Characterisation and purification of an aggrecanase made by injured synovium

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Abstract

Freshly dissected porcine synovial tissue in culture produces an enzymatic activity that cleaves cartilage aggrecan generating ARGS- and AGEG- bearing neo-epitope fragments. The aggrecanolytic activity was abolished when synovial tissue was cultured in the presence of cycloheximide. The enzyme(s) were sensitive to N-terminal inhibitory domain of tissue inhibitor of matrix metalloproteinase (N-TIMP-3) and general matrix metalloproteinase inhibitor (GM6001) suggesting they may belong to a disintegrin and metalloproteinase (ADAM) or ADAM with thrombospondin motifs (ADAMTS) family of enzymes. Cation exchange chromatography was used to partially purify aggrecanase(s) from synovial tissue culture medium (SYCM). Two active species have been separated from the partially purified material using size-exclusion chromatography. The smaller species had a molecular weight of 35-40 kDa while the larger enzyme had an apparent molecular weight greater than 2000 kDa. Low density lipoprotein receptor-related protein (LRP1) didn't appear to be involved in the formation of higher molecular weight complex. The smaller species was further chromatographed on a SMART mono Q column. The sequential chromatography gave approximately 400-fold enrichment of the enzyme. The concentration of the enzyme was estimated by titration with recombinant N-TIMP-3, which was expressed and purified from *E.coli*. The N-TIMP-3 was electrostatically coupled to Ni^{2+} agarose beads. The beads were then used to affinity purify the enzyme from mono Q fractions. The affinity-purified material was electrophoresed and protein bands were selected for mass spectrometry. No ADAMTS enzyme was identified in the candidate bands. Further improvements will be made to the purification procedure to identify the synovial aggrecanase.

TO MY GRANDFATHER

MUHAMMAD HUSSAIN

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Statement of Originality and Copyright

I confirm that all the experimental work presented in this thesis has been performed by me except mass spectrometric analysis of protein bands, which were analysed in the laboratory of Prof. Benedikt Kessler, Henry Welcome Building for Molecular Physiology, University of Oxford. The first 2 years of PhD work was carried out while Kennedy Institute of Rheumatology was based in London as part of Imperial College London. The remaining two years were spent working at Kennedy Institute of Rheumatology as an off-site campus of University of Oxford pending the construction of new building at Old Road Campus, University of Oxford. The award of PhD degree is based on the initial registration with Imperial College London. Hence the PhD thesis has been submitted to Imperial College London. The Kennedy Trustees funded this work.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Abbreviations

ACL	Anterior cruciate ligament
ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motif
AGEG-	AGEG bearing N-terminal fragment of aggrecan
APP	Amyloid precursor protein
ARGS-	ARGS bearing N-terminal fragment of aggrecan
CBB	Coomassie Brilliant Blue
CHX	Cycloheximide
COMP	Cartilage oligomeric matrix protein
CPB2	Carboxypeptidase B2
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
F/T	Freeze thawed
FGF	Fibroblast growth factor
FT	Flowthrough
GAGs	Glycosaminoglycan
HSPs	Heat shock proteins
IGD	Interglobular domain
IL-1α	Leukemia inhibitory factor
LPS	Lipopolysaccaride
LRP1	Low density lipoprotein receptor related protein 1
MAP1	Methionine amino peptidase 1
MMP1ΔC	Catalytic domain of matrix metalloproteinase 1
MMPs	Matrix metalloproteinases
MW	Molecular weight
NCAM	Neuronal cell adhesion molecule
N-TIMP-3	N-terminal domain of TIMP-3
NO	Nitric oxide
OA	Osteoarthritis
OSM	Oncostatin M
PGE$_2$	Protaglandin E$_2$
PVDF	Polyvinyldifluoride
rIGD	recombinant interglobular domain
RA	Rheumatoid arthritis
sLRP1	shed form of LRP1
SYCM	Synovial tissue culture medium
TGFβ	Transforming growth factor β
TIMP	Tissue inhibitor of matrix metalloproteinase
TIMP-3	Tissue inhibitor of matrix metalloproteinase-3
TNFα	Tumour necrosis factor α
α-2M	α-2 macroglobulin
β-ME	β mercaptoethanol

Chapter 1

Introduction

1.1 Background

Osteoarthritis (OA) is a disabling arthropathy characterised by cartilage degeneration whose pathogenesis is not understood. Injuries to synovial joints such as ligamentous tears, predispose to OA. Understanding the molecules induced upon joint injury should give insights into cartilage physiology and its degeneration.

