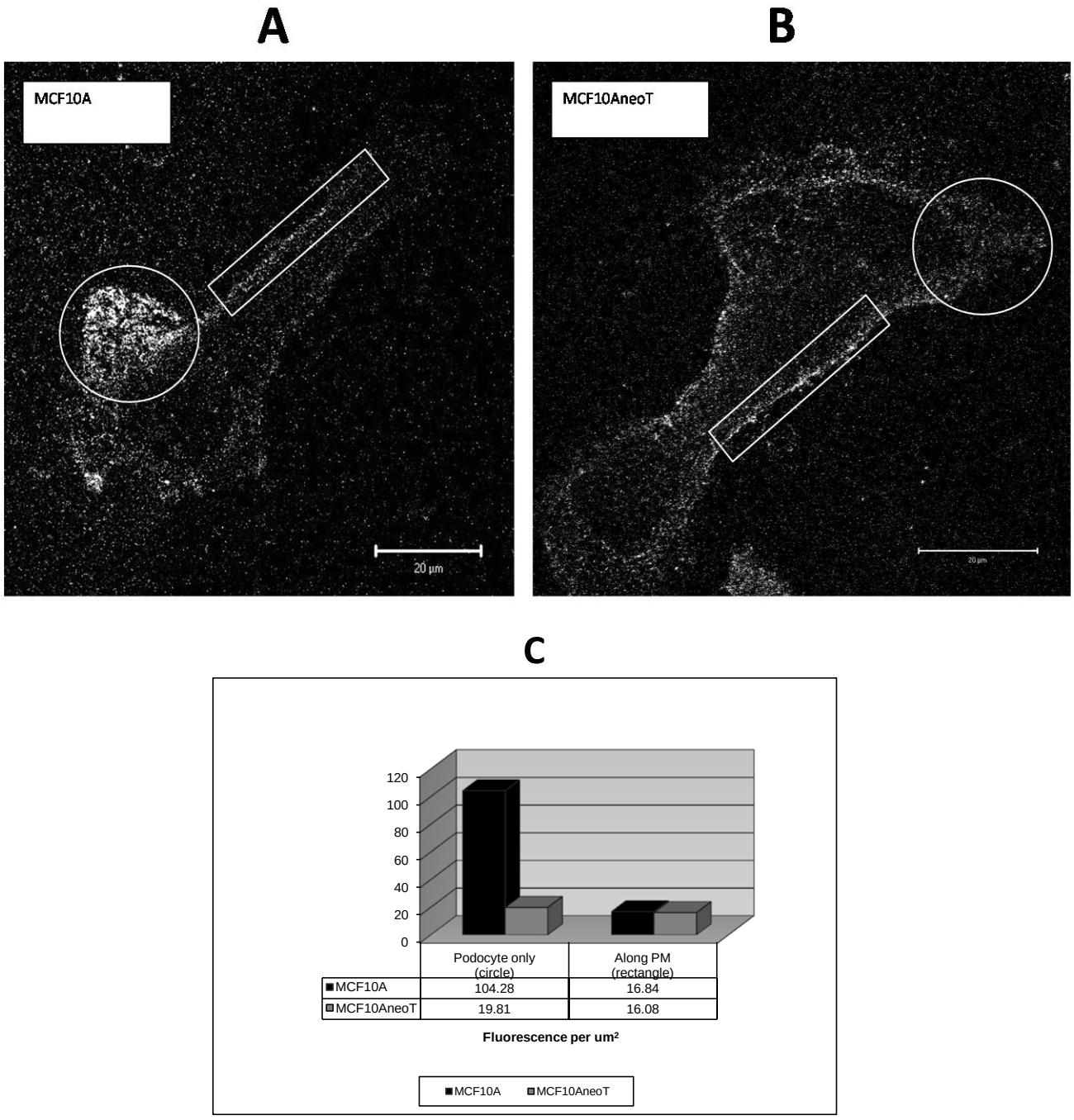

Figure 30. Localisation and distribution pattern of MT1-MMP in MCF10A (A) and MCF10A-neoT (B) human cell lines, labelled with anti-PROcat IgY, and viewed via confocal microcopy.

The cells were labelled with anti-PROcat IgY (40µg/ml). The distribution of MT1-MMP was confirmed using the imageJ analysis tool, which measured the relative fluorescence at a particular region (C).

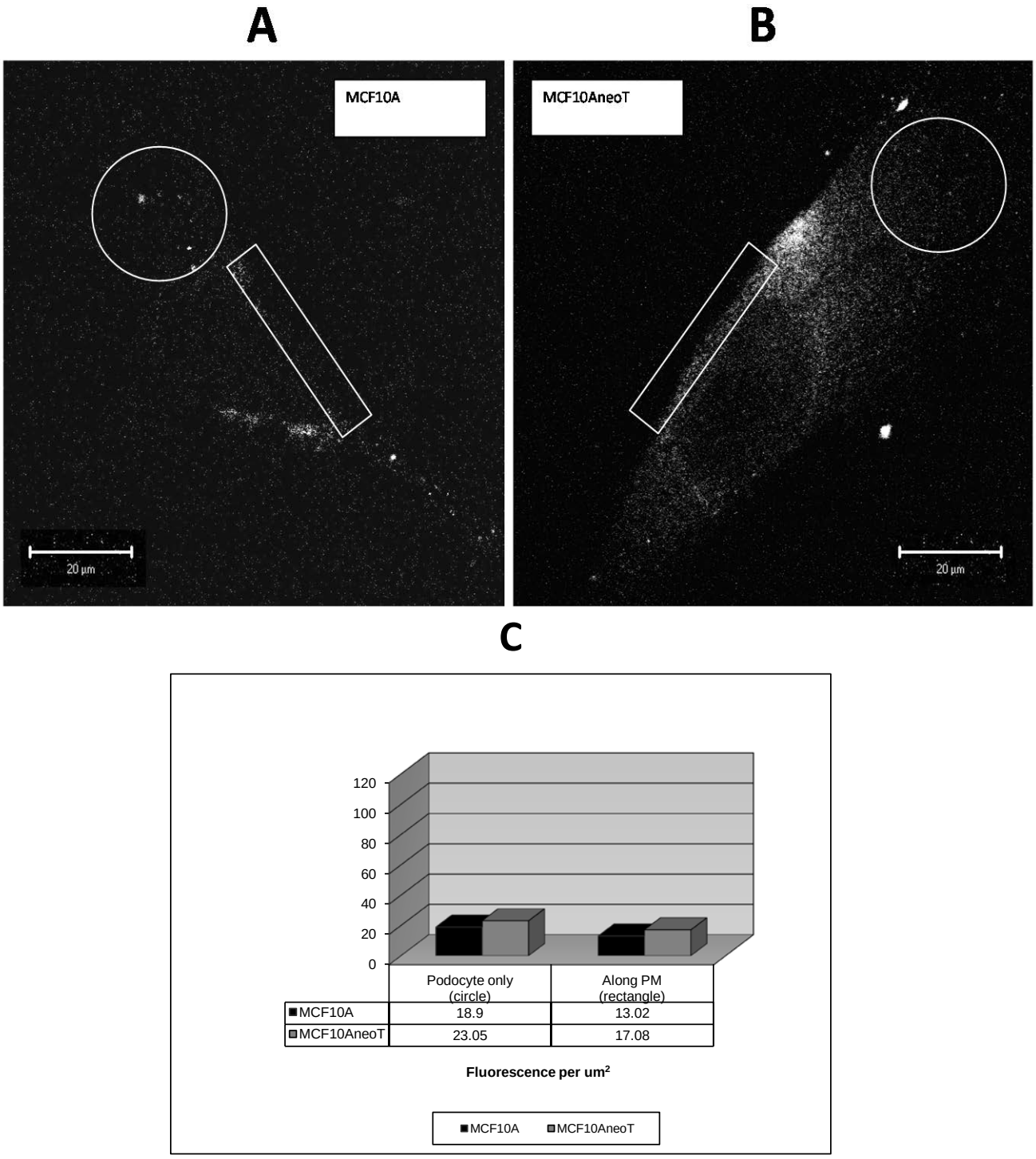

Figure 31. Localisation and distribution pattern of MT1-MMP in MCF10A (A) and MCF10A-neoT (B) human cell lines, labelled with anti-PEXcat IgY, and viewed via confocal microcopy.

The cells were labelled with anti-PEXcat IgY (40µg/ml). The distribution of MT1-MMP was confirmed using the imageJ analysis tool, which measured the relative fluorescence at a particular region (C).

5.12 Localisation of MT1-MMP in MCF10A-neoT cell line

MT1-MMP turnover is controlled by intracellular trafficking of the enzyme through the endoplasmic reticulum (ER), trans Golgi network (TGN) via some as yet unidentified cellular compartments to and from the cell surface (Remacle *et al*., 2003; Jiang *et al*., 2004). The enzyme would, therefore, be anticipated to be found inside the cell as well associated with the ER and TGN, as well as in vesicular compartments.

5.12.1 Reagents

Blocking agent (FBG): 1% (v/v) fish skin gelatin, 0.8% (m/v) BSA, 20 mM gelatin in PBS. (Section 2.14)

Phosphate buffered saline (PBS), pH 7.4. (Section 2.14)

1% (m/v) Bovine serum albumin (BSA) in PBS (BSA-PBS). (Section 2.14)

1% (v/v) Glutaraldehyde in PBS. (Section 2.14)

2% (m/v) Uranyl acetate. (Section 2.14)

1M NaOH. (Section 2.14)

Lead citrate. (Section 2.14)

5.12.2 Procedure

The MCF10A-neoT cell line was labelled with anti-MT1-MMP (PROcat) (40 µg/ml) and anti-MT1-MMP (PEXcat) (40 µg/ml) IgY as described in Section 2.14.

5.12.3 Result

Labelling with both the anti-PROcat and anti-PEXcat antibodies showed a very similar labelling distribution pattern, although there was more labelling observed with the anti-PROcat IgY (Figure 32) in the respective areas than the anti-PEXcat IgY (Figure 33). On the cell surface, the antibodies were localised primarily on the edges of protrusions resembling lamellipodia, as would be anticipated in invading cells, though some MT1-MMP did seem to be generally associated with the outer cell membrane (i.e. not only in lamellipodia) (Figure 32 and 33) (larger arrows). Inside the cell the antibodies were present either on the membrane or within vesicles of varying size as well as associated with tubule-like structures

possibly resembling the ER. This distribution pattern seems to concur with described intracellular trafficking of MT1-MMP and suggests the antibodies possibly recognise the mature, processed and latent forms of the enzyme.

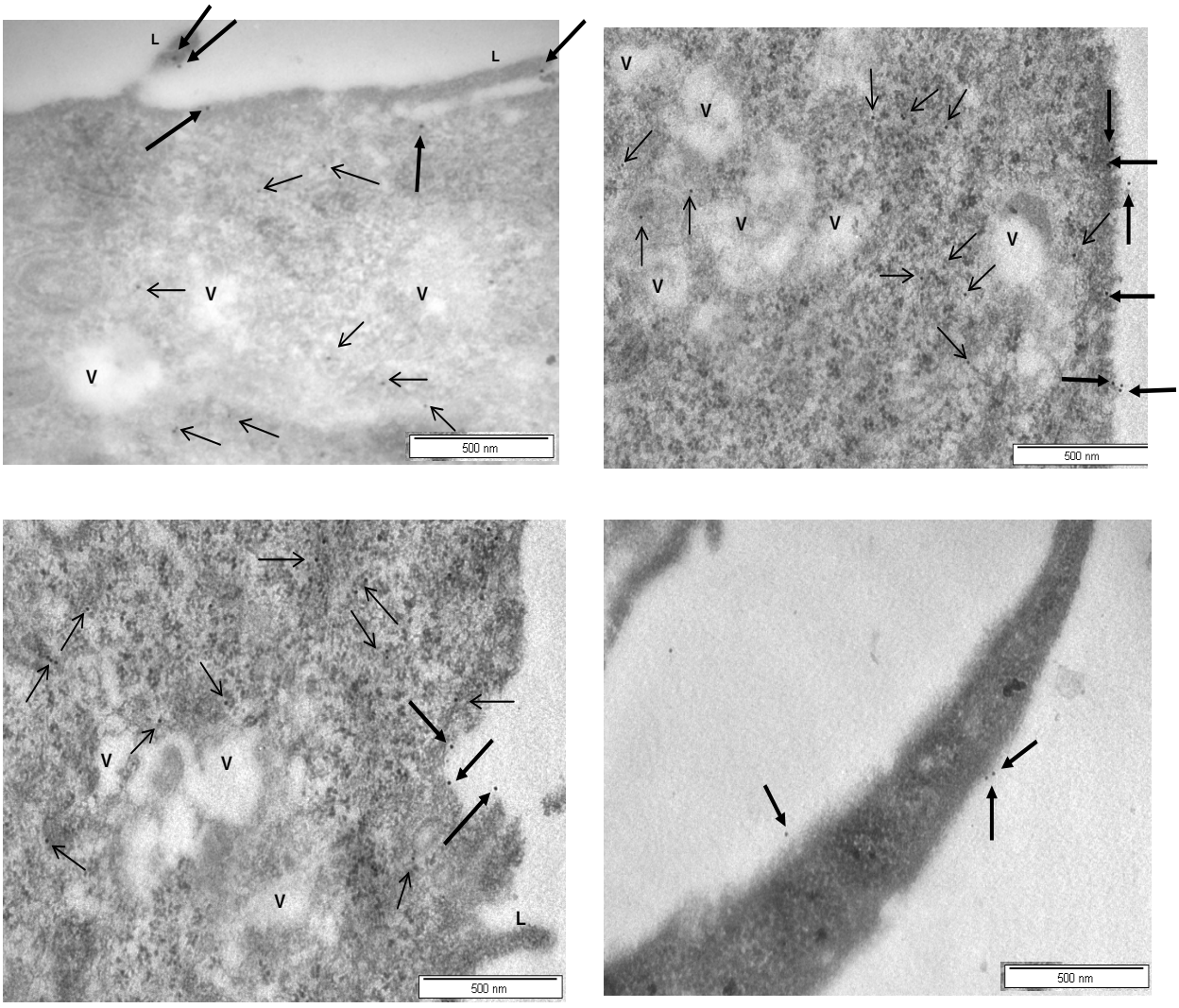

Figure 32. Immunolabelling for MT1-MMP PROcat in MCF10A-neoT cell line.

The tissue was labelled with anti-MT1-MMP (PROcat) IgY (40 µg/ml). The labelling was identified with a secondary rabbit anti-chicken linker antibody and protein A gold (10 nm). ➡ indicates labelling on the surface or on lamellipodia and ⟶ indicates labelling associated with vesicular bodies or tubule–like structures (ER). V, vesicular bodies; L, lamellipodia; ER, endoplasmic reticulum; M, mitochondrion.