In Introduction, I will summarise the physiology of the synovial joint with main focus on the cartilage and synovial tissues. The features of OA and its pathogenesis will be discussed. The known proteinases and cytokines involved in cartilage breakdown will be reviewed towards the end of the introduction section. Lastly, I will describe what is known of the responses of tissues to injury.

1.2   Cartilage structure and function

A synovial joint consists of two articulating surfaces, coated with hyaline cartilage, and a joint capsule lined with synovial tissue (Fig.1.1). The synovium produces synovial fluid, which acts both as a lubricant and a source of nutrients for the articular cartilage. Articular cartilage is composed of predominantly (65 to 80 %) water and of its dry weight, 70 % is collagen, 20 % is proteoglycans and 10 % is other proteins (Sharma *et al.* 2008).
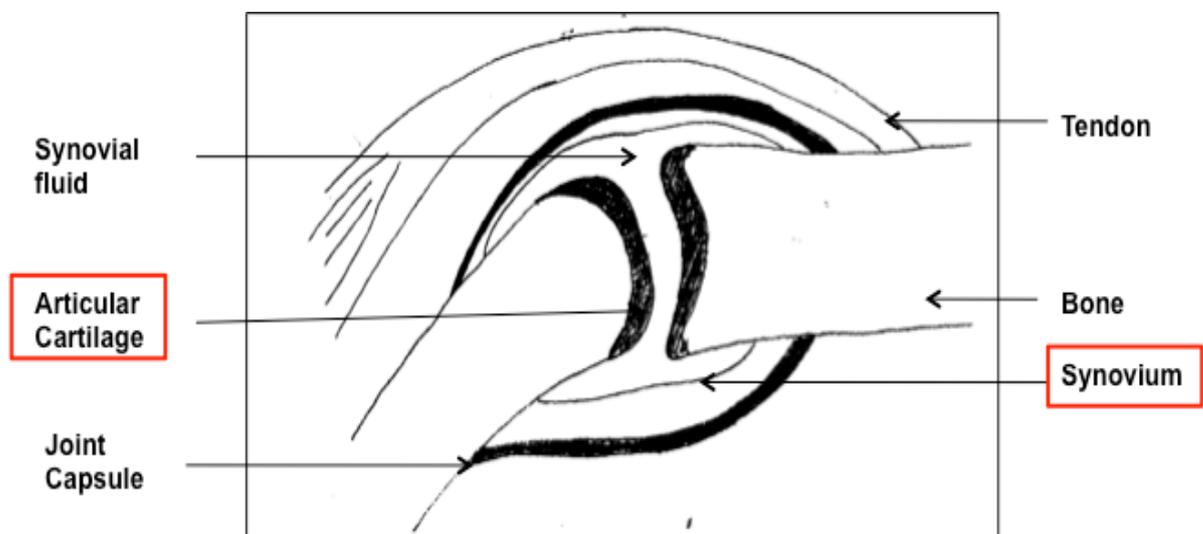
Fig. 1.1. The basic structure of synovial joint (longitudinal section). Adapted from Dr. Kim Midwood's lecture on 'The Synovium: anatomy, physiology and pathology' delivered at Kennedy Institute of Rheumatology on 24th February, 2010.

The chondrocyte: Chondrocytes are the only cells found in the cartilage. They are embedded in the ECM, which is a system of insoluble fibers and soluble polymers. They are derived from mesenchyme (Goldring *et al.* 2006) and are distributed as single cells or in small clusters (Fig. 1.2A). Cartilage is avascular and aneural. The chondrocytes are metabolically active cells which exist in relative hypoxia, and depend mainly on anaerobic respiration (Archer *et al.* 2003). Their turnover in adult cartilage is thought to be very low since dividing cells are rarely seen.

Collagen: Collagen type II is the main structural element of the ECM and forms 90 % of the fibrous network in the cartilage (Textbook of Rheumatology, Kelly , W.N pp 1-12).

The type II $\alpha1$ chains of collagen are synthesized as pro-collagen chains, which contain N-terminal and C-terminal propetides (Olsen 1995). The three compatible $\alpha1$ chains are folded into their triple helical conformation(Lees *et al.* 1997). The $\alpha1$ chains of collagen are proteolytically processed at the N and C terminal ends just after secretion by the chondrocytes (Olsen 1995). The release of the C-propeptide of type II procollagen is a marker of new synthesis of the molecule (Nelson et al., 1998). The proteolytically processed collagen molecules then associate in a highly ordered fashion to form fibrils, which in time become stabilized through cross-links between molecules. Bunches of collagen fibrils form fibres, which are responsible for the form and tensile strength of cartilage. Articular cartilage can be ordered into different zones, which are characterized by the orientation of the collagen fibers (Fig. 1.2B). The superficial layer of cartilage is in contact with synovial fluid and the collagen fibrils are ordered tangentially to the direction of the movement. Beneath the superficial layer is the middle layer. The collagen fibers are perpendicular throughout the middle zone of cartilage. This anchors cartilage to the bone. The tidemark refers to the region where calcified cartilage begins, below which is the bone.

(A)

(B)
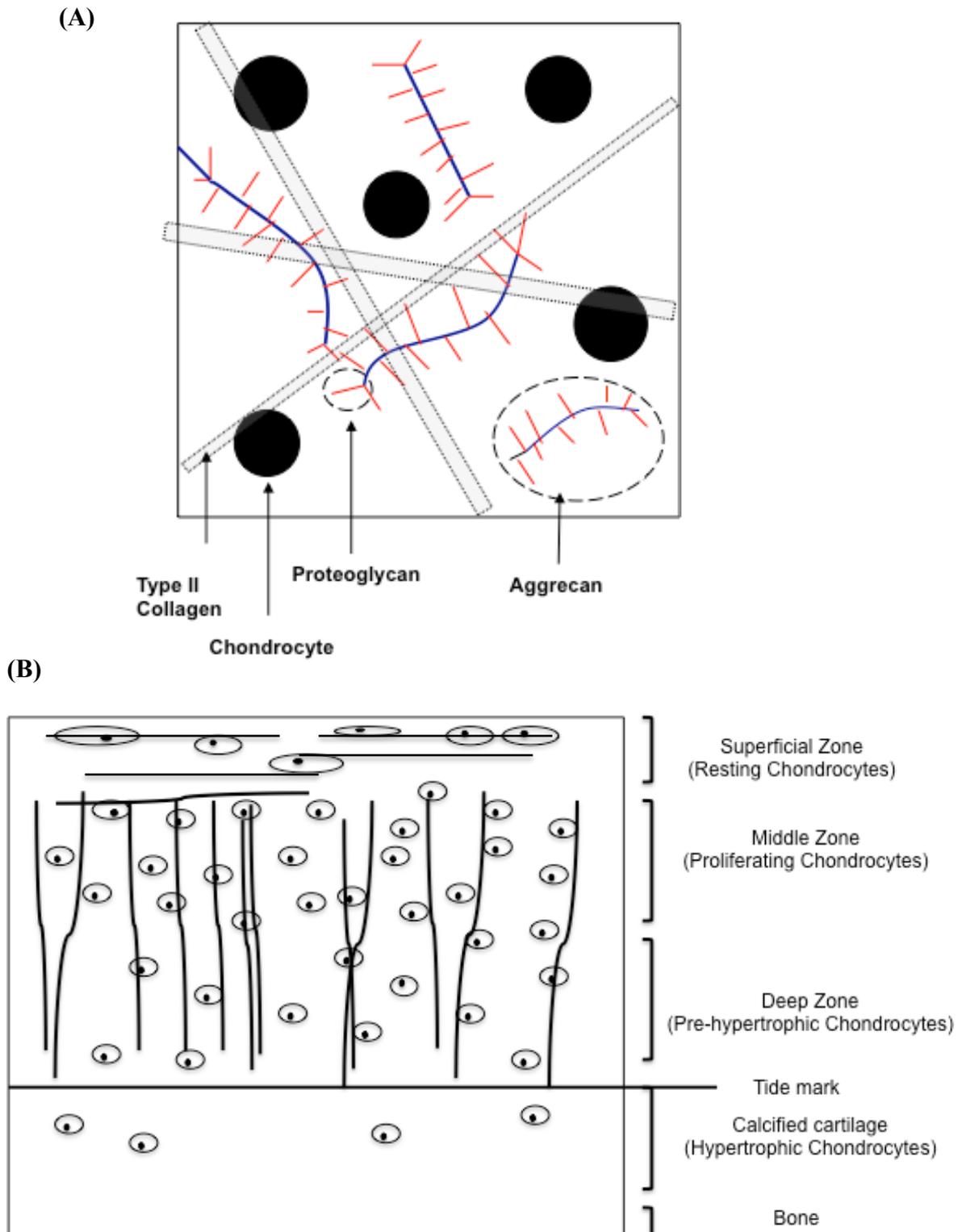
Fig.1.2 Articular cartilage structure (A) Schematic representation of the structure of the cartilage (B) Schematic representation of a longitudinal section through hyaline cartilage showing all the cartilage zones. The black lines represent the collagen fibril layout.