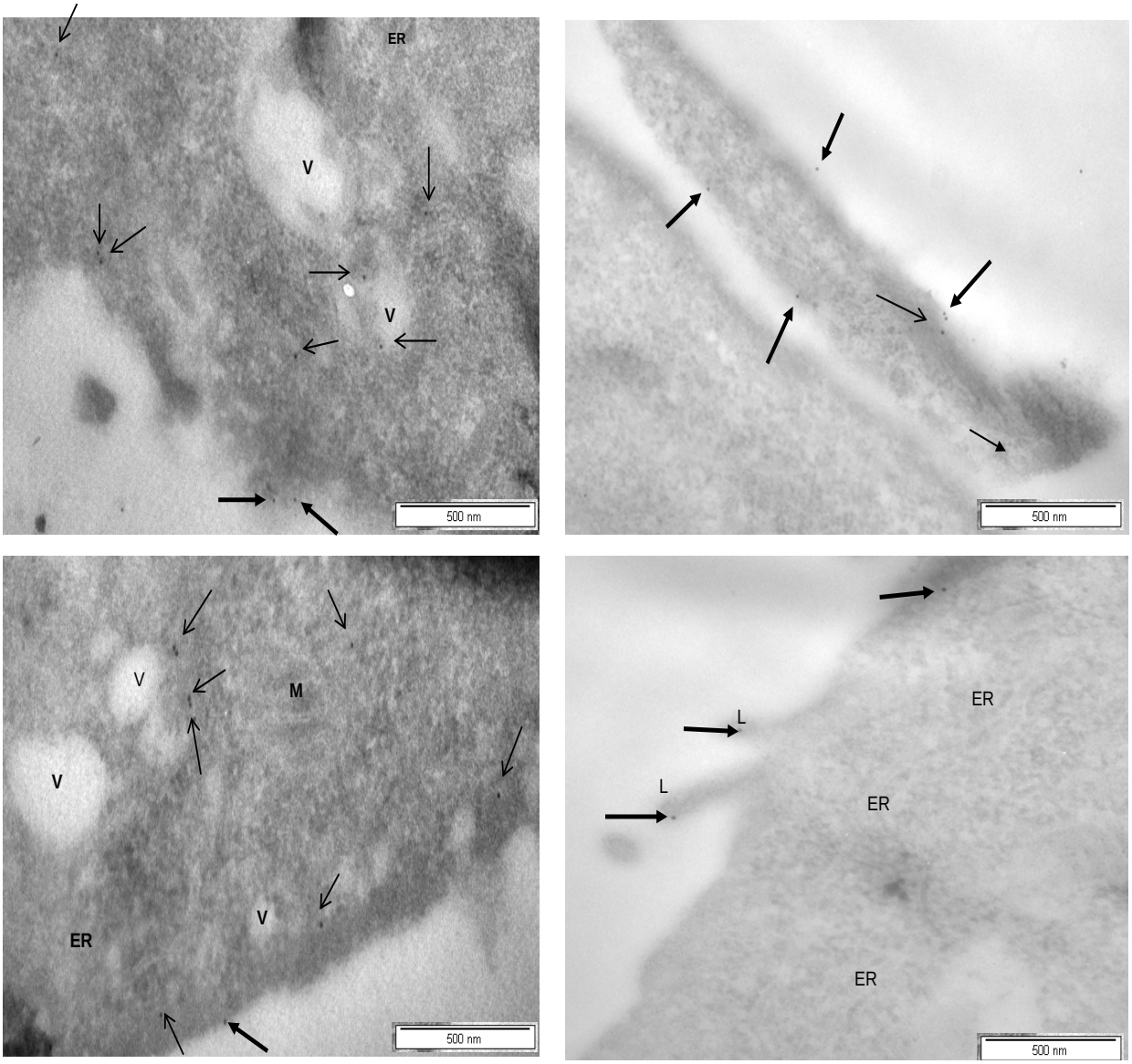

Figure 33. Immunolabelling for MT1-MMP PEXcat in MCF10A-neoT cell line.

The tissue was labelled with anti-MT1-MMP (PEXcat) IgY (40 µg/ml). The labelling was identified with a secondary rabbit anti-chicken linker antibody and protein A gold (10 nm). ⟶ indicates labelling on the surface or on lamellipodia and ⟶ indicates labelling associated with vesicular bodies or tubule-like structures (ER). V, vesicular bodies; L, lamellipodia; ER, endoplasmic reticulum; M, mitochondrion.

5.13 Discussion

Antibodies are widely used in diagnostics and biological research and have proved valuable in defining events during cancer progression and identifying targets and formulating anti-cancer strategies. Raising antibodies against MT1-MMP and other MMPs is notoriously difficult. The challenge in this study was to choose MT1-MMP domains which had the potential to induce specific and immunoinhibitory antibodies and, which also inhibit invasion of cancer cells *in vitro* and *in vivo*.

Clustal analysis showed chickens to be the most suitable host for antibody production against human MT1-MMP, as this mammalian antigen has low sequence homology with chicken MT1-MMP (Chapter 3) (Section 3.1.1). For the stimulation of human antibodies against human MT1-MMP as an anti-cancer approach, however, such analysis indicates that this may be more of a challenge than anticipated due to high homology between species across the various MMP classes.

As far as can be established, chickens have not previously been used for antibody production against this antigen and most subsequent approaches used in this study seem to have a degree of novelty. SDS-PAGE band separation of the crude inclusion body proteins, band excision and use for inoculation, gave poor results compared to the S-Sepharose purified protein injected in alum adjuvant, but seems to be a novel approach in chickens. Acrylamide alone has been reported to be a suitable adjuvant to induce a good immune response in rabbits (Weintraub and Raymond, 1963). Tracy *et al*. (1983) raised monoclonal antibodies against a protein antigen in acrylamide in mice, but included FCA to facilitate antibody production. In chickens, however, the acrylamide-complexed antigen did not seem to stimulate antibodies well, as similar antigen in alum seemed to be more successful. Perhaps chickens are not a suitable host for raising antibodies where acrylamide is used as the primary adjuvant.

Although alum adjuvant has been reported to induce relatively low antibody titres (Yamanashi *et al*., 2003), in this study it gave variable results, depending on the target domain, as would be anticipated. The success of generation of antibodies, however, seemed to reflect the homology assessed using the Clustal X analysis program (Thompson *et al*.,

1997). The propeptide domain, included in the PROcat domain, judged to be the most immunogenic in chickens, was, in fact, shown by ELISA to induce the highest immune response. Therefore, it would seem that the propeptide domain is a useful domain to include in the raising of antibodies. However, it is difficult to assess the contribution of variables, such as the variable amount of contaminating *E. coli* proteins in the antigen injected. Various chickens may also have had varying levels of exposure to *E. coli* during their lifetime, a factor that could more or less accentuate the antibody response to a target antigen injected with contaminating *E. coli* proteins (along with the alum adjuvant). Although the majority of the *E. coli* contaminating proteins were removed, following S-Sepharose purification of the PROcat MT1-MMP antigen, the presence of a few remaining minor *E. coli* proteins may have facilitated the successful induction of the observed antibody response to the PROcat antigen in alum. If this was a factor, this could be checked by quantitatively assessing the pre-immune response to *E. coli* proteins and correlating this with the immune response to the target antigen domain of MT1-MMP.

The hemopexin-like domain seems to be the least immunogenic, despite this domain having a lower sequence homology compared to the catalytic domain, as inoculation of expressed protein, containing both the hemopexin-like and catalytic domains did not seem to induce antibody production in chickens to any great extent. This is difficult to judge without attempting to raise antibodies against the catalytic domain alone.

Although the propeptide, catalytic and hemopexin-like domains are relatively highly conserved across MMP classes (Massova *et al.*, 1998), all three MT1-MMP antibodies did not seem to cross react with two other MMPs with the most similar domains, proMMP-2 and proMMP-9 (in human blood). Weak cross reactivity was observed with anti-PROcat IgY against a protein, suspected to be either mature MMP-2 (62 kDa) or latent, soluble MT1-MMP (63 kDa) or was the result of non-specific binding of an unknown protein (which was also detected by the pre-immune preparation of another chicken) (Figure 26 B, pre-immune, lane 1). Although latent soluble MT1-MMP has previously been observed in supernatants of mesengial cells, and this suggests that the latent MT1-MMP may occur in a soluble form (Kazes *et al.*, 1998), it should have shown some activity in the zymogram (Figure 21). This

was not the case, suggesting the previous conclusion. Since anti-MMP-2 antibodies and zymograms (Figure 21) only detected proMMP-2 (72 kDa) (Figure 26) and not mature MMP-2 (62 kDa) in the same human blood, this strongly suggests that the antibodies were recognising a non-gelatinolytic 62 kDa latent, soluble form of MT1-MMP or non-specific antigen, not the 62 kDa form of MMP-2. Weak recognition of this antigen, in western blots, is possibly indicative of low levels of the non-specifically recognised protein (or latent MT1-MMP) in human blood.

In western blots the anti-PROcat and the anti-PEX IgY recognised the recombinant forms of MT1-MMP but not the native enzyme in the MCF10A and MCF10A-neoT and mouse macrophage homogenates. The anti-PEXcat IgY, in contrast, was more successful in detecting native protein (43 kDa processed MT1-MMP) in the human and mouse macrophage cell lines. There seems to be no reason why the anti-PEXcat antibody should not have recognised its target protein, unless the antigen was loaded at a level that was too low for detection using the antibody at the concentration at which it was used. The native proteins in the cell culture pellets, on the other hand, were loaded much more heavily as the content of target antigen was not known. This discrepency could have resulted in a lack of recognition of the original target antigen. These blots should, therefore, be repeated for the anti-PEXcat antibodies and the target antigen loaded at higher concentrations.

The 43 kDa processed form of MT1-MMP is produced following proMMP-2 activation and usually lacks a large portion of its catalytic domain, as this is removed during maturation to the 43 kDa form (Lehti *et al*., 1998). What remains is the zinc-binding site of this domain, which in the 3-D conformation of the proenzyme, is usually blocked by the propeptide domain. If the PROcat expressed protein adopted this conformation when injected, the resultant antibodies may not recognise the zinc-binding site of the catalytic domain but should recognise the remaining portion of the catalytic domain (which is removed in the 43 kDa form). This may explain why there is no recognition of the 43 kDa processed MT1-MMP bands in the human and mouse cell line blotted protein. There is, however, a domain of the expressed catalytic site that would have been exposed when injected and this may explain why the anti-PROcat antibodies recognise the PEXcat antigen (50 kDa), which still

contains the unblocked region of the catalytic domain. This may also have an impact on the immunoinhibitory effect of these antibodies raised against the PROcat domain as these would target only a part of the catalytic domain and this is the major domain involved in enzyme activity.

The recognition of the human and mouse 43 kDa MT1-MMP suggests that there is a degree of sequence homology between human and murine MT1-MMP. This was seen in Clustal X analysis where the mouse had an observed homology of 97% with the human (Section 3.1.1). It is, therefore, tempting to speculate that, in order to raise antibodies against human MT1-MMP in humans to inhibit cancer invasion, there is sufficient difference in the PEX region of the chicken and mouse MT1-MMP domain to use either of these domains to elicit cross-reacting antibodies that could bind and inhibit human MT1-MMP. This strategy may prove useful if the PEXcat antibodies prove to be effective in inhibiting MT1-MMP activity and preventing tumour cell invasion in the chicken invasion model system.

As previously mentioned in connection with the recognition pattern seen in the blots, the PROcat domain may obscure the zinc-binding site of the catalytic domain during the generation of antibodies, resulting in only partially targeting of the catalytic domain, and, therefore, it seemed unlikely that the PROcat antibodies would result in effective inhibition. It was also anticiptated that crude anti-PROcat antibodies may inhibit the renatured PROcat enzyme better than the purified antibody, as these recognised the renatured catalytic domain of PROcat MT1-MMP better than the purified antibodies. Such a characteristic would be very favourable *in vivo* where antibodies are present in the crude form. This was not the case, however, as the purified IgY generally gave the highest inhibition of enzyme activity compared to crude IgY. A marked difference was seen in the case of the anti-PEXcat antibodies, where purification resulted in inhibition of activity going from 50% to 88% becoming the most inhibitory of the three antibodies. This implies that the anti-PEXcat antibodies recognise the catalytic domain in the renatured PROcat protein used for for immunoinhibition assays (the PROcat renatured protein has no hemopexin-like domain, and is processed to a 25/23 kDa form by removal of the propeptide domain, therefore, this conclusion). Since high concentrations (or low dilutions) of anti-PROcat antibodies seem to

be required for immunoinhibition, confirms the conclusion that these antibodies recognise only part of the catalytic domain i.e. react fairly weakly.

The hemopexin domain, in contrast, is located on the C-terminal of the catalytic domain. This gives full exposure of the active site cleft of the catalytic domain in the PEXcat antigen during antibody production. This, or distortion of the catalytic domain by binding of the hemopexin-like domain, may explain why these antibodies may bind, either to the active site cleft of the catalytic domain or hemopexin-like domain, and result in the best inhibitory effects on enzyme activity. These results seem to support the conclusion that the lack of recognition for the original immunogen in western blots was due to the loading of the target PEXcat immunogen and not due to a lack of recognition of the catalytic domain.

The discrepancy in the recognition of renatured PROcat antigen in the western blots by anti-PROcat, anti-PEXcat and anti-PEX antibodies may be as a result of the method used to isolate the antibodies. Since antibodies were purified using the PEG isolation procedure (Polsen *et al*., 1980), antibody loss may have occurred at each step, and may account for the lack of recognition of some bands by the purified IgY. Staak *et al*. (2001) also reported that any residual PEG, following the IgY isolation procedure may also interfere with immunological assays and this may also be the cause for the inconsistent results. Incorporation of cold ethanol into the PEG isolation procedure (Polsen *et al*., 1985) may be used to remove the residual PEG and has been reported to result in a higher purity than the conventional PEG method (Staak *et al*., 2001) and perhaps should be used in the furture. Polsen (1990) also introduced chloroform into the PEG isolation procedure. Chloroform was used to selectively remove the lipid fractions from the soluble proteins before PEG was used to precipitate the IgY. This improved procedure, considerably increased the IgY yield, compared to using the two consecutive precipitations with PEG, and so perhaps would have been preferable to use in this study.

The anti-PEX IgY induced the poorest inhibitory response, as anticipated, as the PEX domain does not occur in the renatured PROcat enzyme used for immunoinhibition. It was surprising, however, that some immunoinhibition (35%) was observed (however, this inhibition was seen with a highly concentrated preparation and possibly may represent weak

cross-reactivity with a common conformational epitope). Antibodies to the PEX antigen also gave the poorest recognition of MT1-MMP on western blots and, hence, these were omitted from immunolabelling studies. Instead antibodies to the catalytic domain, in conjunction with the hemopexin-like domain, seems to be more suitable and were employed.

In order to explain the fluorescent labelling, it must be remembered that, in the protein target, proMT1-MMP consists of the propeptide (PRO), cataltyic (cat) and hemopexin-like (PEX) domains, and the mature enzyme consists of the catalytic and hemopexin-like domains. Assuming that the anti-PROcat and anti-PEXcat antibodies recognise only MT1-MMP, and all target domains are recognised with the same avidity by their respective antibodies, the labelling intensity will be proportional to the antigen concentration. The anti-PEXcat antibodies should recognise both the proenzyme and mature enzyme equally in both cell lines (both have a hemopexin-like and catalytic domain in common) and give an indication of the total amount of enzyme present in both cell lines. The increased labelling in the MCF10A-neoT cell line (Figure 31 C) with the anti-PEXcat antibodies, therefore, means that mature or precursor MT1-MMP is possibly more abundant in the MCF10A-neoT cell line than in the MCF10A cell line (we cannot differentiate whether this is proenzyme or mature enzyme being targeted by the anti-PEXcat antibodies and this labelling would be irrespective of the relative amount of recognition of hemopexin-like and catalytic domain regions as the same antibody is being used in both cell lines). The MCF10A-neoT cell line is characterised by being highly migratory and invasive (Ochieng *et al*., 1991). The increased levels of MT1-MMP in this cell line may be present and responsible for this invasive phenotype if it were the mature form.

The anti-PROcat antibodies, on the other hand, should recognise the proenzyme with double the labelling intensity, compared to the mature enzyme (as these antibodies would recognise the propeptide and catalytic domains in the poenzyme and only the catalytic domain in the mature enzyme). Since the anti-PROcat antibodies showed at least three times more labelling intensity than the anti-PEXcat antibodies, specifically in the podocytes of the MCF10A cell line and, as these antibodies may preferentially target proMT1-MMP rather than the mature enzyme, this suggests there is more proMT1-MMP in the podocytes of the

MCF10A compared to the plasma membrane of the MCF10A cells and compared to both regions in the MCF10A-neoT cell line. This data also suggests that the anti-PROcat antibodies recognised the propeptide domain more intensely than the catalytic domain, as the total labelling with this antibody should have been the same if the antibody recognised both domains equally. This also agrees with previous immunoinhibition studies where this antibody showed lower inhibition of the catalytic domain of renatured PROcat MT1-MMP, possibly due to the antibody recognising the PRO domain better than the catalytic domain. Since MMP-2 may be localised as a complex with MT1-MMP and TIMP-2 in lamellipodia on invading cells (Chen and Wang, 1999), perhaps the increased labelling intensity in the podocytes of the MCF10A cells may be due to the antibodies cross reacting with MMP-2. The fact that this increase was not reflected in the podocytes of the MCF10A-neoT cells, where MMP-2 levels would be anticipitated to be higher, suggests that either MMP-2 is not localised in the lamellipodia of the MCF10A-neoT cell line or that this recognition by the anti-PROcat antibodies was specific to proMT1-MMP. If the latter is true, this would clarify that the detection of a 62 kDa protein in the western blots was infact latent, soluble MT1-MMP or non-specific binding, as a similar band was also recognised with the pre-immune IgY.

Since the anti-PEXcat antibodies should recognise both forms of MT1-MMP (i.e. pro-and mature MT1-MMP) these antibodies too should have detected high levels of proMT1-MMP in the podocytes of the MCF10A cell line. The fact that the anti-PEXcat antibodies also did not reflect elevated levels of labelling in the podocytes suggests that the recognition of the hemopexin-like and catalytic domains by the anti-PEXcat antibodies is not the same overall, or is not the same in the two cell lines, or that the catalytic domain, in a precursor form, is not recognised in the proenzyme as either the PRO piece occludes this binding site or the catalytic domain is not recognised as much as the hemopexin-like domain by the PEXcat antibodies. This conclusion seems to be supported by conclusions made by interpretation of the binding and recognition of the 43 kDa form in the western blots (Section 5.9) where all forms should be recognised but only once a large portion of the catalytic domain has been lost is the recognition of the hemopexin-like domain gained, i.e. the hemopexin-like domain is recognised more than the catalytic domain.

Although more pronounced with the anti-PROcat antibodies, the labelling pattern observed in the MCF10A cell line, overall, agrees with previous reports which showed the localisation of MT1-MMP primarily in lamellipodia and at the basal surface of the MCF10A cell line (Gilles *et al*., 2001). In the MCF10A-neoT cell line, however, MT1-MMP seemed more evenly distributed on the cell membrane and in lamellipodia. In these cells, MT1-MMP would be expected to be localised at the leading edge of the cell and/or in lamellipodia, to promote invasion. This data, therefore, suggests that MT1-MMP trafficking is possibly slightly disrupted in the MCF10A-neoT cell line.

In fixed and processed tissue for electron microscopy, anti-PEXcat IgY labelled small vesicles, possibly cell sorting vesicles. These usually transport the mature enzyme from the TGN to the cell surface but may also transport processed MT1-MMP from the cell surface into the cell, via clathrin coated pits or calveolae, for subsequent intracellular digestion, or back to other regions of the cell surface and lamellipodia where they can carry out their function (Remacle *et al*., 2003). This agrees with previous western blotting data which showed that these antibodies detected processed forms of MT1-MMP in human and mouse cell lines. If the observed labelling in the tubule-like structures, speculated to be ER, are ER, this suggests that the anti-PEXcat antibodies also recognised proMT1-MMP. This confirms earlier assumptions that anti-PEXcat antibodies should detect all forms of MT1-MMP. However, since these antibodies previously did not seem to detect the catalytic domain, here they were more likely binding to the hemopexin-like domain of proMT1-MMP. The anti-PROcat antibodies were previously shown to recognise proMT1-MMP more than the mature enzyme (i.e. the antibodies preferentially recognise the propeptide domain more than the catalytic domain). It was, therefore, not surprising that these antibodies recognised the ER. Labelling of small vesicles and the plasma membrane by these antibodies, however, was unexpected, as mature/processed enzyme is usually transported to and from the plasma membrane and lamellipodia. The only way to make sense of this labelling is to suggest that these antibodies may be labelling proMT1-MMP-containing vesicles transporting the proenzyme to the cell surface (and lamellipodia), as previously reported (Cao *et al*., 1998; Yana and Weiss, 2000). The fact that in the western blot only the anti-PEXcat IgY detected processed MT1-MMP, does not mean that the PROcat and PEXcat antibodies may not

recognise differently processed MT1-MMP forms in fixed tissue, as each system, whether the antigen is fixed or blotted, may expose antigenic eptitopes differently.

The distribution of MT1-MMP is controlled by the intracellular trafficking of the enzyme from the Golgi through vesicular bodies to the cell surface and the fate of the enzyme is determined by the MT1-MMP cytoplasmic tail. The vesicles move along the microtubules under the assistance of the kinesin motor protein and under the guidance of Rab11 (Ichikawa *et al*., 2000). During migration, MT1-MMP-containing vesicles may be directed towards protrusions (lamellipodia) that form under the influence of Ras-Rac/Rho (Ridley *et al*., 1992). The two effector Rac/Rho proteins are in a reciprocal balance, to organise the cell shape during migration (mesenchymal, rounded and spindle shape), and, during subsequent cell spreading to flatten the cell in an epithelial shape (Bishop *et al*., 2000).

Due to the constant stimuli from mutationally activated Ha-*ras* (V12), the cytoskeleton of the transfected cell retains its spindle shape, characteristic of a migratory cell (Zondag *et al*., 2000). From our results, it would seem that the Ha-*ras* (V12) transfection also alters the distribution of MT1-MMP proenzyme, shifting localisation instead of towards the leading edge of the cell and in lamellipodia, towards the plasma membrane in general via effects on the cytoskeleton (Paine *et al*., 1992). The presence of dysfunctional p53 in the MCF10A-neoT cell line, which also effects arrangement of the cytoskeleton and the distribution of organelles and vesicular bodies, possibly also contributes to the altered distribution of MT1-MMP in the MCF10A-neoT cell line. Alkalinisation of the cytoplasm of the MCF10A-neoT cells also could have an influence on the cytoskeletal layout (Jackson, unpublished data) and possibly the direction of movement of vesicles carried along the microtubules. Both the altered cytoskeletal stimulation and the cytoplasmic alkalinisation in the transfected cells may have influenced the polarisation and trafficking of the vesicles containing MT1-MMP, resulting in a wider distribution of this membrane-bound enzyme, to areas other than the lamellipodia. This is an unanticipated finding.

In summary, the expressed PROcat domain of MT1-MMP seemed to induce the highest antibody response in chickens, although these antibodies seemed to preferentially target the propeptide domain more than the catalytic domain of proMT1-MMP. We also conclude that

the cross reactivity of these antibodies with a protein in human blood in the western blots is more likely latent, soluble MT1-MMP (62 kDa), or a non-specifically bound protein, although additional immunoblot studies would need to be performed to confirm this. Since proMT1-MMP may be transported, via vesicles, to the cell surface, this may account for the labelling observed in vesicles and on the plasma membrane/lamellipodia as well as the ER of fixed tissue. Localisation of proMT1-MMP on the cell surface is thought to be involved in proMMP-2 activation (Cao *et al*., 1998), and so targeting the propeptide and catalytic domain, together, may assist in inhibiting proMMP-2 activation and tumour cell invasion. Despite this, the anti-PEXcat antibodies induced a higher immunoinhibitory response against the renatured PROcat enzyme compared to the anti-PROcat antibodies and indicated that these antibodies recognised the catalytic domain of MT1-MMP. This is inspite of the western blots and immunofluorescence labelling suggesting that the anti-PEXcat antibodies possibly recognised only the hemopexin-like domain. These antibodies were also better at detecting native, processed MT1-MMP, although they generally labelled the fixed tissue (in EM and immunofluorescence labelling) less intensely than the anti-PROcat antibodies. In the fixed tissue, however, it was concluded that the anti-PEXcat antibodies labelled all forms of MT1-MMP most likely via recognition of the hemopexin-like domain. This data suggests, therefore, that the PEXcat antigen would possibly be more suitable for inducing antibodies to inhibit tumour cell invasion via inhibition of MT1-MMP. Since the anti-PROcat antibodies resulted in more intense labelling overall, compared to the anti-PEXcat antibodies, suggests these antibodies may be more useful for labelling to determine the localisation and distribution pattern, particularly of proMT1-MMP. The weak response induced by the anti-PEX IgY, overall, suggests that the hemopexin-like domain alone is not suitable in antibody production (immunoinhibitory activity could not, however, be assessed and should, therefore, be checked using active, renatured MT1-MMP comprising the PEX domain).

It is difficult to categorically determine the specificity of these antibodies in all applications especially as they may behave differently, depending upon whether the antigen is fixed or blotted onto nitrocellulose. Possibly the best strategy to improve specificity would be to transfect the MCF10A cells with green fluorescent protein (GFP)-tagged MT1-MMP and

check the colocalisation with the test antibodies, detected with a secondary antibody, distinguishable from GFP.

Therefore, the hemopexin-like and catalytic domains of MT1-MMP (PEXcat) are perhaps the most suitable targets for use in immunoinihibition studies. Additional studies using a chicken embryo model system would confirm which MT1-MMP antibodies would be the most inhibitory, however.

Chapter 6

Discussion and application

MT1-MMP is one of the best characterised MMPs and since the time it was first isolated in 1994 by Sato and co-workers, it has been studied more frequently than any of the other MMPs. The keen interest in studying this enzyme is attributed to its role in remodelling the ECM for normal growth and development but also its involvement in skin and breast cancer invasion and metastasis. In fact, MT1-MMP is up-regulated by various cytokines and growth factors during tumour progression. These allow the enzyme to initiate primary tumour formation and participitate in the development of secondary tumours (Overall and Lopez-Otin, 2002).

MT1-MMP is the primary activator of proMMP-2 and, together, these proteases attack the connective tissue providing channels through which the invading cells can migrate to distant sites (Sato *et al*., 1996; Sabeh *et al*., 2004). Studies on knockout mice have shown that reduced MT1-MMP expression hinders proMMP-2 activation and subsequently tumour invasion (Sabeh *et al*., 2004). This emphasises the significance of these proteases in tumour progression but also stresses the importance of targeting MT1-MMP to stop proMMP-2 activation and invasion. The activation of proMMP-2 also leads to the downstream activation of other MMPs, which further assists in tumour invasion and metastasis (Fridman *et al*., 1995; Knauper *et al*., 1996; Cowell *et al*., 1998; Toth *et al*., 2003). Being membrane type, MT1-MMP is positioned on the surface of the cell and when the cell is triggered into migration, MT1-MMP co-localises with a CD44 receptor protein at the migration front of the invading cell and in lamellipodia, facilitating invasion (Mori *et al*., 2002). The hemopexin-like domain is essential for binding to CD44 and, thus, is a critical domain in the localisation of MT1-MMP at the invasion front (Mori *et al*., 2002).

Various approaches may be adopted to stop MT1-MMP-associated tumour invasion. One approach is to target MT1-MMP gene transcription by blocking signal transduction pathways (e.g. ERK pathway), cytokines (i.e. interferon-γ, interferon-α and interferon-β) and transcription factors (i.e. AP-1 and NF-κB) all of which are involved in the expression

of the genes coding for MT1-MMP (Westermarck and Kahari, 1999; Overall and Lopez-Otin, 2002; Folgueras *et al*., 2004). The ERK pathway, cytokines and transcription factors are also required in the expression of many other MMPs, however, and blocking these could result in adverse side effects and influence many natural physiological processess. The transcription factors may also be induced by a range of different substances (i.e. agonists and hormones) and so it would be difficult to stop their production.

Another approach could be to block proMT1-MMP activation using TIMP-2 (Westermarck and Kahari, 1999). This could be acheived by up-regulating the expression of TIMP-2 and delivering the MMP inhibitor to the tumour site using gene therapy (Folgueras *et al*., 2004). TIMP-2, however, also promotes the up-regulation and activation of proMMP-2 during tumour development (Toth *et al*., 2000) and also may inhibit other MMPs (Will *et al*., 1996), and so the use of this inhibitor to hinder MT1-MMP activity may also have adverse effects on the normal biological function of MMPs in tissue. Therefore, the non-specific nature of these inhibitors means the TIMP-2 inhibitor approach may not be suitable for use in the development of anti-cancer and therapeutic strategies.

MT-MMPs are activated intracellularly by a furin-like proprotein convertase, which binds at the furin cleavage site (Sato *et al*., 1996). Targeting this activation pathway could also assist in stopping MT1-MMP-associated tumour invasion. Attempts to prevent the binding of furin have been made (Folgueras *et al*., 2004). Inhibiting the furin-like convertase using a specific inhibitor, such as alpha 1-PDX, has also been shown to suppress MT1-MMP activation and tumour invasion in head and neck squamous carcinoma cells and shows promise in cancer therapy (Bassi *et al*., 2001). The furin-like protease is also involved in the activation of other proenzymes and inhibiting its function may detrimentally affect other biological processes (Bennett *et al*., 2000; Leighton and Kadler, 2003).

Other anti-cancer strategies already employed to inhibit MT1-MMP-associated tumour invasion include the use of macromolecules that act as carriers of anti-cancer drugs (Folgueras *et al*., 2004). Such drugs are designed to be released upon cleavage of the carrier protein by MT1-MMP. Similarly, bioactive molecules have also been coupled to carrier

proteins that induce an anti-tumour immune response upon MMP cleavage of the carrier (Folgueras *et al*., 2004).