Proteoglycans: 90% of the proteoglycan mass of cartilage is made up by aggrecan (Sharma *et al.* 2008). Each aggrecan molecule consists of a core protein with three globular domains. The core protein has numerous covalently attached sulphated glycosaminoglycan chains (GAGs) of keratan and chondroitin sulphates (Roughley 2006). Aggrecan molecules aggregate with hyaluronan and this interaction is stabilised by link protein. Aggrecan thus forms huge (> 20 aggrecan monomers) aggregates, which fill the interfibrillar space of the matrix (Roughley 2006). The glycosaminoglycan chains are highly hydrophilic. This creates a swelling pressure, which enables the cartilage to resist compression. The effective pore size of cartilage is low: proteins larger than albumin and smaller negatively charged proteins tend to be excluded from the matrix (Buckwalter *et al.* 1998). Proteinases secreted by the chondrocyte are able to cleave aggrecan at multiple sites as shown in Figure 1.3, leading to its loss from the matrix (Karsenty 2005) . Aggrecan is constantly synthesised in adult cartilage to counteract this loss.

Decorin, biglycan, fibromodulin and lumican are small proteoglycans found in the ECM which have functions in its organisation and stabilisation (Roughley 2006). The glycoprotein cartilage oligomeric matrix protein (COMP) is important for binding and stabilising collagens (Posey *et al.* 2008). Fibronectin is another glycoprotein of the ECM: it can bind integrins as well as collagen and heparan sulphate proteoglycans (Hynes 1990).

	ADAMTS cleavage sites		MMP cleavage sites
A	...RNITETE^{373} ^{374}ARGSVIL...	1	...FVDIPEN^{341} ^{342}FFGVGGE...
B	...STASELE^{1545} ^{1546}GRGTIGE...	2	...TAFSTED^{441} ^{442}LVVQVTA...
C	...TTFKEEE^{1714} ^{1715}GLGSVEL...	3	...HAFCFRG^{666} ^{667}ISAVPSP...
D	...QAPTAQE^{1819} ^{1820}AGEGPSG...	4	...PSGVEEW^{690} ^{691}IVTQVVP...
E	...RSFVSQE^{1919} ^{1920}LGQRPFT...		
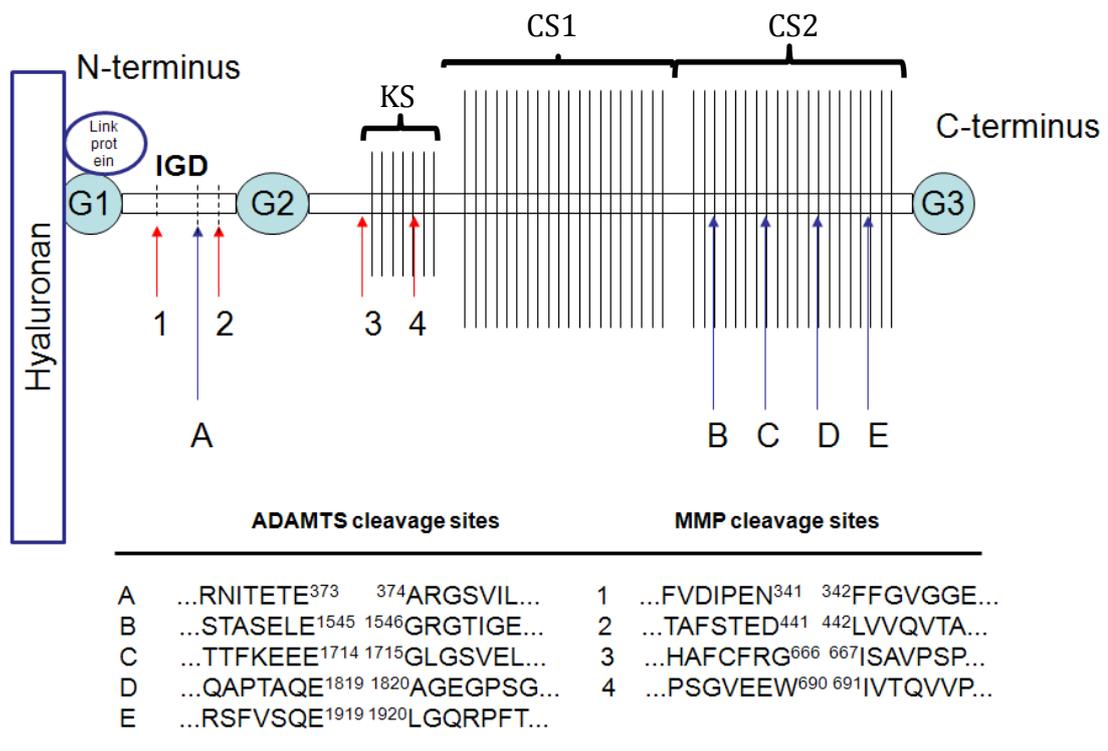
Fig 1.3: Schematic representation of human aggrecan with ADAMTS and MMP cleavage sites. G1, G2, G3 = Globular Domain 1, 2, 3; CS1 = Chondroitan sulphate 1, CS2= Chondroitan sulphate 2, KS = Keratan sulphate. Hyaluronan is a polysaccharide of glucoronic acid and N-acetylglucosamine. ADAMTS cleavage sites are indicated with letters and MMP cleavage site with numbers. Diagram modified from (Sawaji *et al.* 2008).

Pericellular matrix: The region of matrix immediately surrounding the chondrocyte is known as the pericellular matrix. This is structurally and biochemically distinct from the 'territorial' ECM (matrix surrounding the pericellular matrix). It is abundant in perlecan (a heparan sulphate proteoglycan) and type VI collagen. Perlecan binds other matrix molecules and growth factors such as FGF-2 (Vincent *et al.* 2006). Cell-matrix interactions are likely to allow the chondrocyte to respond to mechanical forces. Examples of interactions between cell surface molecules and the ECM include integrins binding to collagen and fibronectin, the tyrosine kinase receptor DDR-2 binding to collagens, and the cellular receptor CD44 binding to hyaluronan (Vogel *et al.* 1997; Xu *et al.* 1997; Knudson *et al.* 2004).