Attempting to design a specific inhibitor that may target specific sites within the active site cleft of MT1-MMP and, thus, prevent the enzyme from binding to a given substrate, may still be a suitable approach to stop tumour invasion (Folgueras *et al*., 2004). Similarily, the design of molecules that bind to a MMP substrate and, thus, compete with the enzyme for the ECM substrate may also be a useful approach to inhibit MT1-MMP-associated tumour invasion (Folgueras *et al*., 2004). Such concepts have already been attempted by synthesising broad spectrum inhibitors, such as hydroxamic acids (Rosello *et al*., 2005). These inhibitors minimise MT1-MMP activity by competing with the enzyme for specific binding sites on the substrate, thereby slowing down the enzyme's activity (Folgueras *et al*., 2004).

Since MT1-MMP cleaves a large repertoire of substrates, however, these inhibitors may not be able to prevent MT1-MMP from binding to all known ECM substrates. Synthetic inhibitors are also expensive and often the most effective dosage leads to toxicity and serious side effects. It seems that many of these synthetic inhibitors are only effective in early stages of tumour progression and possibly do not inhibit tumour metastasis (Overall Lopez-Otin, 2002). MT1-MMP, however, is prevalent at all stages of tumour progression. In addition, the action of some inhibitors can further promote tumour progression by increasing the expression of other MMPs (i.e. MMP-9) (Maquoi *et al*., 2002), whilst others suppress anti-angiogenic proteases, the latter of which would normally reduce tumour progression (Vazquez *et al*., 1999). Broad sepctrum hydroxamate inhibitors may also inhibit the activation of cytokines, i.e. tumour necrosis factor (TNF)- α, which plays a critical role in ECM remodelling and cell signalling (Rosenberg, 2002; Dittmar *et al*., 2009). The non-specific action of synthetic inhibitors on various enzymes and cytokines, therefore, may subsequently effect other normal biological processes.

Natural inhibitors, such as the green tea polyphenol (-)-epigallocatechin 3-O-gallate (EGCG), have also frequently been explored and reported to suppress tumour growth and angiogenesis by inhibiting proMT1-MMP and proMMP-2 activation with no adverse side

effects or toxicity (Oku *et al*., 2003; Yamakawa *et al*., 2004). EGCG has also been reported to inhibit interleukin-1β-induced MMP-1 and MMP-13 activity and expression and is effective at low dosages (Ahmed *et al*., 2004). Like the synthetic inhibitors, these natural inhibitors, therefore, may target both cytokines and MMPs and that they too may effect other normal physiological functions.

Another useful strategy, which was the main focus of this study, is to immunologically inactivate, specifically, MT1-MMP by stimulating short-term antibody production against different regions of the enzyme in the hope of generating immunoinhibitory antibodies, which may stop human tumour cell invasion and metastasis (Overall and Lopez-Otin, 2002). Galvez *et al*. (2001) showed that monoclonal antibodies against the catalytic domain of MT1-MMP inhibited endothelial cell invasion and progression. This suggests such a domain should be targeted to stop invasion. Targeting the catalytic domain of MT1-MMP could also prevent TIMP-2 from binding, a process required for proMMP-2 activation (Strongin *et al*., 1995; Will *et al*., 1996). As observed in this study, the catalytic domain is highly conserved amongst MMPs and is, therefore, difficult to target specifically. The hemopexin-like domain assists the catalytic domain in its function and promotes invasion by localising MT1-MMP at the migration front of invading cells (Tam *et al*., 2002) and, therefore, together with the catalytic domain, may be a suitable target to inhibit MT1-MMP-associated tumour invasion. To induce a more potent inhibitory response, the best approach was anticiptated to be to target the catalytic domain coupled with the propeptide domain, as this domain too has been implicated in promoting tumour invasion by assisting in the activation of proMMP-2 (Cao *et al*., 1998). Together with the catalytic domain, it was thought, therefore, to be a good target for immunotherapy.

In this study, although antibodies raised against the propeptide and catalytic (PROcat) domains of MT1-MMP appeared to be highly antigenic in chickens, the PROcat antibodies were less immunoinhibitory than the PEXcat antibodies, when used to immunoinhibit renatured PROcat MT1-MMP activity. The fact that high antibodies titres were produced, suggests, that the propeptide domain of MT1-MMP is useful to include in the production of antibodies against highly conserved domains, such as the catalytic domain. It was concluded

from the western blots, immunofluorescence (and EM) labelling and immunoinhibition assay, that these antibodies targeted the propeptide domain more than the catalytic domain and that the partial recognition of the catalytic domain (i.e. these antibodies were unable to recognise and bind to the zinc-binding site of the catalytic domain) may have explained why lower immunoinhibition was observed with these antibodies and why higher antibody concentrations were required to achieve an immunoinhibition comparable with that of the PEXcat antibodies. In addition to the higher immunoinhibition achieved with the PEXcat antibodies, these antibodies also appeared to recognise all forms of MT1-MMP. Antibodies targeting the hemopexin-like domain (PEX) of MT1-MMP were poorly immunogenic in chickens and failed to detect native MT1-MMP in cell homogenates. However, immunoinhibitory activity still needs to be assessed for inhibition of MT1-MMP activity for prevention of MT1-MMP-assocaited tumour cell invasion. The strong immunoinhibitory effect of the anti-PEXcat MT1-MMP antibodies combined with their detection of possibly all forms of MT1-MMP, suggested that targeting the hemopexin-like and catalytic domains of MT1-MMP may result in highly specific and immunoinhibitory antibodies, possibly suitable for inhibiting MT1-MMP-associated tumour cell invasion and metastasis.

Fluorescence immunolabelling of MCF10A and MCF10A-neoT cell lines with anti-PROcat and anti-PEXcat antibodies also indicated that proMT1-MMP is prevalent in the lamellipodia of the MCF10A cell line. This localisation of the proenzyme may regulate cell migration in this cell line. Although it was more difficult to to determine which form of MT1-MMP was being targeted, the anti-PEXcat antibodies strongly indicated that MT1-MMP is highly expressed in the MCF10A-neoT cell line compared to the MCF10A cell line. This is consistant with previous studies and may assist in invasion of this cancer cell line. The unanticipated labelling of MT1-MMP, from a concentrated association with lamellipodia (as seen in the normal MCF10A cell line labelled with the anti-PROcat antibodies), to a more evenly dispersed distribution in lamellipodia and along the plasma membrane (as seen in the MCF10A-neoT cell line), suggests that the *ras*-transfection of the MCF10A-neoT cell line effects intracellular trafficking of MT1-MMP. The cytoskeleton provides a microtubular pathway along which kinesin and dinesin microtubule-associated motor proteins transport vesicular bodies to various destinations (Ichikawa *et al*. 2000).

Therefore, it seems that Ha-*ras* specifically affects the function of kinesin and dinesin motor proteins.

An innovation in the methods used to express the various domains in this study was the use of the JM109 (DE3) strain of *E. coli*. This strain was shown here to be a suitable host to express PROcat MT1-MMP using the T7 promoter and in the presence of an IPTG inducer, a modification of the method of Lichte *et al*., 1996. Compared to the BL21 (DE3) strain of *E. coli*, previously used to express PROcat MT1-MMP (Lichte *et al*., 1996), the JM109 (DE3) strain here, appeared to result in higher inclusion body yields. Another modification in the procedure proposed by Lichte *et al*. (1996) included purification of PROcat MT1-MMP on a S-Sepharose and Q-Sepharose column instead of using gel filtration. Although the S-Sepharose column appeared to remove most of the presumptive *E. coli* contaminating proteins, PROcat MT1-MMP protein seemed to be more soluble when purified on a Q-Sepharose column. The denatured PROcat MT1-MMP protein also appeared to bind better to the Q-Sepharose column compared to the S-Sepharose column. Since there were still presumptive *E. coli* contaminating proteins present following both purification procedures, however, indicated that the conditions used for ion exchange chromatography were possibly not the most ideal and could possibly be further improved, perhaps by loading less protein to the column or changing the buffer constituents or pH. The presence of trace levels of *E. coli* in the antigen to be used to raise antibodies in chickens, however, may not have made any difference to the resulting antibodies, as the chickens used in this study to raise antibodies against the MT1-MMP antigens appeared to have had prior exposure to *E. coli* proteins and, thus, innoculating MT1-MMP antigens, contaminated with a few minor *E. coli* proteins, may have not effected the results.

In addition to the choice of renaturation procedure and the plot used to determine K_m, the K_m value finally established (as discussed in Section 4.4) may also have been effected by the presence of these *E. coli* proteins. The PROcat MT1-MMP was renatured using a stepwise gradient dialysis (Koo *et al*., 2002) and was shown to renature less effectively into the native enzyme than when the one step renaturation procedure was employed (Lichte *et al*.; 1996) as shown by its higher K_m. Compared to the one-step renaturation procedure, which quickly

removed the urea and β-mercaptoethanol, the stepwise renaturation procedure resulted in the gradual removal of the urea and β-mercaptoethanol, which possibly prevented the enzyme from precipitating, but possibly also only allowed it to partially renature into its native conformation. This was, thus, the first time that the PROcat domains of MT1-MMP were expressecd in JM109 (DE3) *E. coli*, purified using ion exchange chromatography and renatured using a step-wise gradient dialysis procedure.

Since a PROcat MT1-MMP preparation, free of *E. coli* proteins, was, thus, difficult to achieve and additional electrophoretic separation and injection in acrylamide seemed an alternative approach, this was attempted, but appeared to be inefficient in inducing an antibody response. Alum, in contrast, was shown to be efficient in inducing a suitable immune response in chickens. Compared to other adjuvants (i.e. Freund's adjuvant), alum adjuvants require higher antigen concentrations to promote antibody production (Yamanishi *et al*., 2003), however. Perhaps antibody titres could have improved by increasing antigen levels.

6.1. The way forward

To test the immunoinhibitory effect of the anti-PEXcat MT1-MMP antibodies further, these antibodies may be introduced, via inoculation of laying hens, into the chicken embryo model system (Davison and Solomon., 1980; Kim *et al*., 1998). If these antibodies prove to be successful in inhibiting invasion of the innoculated MCF10A-neoT cancer cells, the PEXcat domains of MT1-MMP may constitute a suitable target for use in cancer therapy studies to inhibit cancer progression.

The next approach, therefore, would be to test the anti-PEX IgY against a suitable active construct containing the PEX domain, and finally perform immunoinhibitory studies in humans using all domain potentially eliciting immunoinhibitory responses in humans. Raising human antibodies against human MT1-MMP, however, may prove to be a more difficult task, as the domains of MT1-MMP are highly conserved across MMP classes (Folgueras *et al*., 2004) and, thus, the human immune system may not detect human MT1-MMP antigen as being foreign unless it was somehow altered. Perhaps conjugating human MT1-MMP with a carrier protein (i.e. keyhole limpet hemocyanin) may help to induce an

antibody response against the human enzyme. The human immune system, may also, however, induce an immune response against the conjugate and remove it from the circulatory system. Recombinant DNA technology may

be used to express a mutated form of human MT1-MMP to induce an antibody response. There is, however, also a concern that targeting human MT1-MMP in humans may result in autoimmune disease.

If antibodies against MT1-MMP raised in humans is perhaps impossible, antibodies raised against the PROcat and PEXcat domains of MT1-MMP, in this study, may be useful in the characterisation of different cell lines using immunocytochemistry and western blotting. Such characterisation studies may include the use of these antibodies to identify and determine the distribution pattern and spatial localisation of MT1-MMP in various cell lines. This would provide useful information regarding the behaviour of the enzyme in normal versus cancer cell lines.

Since proMT1-MMP may also be involved in proMMP-2 activation and the anti-PROcat MT1-MMP antibodies preferentaily targeted this form of MT1-MMP, it may, therefore, be useful to perform additional studies with this antibody to see if they inhibit proMMP-2 activation. If the anti-PROcat antibodies inhibit MMP-2 activity, in addition to MT1-MMP, such antibodies may result in a much improved inhibition of tumour invasion. Such effects may be tested in a chicken invasion model system, as previously described. Although the anti-PROcat antibodies detected a possible latent, soluble form of MT1-MMP in human blood (or a non-specific protein), these results would need to be confirmed using additional immunoblot studies with anti-MMP-2, which specifically targets the 62 kDa form of mature MMP-2.

The discrepencies in the western blot results between the crude and purified anti-PROcat MT1-MMP antibodies suggests that the PEG isolation procedure, used to isolate IgY in this study, need to be re-assessed and optimised further to improve the quality of the isolation. Introducing chloroform in the IgY isolation procedure, to remove the lipid fraction, prior to

precipitation with PEG (Polsen, 1990), may perhaps improve the IgY yields and may also improve the quality of these antibodies. This, therefore, needs to be investigated.

In this study, PROcat MT1-MMP needed to be expressed for antibody production and as a target for immunoinhibition, to allow antibodies to be tested for their immunoinhibitory effect. Besides this, the expressed and renatured PROcat MT1-MMP may also be used to create new insight into the structure-function relationship of the enzyme, which is important in the design of therapeutic drugs and inhibitors in cancer therapy. Soluble MT1-MMP has already been used to understand further its mechanism of activation, its proteolyitc activity and its association with TIMP-2 (Valtanen *et al*., 2000). Only the crystal structure of the catalytic domain in complex with TIMP-2 has been resolved (Fernandez-Catalan *et al*., 1998). In this study PROcat MT1-MMP was succesfully expressed using a T7 polymerase pET expression system in JM109 (DE3) strain of *E. coli*. Perhaps the same recombinant DNA technology can be employed to express whole MT1-MMP, which can subsequently be used in crystallisation studies to determine the tertiary structure of the enzyme. Knowledge of the tertiary structure coupled with availability of the recombinant protein and the antibodies could contribute tremendously towards the development of anti-cancer strategies.

The major contribution of this dissertation has been to identify two possible recombinant protein targets for generating immunoinhibitory antibodies (the PROcat and PEXcat domains). These may now be used in the chicken invasion model to test the concept of whether it is possible to immunoinhibit cancer cell invasion by generating anti-MT1-MMP-activity-inhibiting antibodies in the host. This concept may now be tested.

The aims of this study have largely been achieved now. The broad aim of this study was to attempt to raise highly specific and immunoinhibitory antibodies against different MT1-MMP domains for use in electron and confocal microscopy and chicken CAM invasion studies, to determine the role of MT1-MMP in cancer cell invasion.

Target domains were determined using Clustal X analysis, and recombinant and expressed proteins were used to raise potentially specific and immunoinhibitory antibodies. The specificity of the antibodies against these domains has largely been determined and all

antibodies will be judged to be specific once the identity of the 62 kDa band targeted by the PROcat antibody has been resolved.

The next aim was to test whether, *in vitro*, whether immunoinhibitory antibodies, could be generated against human MT1-MMP. This aim has been achieved *in vitro* and now the immunoinhibitory effects need to be determined *in vivo* in the CAM invasion model system.

References

Ahmed, S., Wang, N., Lalonde, M., Goldberg, V. and Haqqi, T. (2004) Green tea polyphenol epigallocatechin-3-gallate (EGCG) differentially inhibits interleukin-1β-induced expression of matrix metalloproteinase-1 and-13 in human chondrocytes. *JPET.* **308**, 767-773

Aimes, R. and Quigley, J. (1995) Matrix metalloproteinase-2 is an interstitial collagenase: Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native Type 1 collagen generating the specific ¾ and ¼ length fragments. *Americ. Soc. Biochem. Mol. Biol.* **270**, 5872-5876

Arakawa, K., Takenaka, N.,and Sasaki, K. (1970) Ultrasonic Study of Dilute Aqueous Solutions of Urea, Guanidine Hydrochloride and Dioxane. *Bulletin of the chemical company of Japan.* **43**, 636-641

Baker, E. and Leaper, D. (2003) The plasminogen activator and matrix metalloproteinase systems in colorectal cancer:relationship to tumour pathology. *Eur. J.Can*. **39**, 981-988

Baramova, E., Bajou, K., Remacle, A., Hoir, C., Krell, H., Weidle, U., Noel, A. and Foidart, J. (1997) Involvement of PA/plasmin system in the processing of pro-MMP-9and in the second step of pro-MMP-2 activation. *FEBS Lett.* **405**, 157-162

Barrett, A. and Kirschke, H. (1981) Cathepsin B, cathepsin H and cathepsin L. *Methods Enzymol.* **80**, 535

Basile, J., Holmbeck, K., Bugge, T. and Gutkind, J. (2007) MT1-MMP controls tumour-induced angiogenesis through the release of semaphorin 4D. *J. Biol. Chem.* **282**, 6899-6905

Bassi, D., Mahloogi, H., Al-Saleem, L., De Cicco, R., Ridge, J. and Klein-Szanto, A. (2001) Elevated furin expression in aggressive human head and neck tumors and tumor cell lines. *Mol. Carcin.* **31**, 224-232

Batas, B., Schiraldi, C. And Chaudhuri, J. (1999) Inclusion body purification and protein refolding using microfiltration and size exclusion chromatography. *J. Biotech.* **68**, 149-158

Benavides, J., García-Pariente, C., Gelmetti, D., Fuertes, M., Ferreras, M., García-Marín, J. and Pérez, V. (2006) Effects of fixative type and fixation time on the detection of Maedi Visna virus by PCR and immunohistochemistry in paraffin-embedded ovine lung samples. J. *Virological Methods.* **137**, 317-324

Bennett, B., Denis, P., Haniu, M., Teplow, D., Kahn, S., Louis, J., Citron, M. and Vassar, R. (2000) A Furin-like Convertase Mediates Propeptide Cleavage of BACE, the Alzheimer's β-Secretase . *Biol. Chem.* **275**, 37712-37717

Bergmans, H., Van Die, I. and Hoekstra, W. (1981) Transformation in *Escherchia coli*: stages in process. *J. Bacteriol.* **146**, 564-570

Bigg, H. & Rowan, A. (2001) The inhibition of metalloproteinases as a therapeutic target in rheumatoid arthritis and osteoarthritis. *Current Opinion in Pharm.* **1**, 314-320

Birkedal-Hansen, H., Moore, W., Bodden, M., Windsor, L., Birkedal-Hansen, B., DeCarlo, A. and Engler, J. (1993) Matrix metalloproteinases: A review. *Crit. Rev. Oral Biol. Med.* **4**, 197-250

Birrell, G.B., Hedberg, K.K. and Griffith, O.H. (1987) Pitfalls of immunogold labelling: analysis by light microscopy and photoelectron microscopy. *J. Histochem. Cytochem.* **35**, 843-853

Bischoff, K., Shi, L. and Kennely, P. (1998) The detection of enzyme activity following sodium dodecyl sulfate polyacrylamide gel electrophoresis. *Anal. Biochem.* **260**, 1-17

Bishop, A. and Hall, A. (2000) Rho GTPases and their effector proteins. *Biochem. J.* **348**, 241-255

Bode, W., Fernandez-Catalan, C., Tschesche, H. *et al*. (1999) Structural properties of matrix metalloproteinases. *Cell. Mol. Life Sci*. **55**, 639-652

Boon, M., Gerrits, P., Moorlag, H., Nieuwenhuis, P. and Kok, L. (1988) Formaldehyde fixation and microwave irradiation. *Histochem. J.* **20**, 313-322

Boudreau, N. and Weaver, V. (2006) Forcing the third dimension. *Cell.* **25**, 429-431

Bouloumie, A., Sengenes, C., Portolan, G., Galitzky, J. and Lafontan, M. (2001) Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. *Diabetes* **50**, 2080-2086

Bourguignon, L., Singleton, P., Diedrich, F., Stern R. and Gilad, E. (2004) CD44 interaction with Na+-H+ exchanger (NHE1) creates acidic microenvironment leading to hyaluronidase-activation and breast tumour cell invasion. J. Biol. Chem. 279, 26991-27007

Bradford, M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **72**, 248-254.

Brooks, P., Stromblad, S., Sanders, L., von Schalscha,T., Aimes, R., Stetler-Stevenson, W., Quigley, J. and Cheresh, D. (1996) Localisation of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin αvβ3. *Cell*, **85**, 683-693

Brorson, S. a (1998) The combination of high-accelerator epoxy resin and antigen retrieval to obtain more intense immunolablleing on epoxy sections than on LR-white sections for large proteins. *Micron*. **29**, 89-95

Brorson, S. b (1998) Comparison of the immunogold labeling of single light chains and the whole immunoglobulins with anti-κ on LR-white and epoxy sections. *Micron.* **29**, 439-443

Cao, J., Kozarekar, P., Pavlaki, M., Chiarelli, C., Bahou, W. and Zucker, S. (2004) Distinct roles for the catalytic and hemopexin domains of membrane type-1 matrix metalloproteinase in substrate degradation and cell migration. *J. Biol. Chem*. **279**, 14129-14139

Cao, J., Drews, M., Lee, H., Conner, C., Bahou, W. and Zucker, S. (1998) The propeptide domain of membrane type-1 matrix metalloproteinase is required for binding of tissue inhibitor of matrix metalloproteinases and for activation of pro-gelatinase A. *J. Biol. Chem.* **279**, 34745-34752

Cao, J., Sato, H., Takino, T. and Seiki, M. (1995) The C-terminal region of membrane type matrix metalloproteinase is a functional transmembrane domain required for pro-gelatinase A activation. *J. Biol. Chem.***13**, 801-805

Ceuninck, F., Pastoureau, P., Agnellet, S., Bonnet, J. and Vanhoutte, P. (2001) Development of an enzyme-linked immunoassay for the quantification of YKL-40 (cartilage gp-39) in guinea pig serum using hen egg yolk antibodies, *J. Immunol. Methods.* **252**, 153-161

Chen, W and Wang, J. (1999) Specialized Surface Protrusions of Invasive Cells, Invadopodia and Lamellipodia, Have Differential MT1-MMP, MMP-2, and TIMP-2 Localization. *Annals of the New York Academy of Sciences*. **878**, 361-371 (Abstract only)

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T., Higgins, D. and Thompson, J. (2004) Multiple sequence alignment with the clustal series programs. *Nuc. Acids. Res*. **31**, 3497-3500

Chintala, S., Li, W., Lamoreux, M., Ito, S., Wakamatsu, K., Sviderskaya, E., Bennett, D., Park, Y., Gahl, W., Huizing, M., Spritz, R., Ben, S. Novak, E., Tan, J. and Swank, R. (2005) Slc7a11 gene controls production of pheomelanin pigment and proliferation of cultured cells. *PNAS*. **102**, 10964-10969

Choi, J., Choi, K., Auersperg, N. and Leung, P. (2006) Gonadotropins Activate Proteolysis and Increase Invasion through Protein Kinase A and Phosphatidylinositol 3-Kinase Pathways in Human Epithelial Ovarian Cancer Cells. *Can. Res*. **66**, 3912-3920

Coetzer, T., Elliott, E., Fortgens, P., Pike, R. and Dennison, C. (1991) Anti-peptide antibodies to cathepsins B, L and D and type IV collagenase. *J. Immunol. Meth.* **136**, 199-210

Collier, I.E., Wilhelm, S.M., Eisen, A.Z., Marmer, B.L., Grant, G.A., Seltzer, J.L., Kronberger, A., He, C.S., Bauer, E.A. & Goldberg, G.I. (1988) H-*ras* oncogene-transformed human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. *J. Biol. Chem.* **263**, 6579-6587

Compton, S. and Jones, C. (1985) Mechanism of dye response and interference in the Bradford protein assay. *Anal. Biochem*. **151**, 369-374

Cotrim, P., de Andrade, C., Martelli-Junior, H., Graner, E. and Sauk, J. (2002) Expression of matrix metalloproteinases in cyclosporin-treated gingival fibroblasts is regulated by transforming growth factor (TGF)-β1 autocrine stimulation. *J. Periodontol*. **73**, 1313-1322

Cowell, S., Knauper, V., Stewart, M., d'Ortho, M., Stanton, H., Hembry, R., Lopez-Otin, C., Reynolds, J. and Murphy, G. (1998) Induction of matrix metalloproteinase activation cascades based on membrane-type 1 matrix metalloproteinase: associated activation of gelatinase A, gelatinase B and collagenase 3. *Biochem. J.* **331**, 453-458

Creighton, T. (1990) Review article: Protein refolding. *Biochem. J.* **270**, 1-16

Dani, S. and Espindola, R. (2002) A model system for testing gene vectors using murine tumour cells on the chorioallantoic membrane of the chick embryo. *Gen. Mol. Res*. **1**, 167-175

Davison, J. and Solomon, J. (1980) Onset of resistance to pathogenic strains of Campylobacter fetus subspecies jejuni in the chicken embryo. Aspects of developmental and comparative immunology. Pergamon Press, Oxford. pp. 289-294

DeClerck, Y. (2000) Intercations between tumour cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer. *European J. Can.* **36**, 1258-1268

Dennison, C. (2003) A guide to protein isolation, 2nd Ed. Kluwer Academic Publishers, pp 18, 40, 65, 82, 100, 160-161

Dennison, C. and Lovrien, R. (1997) Three phase partitioning: concentration and purification of proteins. *Protein Expres. Purif.* **11**, 149-161

Deryugina, E., Ratnikov, B., Monosov, E., Postnova, T., DiScipio, R., Smith, J. and Strongin, A. (2001) MT1-MMP initiates activation of pro-MMP-2 and integrin αvβ3 promotes maturation of MMP-2 in breast carcinoma cells. *Exp. Cell Res.* **263**, 209-223

Desai, S., Dworecki, B and Cichon, E. (2001) Direct immunodetection of antigens within the precast polyacrylamide gel. *Anal. Biochem.* **297**, 94-98

Dittmar, M., Kiourkenidis, G., Horn, M., Bollwein, S., Bernhardt, G., Rosenberg, G. and Pfefferkorn T. (2004) Cerebral ischemia, matrix metalloproteinases and TNF-alpha: MMP inhibitors may act not exclusively by reducing MMP activity response. *Stroke*. **35**, 338-340

Dohchin, A., Suzuki, J., Seki, H., Masutani, M., Shiroto, H., Kawalami, Y. (2000) Immunostained cathepsin B and L correlate with depth of invasion and different metastatic pathways in early stage gastric carcinoma. *Cancer*. **89**, 482-487

Duffy, M. (1997) Proteases as prognostic markers in cancer. *Clin. Can. Res*. 2, 613-618

Eagleton, M., Ballard, N., Lynch, E., Srivastava, S., Upchurch, G. and Stanley, J. (2006) Early increased MT1-MMP expression and late MMP-2 and MMP-9 activity during angiotensin II induced aneurysm formation. *J. Surgical Res.*

Edman, P. and Begg, G. (1967) A protein sequenator. *European J. Biochem*. **1**, 80-91

Elliott, E., Dennison, C., Fortgens, P. and Travis, J. (1995) Paraformaldehyde fixation of neutrophils for immunolabeling of granule antigens in cryoultrasections. *J. Histochem. Cytochem.* **43**, 1019-1025,

Elshaw, S., Henderson, N., Knox, A., Watson, S., Buttle, D. and Johnson, S. (2004) Matrix metalloproteinase expression and activity in human airway smooth muscle cells. *J. Pharm.* **142**, 1318-1324

Emmert-Buck, M., Roth, M., Zhuang, Z., Campo, E., Rozhin, J., Sloane, B., Liotta, L. and Stetler-Stevenson, W. (1994) Increased gelatinase A (MMP-2) and cathepsin B activity in invasive tumour regions of human colon cancer samples. *Ameri. J. Path.* **145**, 1285-1290

Engvall, E. and Perlmann, P. (1972) Enzyme-linked immunosorbent assay, ELISA: III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J. immunol.* **109**, 129-135

Erdel, M., Trefz, G., Spiess, E., Habermaas, S., Spring, H., Lah, T. and Ebert, W. (1990) Localization of cathepsin B in two human lung cancer cell lines. J. Histochem Cytochem. 38, 1313-1321

Fernandez-Catalan, C., Bode, W., Huber, R. *et al*. (1998) Crystal structure of the complex formed by the membrane type 1-matrix metalloproteinase with the tissue inhibitor of metalloproteinases-2, the soluble progelatinase A receptor. *EMBO Journal* **17**, 5238-5248

Fernandez-Patron, C., Hardy, E., Sosa, A., Seoane, J. and Castellanos, L. (1995) Double Staining of coomassie blue-stained polyacrylamide gels by imidazole-sodium dodecyl sulfate-zinc reverse staining: sensitive detection of coomassie blue-undetected proteins. *Anal. Biochm.* **224**, 263-269

Finkelstein, J., Antony, E., Hingorani, M. and O'Donnell, M. (2003) Overproduction and analysis of eukaryotic multiprotein complexes in Escherichia coli using a dual-vector strategy *Anal Biochem.* **319**, 78-87

Foda, H. and Zucker, S. (2001) Matrix metalloproetainses in cancer invasion, metastasis and angiogenesis. *DDT* **6**, 478-482

Foekens, J., Look, M., Bolt-de Vries, J., Meijer-van Gelder, M., van Putten, W. and Klijn, J. (1999) Cathepsin D in primary breast cancer: prognostic evaluation involving 2810 patients. *BR J. Cancer*. **79**, 300-307

Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, F., Leist, M. and Jaattela, M. (2001) Cathepsin B acts as a dominant execution protease in tumour cell apoptosis induced by tumour necrosis factor. *J. Cell Biol*. **153**, 999-1009

Folgueras, A., Pendas, A., Sanchez, L. and Lopez-Otin, C. (2004) Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *Int. J. dev. Biol*. **48**, 411-424

Forget, M., Desrosiers, R. and Beliveau, R. (1999) Physiological role of matrix metalloproteinases: implications for tumor growth and metastasis. *Can. J. Physiol. Pharamacol*. **77**, 465-480

Freitas, S., Meduri, G., Le Nestour, E., Bausero, P. and Perrot-Applanat, M. (1999) Expression of matrix metalloproeinases and ther inhibitors in blood vessels in human endometrium. *Biol. Reprod.* **61**, 1070-1082

Frere, J. (2006) Beta-lactamases and bacterial resistence to antibiotics. *Mol. Microbiol*. **16**, 385-395

Fridman, R., Toth, M., Pena, D. and Mobashery, S. (1995) Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). *Can. Res*. **55**, 2548-2555