1.3 The synovium (Mark C., and Allan Silman., Book of Rheumatology 3rd Edition)

Synovium is a vascular connective tissue that lines the non-cartilaginous surfaces in the joint cavity. It is continuous with the joint capsule, a tough dense fibrous connective tissue that consists of intertwining bundles of collagen attached to the bones via specialised zones (Mark C., and Allan Silman., Book of Rheumatology 3rd edition Chapter 17, p159-165). The capsule forms a sleeve around the joint to seal the joint space and provides stability. There are two distinct layers to the synovium: the intimal cell lining layer which faces the joint cavity, and a sub-intimal connective tissue layer attached to the joint capsule (Fig.1.4) (Archer, C., Bruce Caterson., The Biology of Synovial Joint, Section 3: Synovium p 223) The intima is one or two cells thick, and is sometimes thrown into villi to increase its surface area. The intimal cells lie on a bed of connective tissue and are not joined to each other via tight junctions or desmosomes. There is no basement membrane. Two cell types comprise the intima: type A and type B. Type A are macrophage-like cells with prominent Golgi apparatus and lysosomal vesicles whose main function is assumed to be clearing debris. Type B are fibroblast-like cells having prominent endoplasmic reticulum. They are thought to synthesise components of synovial fluid such as hyaluronan and lubricating molecules such as lubricin (Archer, C., Bruce Caterson., The Biology of Synovial Joint, Section 3: Synovium chapter 14., The Biology of Synovial cells p 225). The sub-synovial layer contains fibroblasts, networks of blood and lymphatic vessels, and nerve fibres (Davies *et al.* 1948). In addition there are resident macrophages, and mast cells, as well as mononuclear leukocytes that pass through the tissue (Davies *et al.* 1948).

A major function of the synovium is to produce synovial fluid. This is an ultrafiltrate of blood plasma to which are added molecules secreted by the synovial cells. Besides its lubricating function, it provides nutrients to the cartilage and perhaps the sub-chondoral bone and removes the waste products of metabolism.
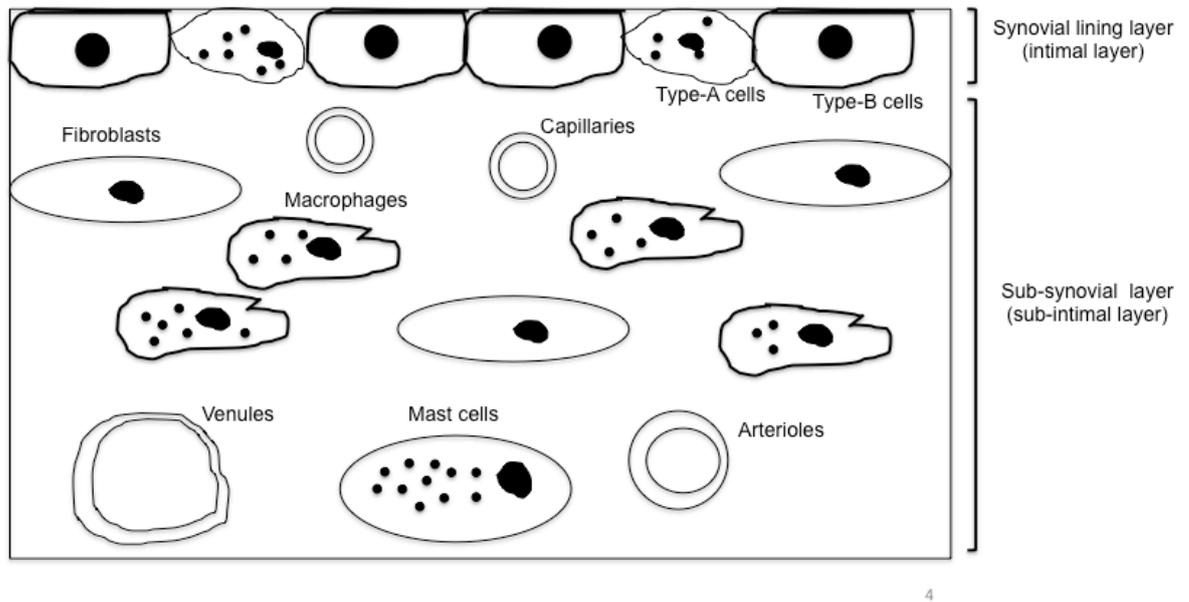
Fig. 1.4 Schematic illustration of synovium showing synovial lining layer resting on sub-synovial layer. The figure is adapted from The Rheumatology by Mark C., and Allan Silman 3rd Edition Chapter 17, p 62 figure 8.6)

1.4 Osteoarthritis

OA is a disease of joints characterised by cartilage degeneration and osteosclerosis with formation of bone cysts and osteophytes (Altman 1991). In the early stages of disease the cartilage surface becomes fibrillated with loss of sulphated proteoglycan (Collins *et al.* 1960). There follows progressive loss of cartilage ECM whose principle components are aggrecan and type II collagen (Mankin *et al.* 1970). Deep vertical fibrillations develop and the chondrocytes become localised in nests. Sometimes regions of intense metachromatic staining surround these, indicating increased proteoglycan synthesis, and suggesting attempted repair of the damaged ECM (Mankin *et al.* 1970). As cartilage destruction continues there is remodelling and abnormal growth of adjacent bone. At the margins of the joints new outgrowths of bone called osteophytes form. The joint becomes increasingly painful and function is lost. Besides the characteristic changes in cartilage and bone, low-grade synovitis may also be present.