Gabriel, O. and Gersten, D. (1992) Staining for enzymatic activity after gel electrophoresis, I. *Anal. Biochem*. **203**, 1-21

Gadéa, G., Lapasset, L., Gauthier-Rouvière, C. and Roux, P. (2002) Regulation of Cdc42-mediated morphological effects: a novel function for p53. *EMBO J*. **21**, 2373-2382

Gálvez, B., Matías-Román, S., Albar, J., Sánchez-Madrid, F. and Arroyo, A. (2001) Membrane type 1-matrix metalloproteinase is activated during migration of human endothelial cells and modulates endothelial motility and matrix remodeling. *J. Biol. Chem*. **276**, 37491-37500

Gibbs, D., Warner, R., Weiss, W., Johnson, K. and Varani, J. (1999) Characterization of matrix metalloproteinases produced by rat alveolar macrophages. *Am. J. Respir. Cell. Mol. Biol.* **20**, 1136-1144

Gilles, C., Polette, M., Coraux, C., Tournier, J., Meneguzzi, G., Munaut, C., Volders, L., Rouselle, P., Birembaut, P. and Foidart, J. (2001) Contribution of MT1-MMP and of human laminin-5 γ2 chain degradation to mammary epithelial cell migration. *J. Cell Sci.* **114**, 2967-2976

Ginestra, A., Monea, S., Seghezzi, G., Dolo, V., Nagase, H., Mignatti, P. and Vittorelli, M. (1997) Urokinase plasminogen activator and gelatinases are associated with membrane vesicles shed by human HT1080 fibrosarcoma cells. Amer. Soc. Biochem Mol. Biol. 272, 17216-17222

Goldring, J.P., and Coetzer, T.H.T. (2003) Isolation of chicken immunoglobulins (IgY) from egg yolk. *Biochem. Mol. Biol. Edu*. **31**, 185-187

Golubkov, V., Chekanov, A., Shiryaev, S., Aleshin, A., Ratnikov, B., Gawlik, K., Radichev, I., Motamedchaboki, K., Smith, J. and Strongin, A. (2007) Proteolyisis of the membrane type-1 matrix metalloproteinase prodomain. *J. Biol. Chem.* **282**, 36283-36291

Golubnitschaja, O., Yeghiazaryan, K., Liu, R., Monkemann, H., Leppert, D., Schild, H., Haefliger, I. and Flammer, J. (2004) Increased expression of matrix metalloproteinases in mononuclear blood cells of normal-tension glaucoma patients *J. Glaucoma* **13**, 66-72

Gomez, D., Alanso, D., Yoshiji, H. and Thorgeirsson, U. (1997) Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur. J. Cell Biol*. **74**, 111-122 (Abstract)

Goretzki, L., Schmitt, M., Mann, K., Calvete, J., Chucholowski, N., Kramer, M., Gunzler, W., Janicke, A. and Graeff, A. (1992) Effective activation of the proenzyme form of urokinase-type plaminogen activator (pro-uPA) by the cytosine protease cathepsin L. *FEBS Lett.* **297**, 112-118

Grindstaff, J., Brodie, E. and Ketterson, E. (2003). Immune function across generations: integrating mechanism and evolutionary process in maternal antibody transmission. *Proc R Soc Lond B* **270**, 2309–2319

Gross, J. and Lapiere, C. (1962) Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proc. N. A. S.* **48**, 1014-1022

Hanes, C. (1932) Studies on plant amylases: the effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochem. J*. **26**, 1406-1421

Hansen, P., Scoble, J., Hanson, B. and Hoogenraad, N. (1998) Isolation and purification of immunoglobulins using thiophilic interaction chromatography *J. Immunol. Methods*. **215**, 1-7

Harlow, E. and Lane, D. (1988) Antibodies: a laboratory manual. CSHL Press. pp 61-67; 93-112

Hasebe, T., Hartman, R., Fu, L., Amano, T. and Shi, Y. (2007) Evidence for a cooperative role of gelatinase A and membrane type-1 matrix metalloproteinase during *Xenopus laevis* development. *Mech. Develop.* **124**, 11-22

Hau, J. and Hendriksen, C. (2005) refinement of polyclonal antibody production by combning oral immunization of chickens with harvest of antibodies from the egg yolk. *ILAR J.* **46**, 294-299

Haupt, L., Thompson, E., Trezise, A., Irving, R., Irving, M. and Griffiths, L. (2006) *In vitro* and *in vivo* MMP gene expression localization by in situ-RT-PCR in cell culture and paraffin embedded human breast cancer cell line xenografts *BMC Cancer* **6**

Henriet, P., Blavier, L. and Declerck, Y. (1999) Tissue inhibitors of matrix metalloproteinases (TIMP) in invasion and proliferation. *APMIS.* **107**, 111-119

Hernandez-Barrantes, S., Toth, M., Bernardo, M., Yurkova, M., Gervasi, D., Raz, Y., Sang, Q. and Fridman, R. (2000) Binding of active (57 kDa) membrane type-1 matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and proMMP-2 activation. J. Biol. Chem. 275, 12080-12089

Heussen, C. and Dowdle, E. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem.* **102**, 196-202.

Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S., Mankani, M., Robey, P., Poole, R., Pidoux, I., Ward, J. and Birkedal-Hansen, H. (1999) MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis and connective tissue disease due to inadequate collagen turnover. *Cell*, **99**, 81-92

Honrubia-Marcos, M., Ramos, A. and Gil, J. (2005) Overexpression of the ftsZ gene from *Corynebacterium glutamicum* (*Brevibacterium lactofermentum*) in *Eschericia coli*. *Can. J. Microbiol*. **51**, 85-89

Hotary, K., Allen, E., Brooks, P., Datta, N., Long, M. and Weiss, S. (2000) Membrane type 1 matrix metalloproteinase usurps tumour growth control imposed by the three-dimensional extrac cellular matrix. *Cell.* **114**, 33-45

Huang, W., Suzuki, K., Nagase, H., Arumugam, S., Van Doren, S. and Brew, K. (1996) Folding and chrachterization of the amino-terminal domain of human tissue inhibitor of metalloproteinases-1 (TIMP-1) expressed at high yield in *E. coli. FEBS* **384**, 155-161

Hudson, L. and Hay, F: (1980) Practical immunology. Blackwell Scientific Publ., Oxford. 192-202

Hurst, D., Schwartz, M., ghaffari, M., Jin, Y., Tschesche, H., Fields, G. and Sang, Q. (2004) Catalytic-and ecto-domains of membrane type 1-matrix metalloproteinase have similar inhibition profiles but distinctive endopeptidase activities. *Biochem. J.* **377**, 77-79

Huhtala, P., Tuuttila, A., Chow, L.T., Lohi, J., Keski-Oja, J. & Tryggvason, K. (1991) Complete structure of the human gene for 92-kDa type IV collagenase. Divergent regulation of expression for the 92- and 72-kilodalton enzyme genes in HT-1080 cells. *J. Biol. Chem.* **266**, 16485-16490.

Ichikawa, T., Yamada, Homma, D., Cherry R., Morrison, I. and Kawato, S. 2000 Digital fluorescence imaging of trafficking of endosomes containing low-density lipoprotein in brain astroglial cells. *Biochem. Biophys. Res. Comm.* **269**, 25-30

Irie, K., Tsuruga, E., Sakakura, Y., Muto, T. and Yajima, T. (2001) Immunohistochemical localization of membrane type 1-matrix metalloproteinase (MT1-MMP) in osteoclasts in vivo. *Tiss. Cell*. **33**, 478-482107,

Itoh, Y., Binner, S. and Hagase, H. (1995) Steps involved in activation of the complex of pro-matrix metalloproteinase 2 (progelatinase A) and tissue inhibitor of metalloproteinases (TIMP)-2 by 4-aminophenylmercuric acetate. Biochem. J. 308, 645-651

Itoh, Y. and Seiki, S. (2006) MT1-MMP: a potent modifier of pericellular microenvironment. *J. Cellul. Physiol*. **206**, 1-8

Jiang, A., Lehti, K., Wang, X., Weiss, S., Keski-Oja, J. and Pei, D. (2001) Regulation of membrane-type matrix metalloproteinase 1 activity by dynamin-mediated endocytosis. *PNAS*

Kang, D., Gho, Y., Suh, M. and Kang, C. (2002) Highly sensitive and fast protein detection with coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Bull. Korean Chem. Soc.* **23**, 1511-1512

Kasez, I., Delarue, F., Hagege, J., Bouzhir-Sima, L., Rondeau, E., Sraer, J. and Nguyen, G. (1998) Soluble latent membrane-type 1 matrix metalloprotease secreted by human mesangial cells is activated by urokinase. *Kidney Int*. **54**, 1976-1984

Kawano, Y., Okamoto, I., Murakami, D., Itoh, H., Yoshida, M., Ueda, S. and Saya, H. (2000) Ras oncoprotein induces CD44 cleavage through phosphoinositide 3-OH kinase and the Rho family of small G proteins. *J. Biol. Chem.* **275**, 29628-29635

Kim, P. and Baldwin, R. (1982) Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding. *Ann. Rev. Biochem.* **51**, 459-489

Kim, J.; Yu, W.; Kovalski, K. and Ossowski, L. (1998) Requirement for specific proteases in cancer cell intravasation as revealed by a novel semi-quantitative PCR-based assay. *Cell* **94**: 353-362

Kim, C. and Lee, E. (2000) Effects of operating parameters in *in vitro* renaturation of a fusion protein of human growth hormone and glutathione S transferasefrom inclusion body. *Process.Biochem*. **36**, 111-117

Kleiner, D. and Stetler-Stevenson, W. (1994) Quantitative zymography: detection of picogram quantities of gelatinases. *Anal. Biochem*. **218**, 325-329

Knauper, V., Will, H., Lopez-Otin, C., Smith, B., Atkinson, S., Stanton, H., Hembry, R. and Murphy, G. (1996) Cellular mechanisms for human procollagenase-3 (MMP-13) activation. *J. Biol. Chem.* **271**, 17124-17131

Knight, C.G., Willenbrock, F. and Murphy, G. (1992) A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS* **296**, 263-266

Kobayashi, H., Schmitt, M., Goretzki, L., Chucholowski, N., Calvete, J., Kramer, M., Gunzler, W., Janicke, F. and Graeff, H. (1991) Cathepsin B efficiently activates the soluble and tumour form of the proenzyme urokinase-type plasminogen activator (pro-uPA). *J. Biol. Chem.* **266**, 5147-5152

Koo, H., Kim, J. and Hwang, I. (2002) Refolding of the catalytic and hinge domains of human MT1-MMP expressed in *Escherichia coli* and its characterization. *Mol. Cells* **13**, 118-124

Kos, J., Stabuc, B., Schweiger, A., Krasovec, M., Cimerman, N., Kopitar-Jerala, N. and Vrhovec, I. (1997) Cathepsins B, H and L and their inhibitors stefin A and cystatin C in sera of melanoma patients. *Clin. Can. Res*. **3**, 1815-1822

Krajewski, S., Zapata, J. and Reed, J. (1996) Detection of multiple antigens on western blots. *Anal. Biochem.* **236**, 221-228

Kreuter, J. and Haenzel, I. (1978) Mode of action of immunological adjuvants: some physiochemical factors influencing the effectivity of polyacrylic adjuvants. *Infect. Immun*.**19**, 667-675

Kridel, S., Sawai, H., Ratnikov, B., Chen, E., Li, W., Godzik, A., Strongin, A. and Smith, J. (2002) A unique substrate binding mode discriminates membrane type-1 matrix metalloproteinase from other matrix metalloproteinases. *J. Biol. Chem.* **277**, 23788-23793

Kröger, M. and Tschesche, H. (1997) Cloning, expression and activation of a truncated 92-kDa gelatinase minienzyme. *Gene.* **196**, 175-180

Kurien, B. and Scofield, R. (2006) Western blotting. *Methods* **38**, 283-293

Kwissa, M. and Lindblad, E. (2003) Codelivery of a DNA vaccine and a protein vaccine with aluminum phosphate stimulates a potent and multivalent immune response. *J. Mol. Med.* **81**, 502-510

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T. *Nature* **227**, 680-685

Lang, R., Braun, M., Sounni, N., Noel, A., Frankenne, F., Foidart, J., Bode, W. and Maskos, K. (2004) Crystal Structure of the Catalytic Domain of MMP-16/MT3-MMP: Characterisation of MT-MMP Specific Features. *J. Mol. Biol*. **336**, 213-225

Langlois, S., Tomasso, G., Boivin, D., Roghi, C., Murphy, G., Gingras, D. and Beliveau, R. (2005) Membrane type1-matrix metalloproteinase induces endothelial cell morphogenic differentiation by a caspase-dependent mechanism. *Exp. Cell Res*. **307**, 452-464

Larsson, A. and Nilsson, B. (1988) Immunization with nanaogram quantities of nitrocellulose-bound antigen, electroblotted from sodium dodecyl sulphate-polyacrylamide gels. *Scan. J. Immunol*. **27**, 305-309

Leber, T and Balkwill, F. (1997) Zymography: a single-step staining method for quantitation of proteolytic activity on substrate gels. *Anal. Biochem.* **249**, 24-28

Lehti, K., Lohi, J., Juntunen, M., Pei, D. and Keski-Oja, J. (2002) Oligomerization through Hemopexin and Cytoplasmic Domains Regulates the Activity and Turnover of Membrane-type 1 Matrix Metalloproteinase. *J. Biol. Chem.* **277**, 8440-8448

Leighton, M and Kadler, K. (2003) Paired basic/furin-like proprotien convertase cleavage of pro-bmp-1 in the trans-golgi network. *J Biol. Chem.* **278**, 184778-18484

Lepage, T. and Gache, C. (1990) Early epression of a collogenase-like hatching enzyme gene in the sea urchin embryo. *EMBO J.* **9**, 3003-3012

Li, H., Bauzon, D., Xu, X., Tschesche, H., Cao, J. and Sang, Q. (1997) Immunological characterisation of cell-surface and soluble forms of membrane type 1 matrix metalloproteinase in human breast cancer cells and fibroblasts. *Mol. Carcinogenesis*. **22**, 84-94 (Abstract only)

Lichte, A., Kolkenbrock, H. and Tschesche, H. (1996) The recombinant catalytic domain of membrane-type matrix metalloproteainse-1 (MT1-MMP) induces activation of progelatinase A and progelatinase A complexed with TIMP-2. *FEBS Lett.* **397**, 277-282

Lin, M., Williams, C., Murray, M., Conn, G. and Ropp, P. (2004) Ion chromatographic quantification of cyanate in urea solutions: estimation of the efficiency of cyanate scavengers for use in recombinant protein manufacturing. *J. Chromatog B.* **803**, 353-362

Lindblad, E. (2004) Aluminium compounds for use in vaccines. *Immun. Cell Biol.* **82**, 497-505

Lindblad, E. (2004) Aluminium adjuvants- in retrospect and prospect. *Vaccine* **22**, 3658-3668Lichte, A., Kolkenbrock, H. and Tschesche, H. (1996). The recombinant catalytic domain of membrane-type matrix metalloproteainse-1 (MT1-MMP) induces activation of progelatinase A and progelatinase A complexed with TIMP-2. *FEBS Lett.* **397**, 277-282

Liu, E., Thant, A., Kikkawa, F., Kurata, H., Tanaka, S., Nawa, A., Mizutani, S., Matsuda, S., Hanafusa, H. and Hamaguchi, M. (2000) The ras-mitogen-activated protein kinase pathway is critical for the activation of matrix metalloproteinase secretion and the invasiveness in v-crk-transformed 3Y1. *Can. Res.* **60**, 2361-2364

Lineweaver, H and Burk, D. (1934) The determinantion of enzyme dissociation constants. *J. Americ. Chem. Soc*. **56**, 658-666

Ma, D., Pan, G. and Han, F. (2002) Diastereoselective Henry reactions of N,N-dibenzyl α-amino aldehydes with nitromethane catalysed by enantiopure guanidines. *Tetrahedron Lett.* **43**, 9401-9403

Machold, C., Schlegl, R., Buchingerb, W. and Jungbauer, A. (2005) Matrix assisted refolding of proteins by ion exchange chromatography. *J. Biotech*. **117**, 83-97

Maidment, J., Moore, D., Murphy G., Murphy, G. and Clark, I. (1999) Matrix metalloproteinase homologues from Arabidopsis thaliana. *J. Biol. Chem*. **274**, 34706-34710

Maisi, P., Prikk, K., Sepper, R., Pirila, E., Salo, T., Hietanen, J. and Sorsa, T. (2002) Soluble membrane-type 1 matrix metalloproteinase (MT1-MMP) and gelatinase A (MMP-2) in induced sputum and bronchoalveolar lavage fluid of human bronchial asthma and bronchiectasis. *APMIS* **110**, 771-782

Makrides, S. (1996) Strategies for achieving high-level expression of genes in Escherichia coli. Microbiol. Rev. 60, 512-538

Malemud, C. (2006) Matrixmetalloproteinases in health and disease: an overview. *Fronteiers in Biosci.*. **11**, 1696-1701

Maquoi, E., Peyrollier, K., Noel, A., Foidart, J. and Frankenne, F. (2003) Regulation of membrane-type 1 matrix metalloproteinase activityby vacuolar H+-ATPases. *Biochem. J.* **373**, 19–24

Markowski, G. and Ramsby, M. (1996) Calibrating gelatin zymograms with human gelatinase standards. *Anal. Biochem*. **236**, 353-356

Marston, F. (1986) The purification of eukaryotic polypeptides synthesized in *Eschericia coli*. *Biochem J*. **240**, 1-12

Massova, I., Kotra, L., Fridman, R. and Mobashery, S. (1998) Matrix metalloproteinases: structures, evolution and diversification. *FASEB J*. **12**, 1075-1095

Mayer, G. and Bendayan, M. (1999) Immunogold signal amplification: application of the CARD approach to electron microscopy. *J. Histochem. Cytochem.* **47**, 421-430

Mayhew, T.M. and Desoye, G. (2004) A simple method for comparing immunogold distributions in two or more experimental groups illustrated suing GLUT1 labelling of isolated trophoblast cells. *Placenta* **25**, 580-584

Middleberg, A. (2002) Preparative protein refolding. *Trends in Biotechnol.* **20**: 437-443.

Moore, W. and Spilburg, C. (1986) Purification of human collagenases with a hydroxamic acid affinity column. *Biochem*. **25**, 5189-5195

Mori, H., Tomari, T., Koshikawa, N., Kajita, M., Itoh, Y., Sato, H., Tojo, H., Yana, I. and Seiki, M. (2002) CD44 directs membrane type-1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. *EMBO* **21**, 3949-3959

Morewood, C., Elliott, E., Dennison, C. and Bruton, A. (1992). Further Modification of the LKB 7800 Series Knifemaker for Improved Reproducibility in Breaking 'Cryo' Knives. *J. Microscopy*. **168**, 111-114.

Mucha, A., Cuniasse, P., Kannan, R., Beau, F., Yiotakis, A., Basset, P. and Dive, V. (1998) Membrane type-1 matrix metalloprotease and stromelysin-3 cleave more efficiently synthetic substrates containing unusual amino acids in their P_1 Positions *J Biol Chem*, **273**, 2763-2768

Mun-Bryce, S. and Rosenberg, G. (1998) Matrix metalloproteinases in cerebrovascular disease. *J. Cerebral Blood flow and metabolism.* **18**, 1163-1172

Munoz-Najar, M., Neurath, K. and Vumbaca, F. (2006) Hypoxia stimulates breast carcinoma cell invasion through MT1-MMP and MMP-2 activation. *Oncogene* **2523**, 79–92.

Murk, J.; Posthuma, G., Koster, A., Geuze, H., Verkleij, A.; Kleijmeer, M. and Humbel, B. Influence of aldehyde fixation on the morphology of endosomes and lysosomes: quantitative analysis and electron tomography. *J. Microscop*. **212**, 81-90

Murphy, G. and Knauper, V. (1997) Relating matrix metalloproteinase structure to function: why the hemopexin domain*? Matrix Biol*. **15**, 511-518

Murphy, G., Knauper, V., Atkinson, S., Butler, G., English, W., Hutton, M., Stracke, J. and Clark, I. (2002) Matrix metalloproteinases in arthritic disease. *Arthritis Res.* **4**, 39-49

Murray, G. (1992) Enzyme histochemistry and immunohistochemistry with freeze-dried or freeze-substituted resin-embedded tissue. *Histochem. J.* **24**, 399-408

Mysliwy, J., Dingley, A., Sedlacek, R. and Grötzinger, J. (2006) Structural characterization and binding properties of the hemopexin-like domain of the matrixmetalloproteinase-19. *Protein Exp Purif.* **46,** 2406-413

Nagase, H. and Woessner, J. (1999) Matrix metalloproteinases. *J. Biol. Chem*. **274**, 21491-21494

Narat, M. (2003) Production of antibodies in chickens. *Food.Technol. Biotechnol*. **41**, 259-267

Newman, G., Jasani, B. and Williams, E. (1983) A simple post-embedding system for the rapid demonstration of tissue antigens under the electron microscope. *Histochem. J.* **15**, 543-555

Nguyen, M. Arkell, J. and Jackson, C. (2000) Three-dimensional collagen matrices induce delayed but sustained activation of progelatinase A in human endothelial cells via MT1-MMP. *Internat. J. Biochem. Cell Biol.* **32**, 621-631

Nomura, T and Katunuma, N. (2005) Involvement of cathepsins in the invasion, metastasis and proliferationof cancer cells. *J. Med. Invest*. **52**, 1-9

Oblander, S., Zhou, Z., Galvez, B., Starcher, B., Shannon, J., Durbeej, M., Arroyo, A., Tryggvason, K. and Apte, S. (2005) Distinctive functions of membrane type 1-matrix metalloproteinase (MT1-MMP or MMP-14) in lung and submandibular gland development are independent of its role in proMMP-2 activation. *Devel. Biol*. **277**, 255-269

Ochieng, J., Basolo, F., Albini, A., Melchiori, A., Watanabe, H., Elliott, J., Raz, A., Parodi, S. and Russo, J. (1991) Increased invasive, chemotactic and locomotive abilities of c-Ha-ras-transformed human breast epithelial cells. *Invas. Metast*. **11**, 38-47

Ohkubo, S., Miyadera, K., Sugimoto, Y., Matsuo, K., Wierzba, K., Yamada, Y. (1999) Identification of substrate sequences for membrane type-1 matrix metalloproteinase using bacteriophage peptide display library. *Biochem. Biophys. Res. Comm.* **266**, 308-313

Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M. and Okada, Y. (1997) Membrane type I matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.* **272**, 2446-2451

Okada, A., Bellocq, J., Rouyer, N., Chenard, M., Rio, M., Chambon, P. and Basset, P. (1995) Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast and head and neck carcinomas. *Proc. Natl. Acad. Sci. USA* **92**, 2730-2734

Oku, N., Matsukawa, M., Yamakawa, S., Asai, T., Yahara, S., Hashimoto, F. and Akizawa, T. (2003) Inhibitory effect of green tea polyphenols on membrane-type a matrix metalloproteinase, MT1-MMP. *Biol. Pharm. Bull.* **26**, 1235-1238

Ong, K., Sun, Z., Cheng, T-c., Wei, Y., Yuan, J. and Yin, R. (2004) Denaturation/renaturation of organophosphorus acid anhydrolase (OPAA) using guanidinium hydrochloride and urea.

Ou, L., Ma, J., Zheng, X., Chen, X., Li, G. and Wu, H. (2006) The expression and refolding of isotopically labeled recombinant Matrilysin for NMR studies. *Protein Exp. Purif.* **47**, 367-373

Overall, C. (2002) Molecular determinants of metalloproteainse substrate specificity. *Mol. Biotech.* **22**, 51-81

Overall, C. and Lopez-Otin, C. (2002) Strategies for MMP inhibition in cancer: Innovations for the post-trial era. *Nature Reviews* **2**, 657-672

Paine, T., Fontanini, G., Basolo, F., Geronimo, I., Elliott, J. and Russo, J. (1992) Mutated c-Ha-ras oncogene alters cytokeratin expression in the human breast epithelial cell line MCF-10A. *Am. J. Pathol.* **140**, 1483-1488

Palmer, T (1981) Understanding enzymes. Ellis Horwood Limited. pp126-127

Palosaari, H., Pennington, C., Larmas, M., Edwards, D., Tjaderhane, L. and Salo, T (2003) Expression profile of matrix metalloprotienases (MMPs) and tissue inhibitors of MMPs in mature human odontoblasts and pulp tissue. *European J. Oral Sci*. **111**, 117-127

Parker, A., Stow, M., Panicker, A. *et al*. (2000) Large-scale expression, refolding, and purification of the catalytic domain of human macrophage metalloelastase (MMP-12) in *Escherichia coli*. *Protein Express. Purif.* **20**, 152-161

Pathak, N., Hu, S. and Koehn, J. (1998) The expression, refolding and purification of the catalytic domain of human colleganse-3 (MMP-13). *Protein Express. Purif*. **14**, 283-288

Patterson, R., Youngner, J., Weigle, W. and Dixon, F. (1962) Antibody production and transfer to egg yolk in chickens. *J. Immunol*. **89**, 272-278

Pei, D and Weiss, S. (1996) Transmembrane-deletion mutants of the membrane-type matrix metalloproteinase-1 process progelatinase A and express intrinsic matrix-degrading activity. *J. Biol. Chem.* **271**, 9135-9140

Plachter, B., Wieczorek, L., Scholl, B., Ziegelmaier, R. and Jahn, G. (1992) Detection of cytomegalovirus antibodies by an enzyme-linked immunosorbent assay using recombinant polypeptides of the large phosphorylated tegument protein pp150. *J. Clin. Micro.* **30**, 201-206

Polette, M. and Birembaut, P. (1998) Membrane type metalloproteinases in tumour invasion. *Int. J Biochem. Cell Biol.***30**, 1195-1202

Polsen, A. (1990) Isolation of IgY from yolk eggs by a chloroform polyethylene glycol procedure. *Immunol. Invest.* **19**, 253-258

Polsen, A., Barbara Von Wechmar, M.,. and Fazakerley, G. (1980) Antibodies to proteins from yolk of immunized hens. *Immunol. Comm.* **9**, 495-514

Polsen, A., Potgieter, G., Largier, J., Mears, G. and Joubert, F. (1963) The fractionation of protein mixtures by linera polymers of high molecular weight. *Biochim. Biophys. Acta.* **82**, 463-475

Ponta, H., Sherman, L. and Herrlich, P. (2003) CD44: From adhesion molecules to signaling regulators. *Nature Reviews Mol. Cell Biol.* **4**, 33-45

Premzl, A., Zavasnik-Bergant, V., Turk, V. and Kos, J. (2003) Intracellular and extracellular cathepsin B facilitates invasion of MCF-10A neoT cells through reconstituted extracellular matrix in vitro. *Exp. Cell Res.* **283**, 206-214

Prouty, W., Karnovsky, M. and Goldberg, A. (1975) Degradation of abnormal proteins in *Eschericia coli*. *J. Biol. Chem.* **250**, 1112-1122

Pryor, K. and Leiting, B. (1997) High-level expression of soluble protein in Escherichia coli using a His_6-tag and maltose-binding protein double-affinity fusion system. Prot. Exp. Purific. 10, 309-319 (Abstract)

Ramchurana, S., Mateusa, B., Holsta, O., Karlssona, E. (2005) The methylotrophic yeast *Pichia pastoris* as a host for the expression and production of thermostable xylanase from the bacterium *Rhodothermus marinus*. *FEMS Yeast Res.* **5**, 839-850

Raymond, S. and Weintraub, L. (1959) Acrylamide gel as a supporting medium for zone electrophoresis. *Sci.* **130**, 711

Read, S and Northcote, D. (1981) Minimisation of variation in the response to different proteins of the coomassie blue G dye-binding assay for protein. *Anal. Biochem.* **116**, 53-64

Remacle, A., Murphy, G. and Roghi, C. (2003) Membrane type-1 matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. *J. Cell Sci*. **116**, 3905-3916

Ribatti, D., Vacca, A., Roncali, L. and Dammacco, F. (1996) The chicken embryo choriallantoic membrane as a model for *in vivo* research on angiogenesis. *Int. J. Dev. Biol*. **40**, 1189-1197

Ridley, A., Paterson, H., Johnston, C., Diekmann, D. and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-410

Roderfeld, M., Buttner, F., Bartnik, E. *et al*. (2000) Expression of human membrane type-1 matrix metalloproteinase in *Pichia pastoris*. *Protein Express. Purif.* **19**, 369-374

Rosenberg, G. (2002) Matrix metallproteinases in neuroinflammation. *GLIA.* **39**, 279-291

Rosenthal, E., Hotary, K., Bradford, C. and Weiss, S. (1999) Role of membrane type-1 matrix metalloproteinase and gelatinase A in head and neck squamous cell carcinoma invasion in vitro. *Otolaryngology-Head and Neck surgery* **121**, 337-343

Rossello, A., Nuti, E., Catalani, M., Carelli, P., Orlandini, E., Rapposelli, S., Tuccinardi, T., Atkinson, S., Murphy, G. and Balsamo, A. (2005) A new development of matrix metalloproteainse inhibitors: twin hydroxamic acids as potent inhibitors of MMPs. *Bioorg. Med. Chem. Lett.* **15**, 2311-2314

Roy, I., Sharma, A. and Gupta, M. (2004) Three-phase partitioning for simultaneous renaturation and partial purification of *Aspergillus niger* xylanase. *Biochim. Biophys*. **1698**, 107-110

Rozanov, D., Deryugina, E., Monosov, E., Marchenko, N. and Strongin, A. (2004) Aberrant, persistent inclusion into lipid rafts limits the tumorigenic function of membrane type-1 matrix metalloproteinase in malignant cells. *Exp. Cell Research.* **293**, 81-95

Sabeh, F., Ota, I., Holmbeck, K., Birkedal-Hansen, H., Soloway, P., Balbin, M., Lopez-Otin, C., Shapiro, S., Inada, M., Krane, S., Allen, E., Chung, D. and Weiss, S. (2004) Tumour cell traffic through the extracellular matrix os controlled by the membrane-anchored collagenase MT1-MMP. *J. Cell Biol*. **167**, 769-781

Sambrook, J. and Russell, D. (2006) Fragmentation of DNA by sonication. Cold Spring Harb. Protoc.