Clinical aspects of OA: OA may occur in any joint, but it most commonly affects the hip, knee, hand and spine. Nearly 5 million people in the UK are estimated to have radiological evidence of moderate to severe OA of their hands, knees or hips which costs £3.197 billion in lost production (Department for Work and Pensions: Analytical Services Division, 2000).

Pharmacological therapy for OA is symptomatic and supportive (Conaghan *et al.* 2008). No disease modifying drugs exist and in advanced OA joint replacement is the only treatment. Strategies for development of new treatments include both cell and pharmacological therapy. The latter broadly involves either boosting cartilage anabolism responses with growth factors such as FGF or blocking catabolic proteinases such as matrix metalloproteinases (MMPs) and aggrecanases with inhibitors (Chuma *et al.* 2004; Fosang *et al.* 2008; Troeberg *et al.* 2008). For such therapies to be effective they would need to

be given early in disease. Generally the disease is diagnosed late when the cartilage damage is extensive.

From a clinical perspective OA is a heterogeneous disease. Hand OA is characterized by polyarticular interphalangeal joint involvement of the fingers. There is formation of Herberden's nodes, knobbly swellings of the distal interphalangeal joints of the fingers, and Bouchard's nodes, swellings of the proximal interphalangeal joints (Mark C., and Allan Silman., Book of Rheumatology 3rd Edition p 22). Women are more likely than men to be affected by this form of OA, especially after the menopause with strong familial predisposition (Pattrick *et al.* 1989). The knee and the hip are the most commonly affected large joints, probably because they are the main weight-bearing joints of the body. Degenerative arthritis of the cervical or lumbar spine is also very prevalent. OA of the shoulders, elbows, feet and ankles are less common.

Risk factors for OA: OA is multifactorial disease in which age, weight, genetic predisposition, malalignment and previous joint trauma all contribute to susceptibility. Obesity predisposes to OA especially of the hip, which is the main load-bearing region of the body. Studies have shown that weight reduction can improve the pain score and function in subjects with OA (Roddy *et al.* 2006). From twin studies, genetic factors have been found to be a strong determinant of the disease, with estimates of OA heritability being greater than 50 % for hip, knee and spine OA (Spector *et al.* 2004). Genome wide scanning and other approaches have identified polymorphisms in gene loci such as the IL-1 gene cluster, the IL-4 receptor, frizzled-related protein-3 and asporin (hip OA) and matrilin-3 (hand OA) that influence disease susceptibility (Felson *et al.* 2000; Loughlin *et al.* 2002). Such approaches have highlighted the problem of heterogeneity in OA populations and have been largely disappointing in identifying polymorphisms, which confer risk to large groups of patients. Other factors which may be important include bone density,

oestrogen deficiency and vitamin D status (Felson *et al.* 2000).

OA as an active or passive disease: One view of OA is that it is a passive wearing out of the material of the articular cartilage. This in part is a result of the poor inherent repair capacity of the tissue. Damage to adult articular cartilage fails to heal spontaneously and defects that penetrate the subchondral bone elicit a repair response that generates a fibrocartilage repair tissue, which is a poor substitute for hyaline cartilage (Hayes *et al* 2001). An alternative view is that OA is the result of active cellular processes. The formation of cell clusters and the early focal loss of proteoglycan staining suggest altered cellular activity (personal observation). The existence of the aggrecan fragments in the synovial fluids from patients with OA suggests increased proteolysis (Lohmander *et al.* 1993) and there is evidence of increased expression of MMPs and collagenases (Billinghurst *et al.* 1997; Aigner *et al.* 2001).