Santama, N., Krijnse-Locker, J., Griffiths, G., Noda, Y., Hirokawa, N. and Dotti, C. (1998) KIF2β, a new kinesin superfamily protein in non-neuronal cells, is associated with lysosomes and may be implicated in their centrifugal translocation. *EMBO J.* **17**, 5855-5867

Sarkar, S., Chaudhuri, S. and Basu, T. (2002) Mechanism of artificial transformation of *E. coli* with plasmid DNA- clues from the influence of ethanol. *Current Sci*. **83**, 1376-1380

Sato, H., Takino, T., Okada, Y., Cao, J., Shinigawa, A., Yamamoto, E. and Seiki, M. (1994) A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature* **370**, 61-65

Sato, H., Takino, T., Kinoshita, T., Imai, K., Okada, Y., Stetler-Stevenson, W. and Seiki, M. (1996) Cell surface binding and activation of gelatinase A induced by expression of membrane type-1 matrix metalloproteinase (MT1-MMP). *FEBS lett.* **385**, 238-240

Sato, H., Kinoshita, T., Takino, T., Nakayama, K. and Seiki, M. (1996) Activation of a recombinant membrane type 1-matrix metalloproteinase (MT1-MMP) by furin and its interaction with tissue inhibitor of metalloproteinases (TIMP)-2. *FEBS Lett* **393**,101-104

Schein, C. and Noteborn, M. (1998) Formation of recombinant soluble proteins in *Escherichia coli* is favoured by lower temperatures. *Biotech.* **6**, 291-294 (Abstract)

Schofer, C., Janacek, J., Weipoltshammer, K., Pourani, J. and Hozak, P. (2004) Mapping of cellular compartments based on ultrastructural immunogold labelling. *J. Struct. Bio.* **147**, 128-135

Seiki, M., Koshikawa, N. and Yana, I. (2003) Role of pericellular proteolysis by membrane-type 1 matrix metalloproteinase in cancer invasion and angiogenesis. *Can. Metastas. Rev*. **22**, 129-143

Shapiro, A., Vinuela, E. and Maizel, J. (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **28,** 815–820

Shapiro, S. (1999) Diverse roles of macrophage matrix metalloproteinases in tissue destruction and tumour growth. *Thrombosis and Haemostasis* **82**, 846-849

Shin, I., Kim, S., Song, H., Kim, H. and Moon, A. (2005) H-Ras activation of Rac-MKK3/6-p38 pathway. *J. Biol. Chem.* **280**, 14675-14683

Sloane, B., Moin, K., Sameni, M., Tait, L., Rozhin, J. and Ziegler, G. (1994) Membrane association of cathepsin B can be induced by transfection of human epithelial cells with c-Ha-ras oncogene. *J. Cell. Sci.* **107**, 373-384

Smejkal, G. and Kaul, C. (2001) Stability of nitroblue tetrazolium-based alkaline phosphatase substrates. *J. Histochem. Cytochem.* **49**, 1189-1190

Soule, H., Maloney, T., Wolman, S., Peterson, W., Brenz, J., Mcgrath, C., Russo, J., Pauley, R., Jones, R. and Brooks, S. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer. Res.* **50**, 6075-6086

Sounni, N., Janssen, M., Foidart, J. and Noel, A. (2003) Membranetype-1matrix metalloproteinase and TIMP-2 in tumour angiogenesis. *Matrix Biol*. **22**, 55-61

Staak, C., Schwarzkorpf, C., Behn, I., Hommel, U., Hlinak, A., Schade, R. and Erhard, M. (2001) Isolation of IgY form yolk. Chicken egg yolk, antibodies, production and application. Springer, pp 65-71

Stanton, H., Gavrilovic, J., Atkinson, S., d'Ortho, M., Yamada, K., Zardi, L. and Murphy, G. (1998) The activation of proMMP-2 (gelatinase A) by HT1080 fibrosarcoma cells is promoted by culture on a fibronectin substrate and is concomitant with an increasein processing of MT1-MMP (MMP-14) to a 45 kDa form. *J. Cell Sci*. **111**, 2789-2798

Stetler-Stevenson, W., Liotta, L. and Kleiner, D. (1993) Extracellular matrix 6: role of matrix metalloproteinases in tumour invasion and metastasis. *Faseb J.* **7**, 1434-1441

Strongin, A., Collier, I., Bannikov, G., Marmer, B., Grant, G. and Goldberg, G. (1995) Mechanism of cell surface activation of 72 kDa type IV collagenase. *J. Biol. Chem.* **270**, 5331-5338

Studier, F., Rosenberg, A., Dunn, J. and Dubendorff, J. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**, 60-89.

Suzuki, K., Kan, C., Hung, W., Gehring, M., Brew, K. and Nagase, H. (1998) Expression of human pro-matrix metalloproteinase 3 that lacks the N-terminal 34 residues in Escherichia coli: autoactivation and interaction with tissue inhibitor of metalloproteinase 1 (TIMP-1). *Biol. Chem.* **379**, 185-191

Tait, L., Soule, H. and Russo, J. (1990) Ultrastructural and immunocytochemical characterization of an immortalized human breast epithelial cell line, MCF-10. *Cancer Res*. **50**, 6087-6094

Tam, E., Wu, Y., Butler, G., Stack, M. and Overall, C. (2002) Collagen binding properties of the membrane type-I matrix metalloproteinase (MT1-MMP) hemopexin-like C domain. *J. Biol. Chem.* **277**, 39005-39014

Testa, J., Brooks, P., Lin, J. and Quigley, J. (1999) Eukaryotic expression cloning with an antimetastatic monoclonal antibody identifies a tetraspanin (PETA-3/CD151) as an effector of human tumour cell migration and metastasis. *Can Res*. **59**, 3812-3820

Tetu, B., Brisson, J., Wang, C., Lapointe, H., Beaudry, G., Blanchette, C. and Trude, D. (2006) The influence of MMP-14, TIMP-2 and MMP-2 expression on breast cancer prognosis. *Breast Cancer Res*. 8

Thant, A., Sein, T., Liu, E., Machida, K., Kikkawa, F., Koike, T., Seiki, M., Matsuda, S. and Hamaguchi, M. (1999) Ras pathway is required for the activation of MMP-2 secretion and for the invasion of src-transformed 3Y1. *Oncogene* **18**, 6555-6563

Thompson, J., Gibson, T., Plewniak, F., Jeanmougin, F. and Higgins, D. (1997) The Clustal-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nuc. Acids Res.* **25**, 4876-4882

Tokumaru, Y., Fujii, M., Otani, Y., Kameyama, K., Imanishi, Y., Igarashi, N. and Kanzaki, J. (2000) Activation of matrix metalloproteinase-2 in head and neck squamous cell carcinoma: studies of clinical samples nd in vitro cell lines co-cultured with fibroblasts. *Cancer Letters* **150**, 15-21

Toth, M., Bernardo, M., Gervasi, D., Soloway, P., Wangs, Z., Bigg, H., Overall, C., DeClerck, Y., Tschesche, H., Cher, M., Brown, S., Mobashery, S. and Fridman, R. (2000) Tissue inhibitor of metalloproteinase (TIMP)-2 acts synreggistically with synthetic matrix metalloproteinase (MMP) inhibitors but not with TIMP-4 to enhance the (membrane type-1)-MMP-dependent activation of pro-MMP-2. *J. Biol. Chem*. **275**, 41415-41423

Toth, M., Herrandez-Barrantes, S., Osenkowski, P., Bernardo, M., Gervasi, D., Yoichiro, S., Meroueh, O., Kotra, L., Galvez, B., Arroyo, A., Mobashery, S. and Fridman, R. (2002) Complex pattern of membrane type-1 matrix metalloproteinase shedding. *J. Biol. Chem*. **277**, 26340-26350

Toth, M., Chvyrkova, I., Bernardo, M., Herandez-Barrantes, S. and Fridman, S. (2003) Pro-MMP-9 activation by the MT1-MMP/MMP-2 axis and MMP-3: role of TIMP-2 and plasma membranes. *Biochem. Biophys. Res. Comm*. **308**, 386-395

Towbin, H., Staehelin, T. and Gordon, J. (1979) electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354

Tracy, R., Katzmann, J., Kimlinger, T., Hurst, G. and Young, D. (1983) Development of monoclonal antibodies to proteins separated by two-dimensional gel electrophoresis. *J. immunol. Methods.* **65**, 97-107

Tsumoto, K., Ejima, D., Kumagai, I. *et al*. (2003) Practical considerations in refolding proteins from inclusion bodies. *Protein Express. Purif.* **28**, 1-8

Ueda, J., Kajita, M., Suenaga, N., Fujii, K. and Seiki, M. (2003) Sequence-specific silencing of MT1-MMP expression suppresses tumour cell migration and invasion: importance of MT1-MMP as a therapeutic target for invasive tumours. *Oncogene* **22**, 8716-8722

Valtanen, H., Lehti, K., Lohi, J. and Keski, J. (2000) Expression and purification of soluble and inactive mutant forms of membrane type-1 matrix metalloproteinase. *Protein Exp. Purif.* **19**, 66-73

Van Wart, H. and Birkedal-Hansen, H. (1990) The cytseine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. USA* **87**, 5578-5582

Vasina, J. and Baneyx, F. (1996) Recombinant protein expression at low temperatures under the transcriptional control of the major *Escherichia coli* cold shock promoter *csp*A. *Appl. Environ. Microbiol*. **62**, 1444-1447

Vazquez, F., Hastings, G., Ortega, M., Lane, T., Oikemus, S., Lombardo, M. and Iruela-Arispe, M: (1999) METH-1, a human ortholog of ADAMTS-1, and METH-2 are members of a new family of proteins with angio-iinhibitory activity. *J Biol Chem.* **274**, 23349–23357

Vincentelli, R., Canaan, S., Campanacci, V., Valencia, C., Maurin, D., Frassinetti, F., Scappucini-Calvo, L., Bourne, Y., Cambillau, C., Bignon, C. (2004) High-throughput automated refolding screening of inclusion bodies *Protein Sci.* **13**, 2782-2792

Visse, R. and Nagase, H. (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure function and biochemistry. *Circulation Res.* **92**, 827

Voller, A., Bartlett, A. and Bidwell, D. (1978) Enzyme immunoassays with special reference to ELISA techniques. *J. Clin. Path.* **31**, 507-520

Vu, T. and werb, Z. (2000) Matrix metalloproteinases:effectors of development and normal physiology. *Genes & Dev*.**14**, 2123-2133

Wang, P., Nie, J. and Pei, D. (2004) The hemopexin-like domain of membrane-type matrix metalloproteinase-1 (MT1-MMP) is not required for its activation of proMMP-2 on cell surface but is essential for MT1-MMP-mediated invasion in three-dimensional type I collagen. *J. Biol. Chem*. **279**, 51148-51155 by gel electrophoresis. *J. Biol. Chem.* **247**, 3242-3251

Weber, G., Bronson, R., Hagan, J., Cantor, H., Schmits, R. and Mak, T. (2002) Absence of the CD44 gene prevents sarcoma metastasis. *Can. Res*. **62**, 2281-2286

Weiner, A., Platt, T. and Weber, K. (1972) Amino-terminal sequence analysis of proteins purified on a nanomole scale

Weintraub, M. and Raymond, S. (1963) Antiserums prepared with acrylamide gel used as adjuvant. *Science.* **142**, 1677-1678

Weng, Y., Hsu, F., Yang, W. and Chen, H. (2006) Optimization of the overexpression of glutamate mutase S component under the control of T7 system by using lactose and IPTG as the inducers *Enzyme Microbial Tech.* **38**, 465-469

Werb, Z. (1997) ECM and cell surface proteolysis: Regulating cellular ecology. *Cell*. **91**, 439-442

Westermarck, J. and Kahari, V. (1999) Regulation of matrix metalloproteinase expression in tumour invasion. *FASEB.* **13**, 781-792

Will, H., Atkinson, S., Butler, G., Smyth, B. and Murphy, G. (1996) The soluble catalytic domain of membrane type 1 matrix metalloproteinase cleaves the propeptide of progelatinase A and initiates autocatalytic activation. *J. Biol. Chem*. **271**, 17119-17123

Wiseman, B., Sternlicht, M., Lund, L., Alexander, C., Mott, J., Bissell, M., Soloway, P., Itohara, S. and Werb, Z. (2003) Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *J. Cell. Biol*. **162**, 1123-1133

Woessner, J. (1991) Matrix metallproteinases and their inhibitors in connective tissue remodeling. *FASEB.* **5**, 2145-2154

Wolf, S. (1995) Introduction to cell and molecular biology. Wadsworth Publishing Company, pp 188.

Wu, X., Gan, B., Yoo, Y. and Guan, J. (2005) FAK-mediated Src phosphorylation of endophilin A2 inhibits endocytosis of MT1-MMP and promotes ECM degradation. *Devel. Cell* **9**, 185-196

Wysocki, A., Staiano-Coico, L. and Grinnell, F. (1993) Wound fluid from chronic leg ulcers contains elevated levels of metalloproteinases MMP-2 and MMP-9. *J. Investig. Dermat.* **101**, 64-68

Yamakawa, S., Asai, T., Uchida, T., Matsukawa, M., Akizawa, T. and Oku, N. (2004) (-)-Epigallocatechin gallate inhibits membrane-type 1 matrix metalloproteinase, MT1-MMP, and tumour angiogenesis. *Can. Lett.* **210**, 47-55

Yamanishi, R., Yusa, I., Bando, N. and Terao, J. (2003) Adjuvant activity in inducing antigen specific IgE antibodies in BALB/c mice: a re-evaluation. *Biosci. Biotechnol. Biochem* **67**, 166-169

Yana, I and Weiss, S. (2000) Regulation of membrane type-1 matrix metalloproteinase activation by proprotein convertases. *Mol. Biol. Cell*. **11**, 2387-2401

Yana, I. and Seiki, M. (2002) MT-MMPs play pivotal roles in cancer dissemination. *Clin. Exp. Met.* **19**, 209-215

Yansura, D. and Henner, D. (1984) Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. *Proc. Natl. Acad. Sci*. **81**, 439-443

Zantek, N., Walker-Daniels, J., Stewart, J., Hansen, R., Robinson, D., Miao, H., Wang, B., Kung, H., Bissell, J. and Kinch, M. (2001) MCF-10A-NeoST: a new cell system for studying cell-ECM and cell-cell interactions in breast cancer. *Clin. Cancer. Res*. **7**, 3640-3648

Zondag, G., Evers, E., Klooster, J., Janssen, L., Van Der Kammen, R. and Collard, J. (2000) Oncogenic Ras down regulates Rac activity, which leads to increased Rho activity and epithelial-mesenchymal transition. *J. Cell. Biol.* **149**, 775-782