That OA is a disease depending on cellular activity is further supported by studies in knockout mice. The cartilage resorbing action of cytokines is thought to be due to cells being stimulated to release proteinases to degrade firstly aggrecan and secondly collagen. Two aggrecanases were purified from IL-1 stimulated bovine nasal cartilage cultures and subsequently identified belonging to a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family. These were ADAMTS-4 and ADAMTS-5 (Abbaszade *et al.* 1999; Tortorella *et al.* 1999). Glasson and her colleagues showed that ADAMTS5, but not ADAMTS4 null mice were partially protected from OA caused by surgical destabilization of medial meniscus (Glasson *et al.* 2005). Mice null for MMP-13, the major murine mouse collagenase, also showed significant protection (Little *et al.* 2009). Similar protection from surgically induced OA has been observed in mice null for MyD88 (an intracellular adaptor protein essential for the formation of IL-1 and TLR receptor signalling complexes) (Tonia Vincent, Kennedy Institute, personal communication).

Chondrocytes are surrounded by a pericellular pool of FGF-2 (Chia *et al.* 2009). FGF-2 knockout mice show accelerated OA compared to wild type controls (Chia *et al.* 2009). Subcutaneous injections of FGF-2 in FGF null mice reversed the OA comparable to wild type controls. Thus the signaling pathways activated by FGF-2 may be important in protecting joints from degeneration over time. These studies showing the course of OA to be influenced by individual genes are consistent with the involvement of active cellular processes.

1.5   Proteinases and inhibitors involved in cartilage ECM catabolism

The remodelling of the cartilage ECM is carried out by proteinases made by cartilage and also perhaps the surrounding tissue. Proteinases are currently classified into 5 groups: serine, threonine, cysteine, aspartate and metalloproteinases (Biochemistry 3rd edition Voet & Voet, Chapter 13). The main enzymes involved in cartilage matrix catabolism are MMPs and ADAMTS enzymes. These are part of the Metzicins (MA) MA clan of metallopeptidases according to the MEROPS peptidase database (Rawlings *et al.* 2006). MEROPS is an on-line database for peptidases and their inhibitors published by Rawlings & Barrett in 1993. The motif HEXXH characterises the clan MA. The two histidines in the consensus sequence coordinate the zinc ion. The amino acid, which forms the last coordinate of the catalytic zinc, differs depending on the different families in the clan. The MA clan is split into two subclans, MA(E) where E stands for glutamic acid and MA(M) where M stands for methionine based on the identity of the last zinc coordinate. The MMPs, ADAMs and ADAMTSs belong to subclan MA(M).

MMPs: Proteoglycan loss from the articular cartilage matrix was first described by Fell and Mellanby in embryonic chick limb bones cultured with retinoic acid (Fell *et al.* 1951). Subsequently the enzymes implicated in mediating this matrix loss were considered to be the lysosomal proteinases such as cathepsins B, D and L (Lucy *et al.* 1961). Later however, it was found that the addition of cysteine proteinase inhibitor E-64 and pepstatin to the chick limb explant cultures stimulated with retinoic acid had no effect on proteoglycan loss suggesting that cathepsins, which are essentially intracellular enzymes active at acidic pH, were not directly influencing proteoglycan loss in the chick limb system (Hembry *et al.* 1982).

Attention then turned to the newly emerging group of MMPs. Collagen's unique structure renders it highly resistant to proteolysis at physiological pH and temperature.

Jerome Gross and Charles Lapiere first described a specific collagenase in 1962 in the tadpole tail during metamorphosis (Gross *et al.* 1962). Specific collagenases cleave collagen at a single site generating ¾ and ¼ collagen fragments. Human collagenase was first described by Evanson and others in cultures of rheumatoid synovium (Evanson *et al.* 1967). The enzyme was later purified from cultures of rheumatoid synovium (Woolley *et al.* 1975) and is now known as MMP-1. General metalloproteinase inhibitors were shown to inhibit IL-1- and retinoic acid stimulated aggrecan release in cartilage (Hembry *et al.* 1982; Caputo *et al.* 1987). MMPs were therefore strong candidates for involvement in proteoglycan turnover as well as collagenolysis. There are currently 23 human members in the MMP family assigned in the MEROPS database. The basic structure consists of a signal sequence, followed by a pro-domain, a catalytic domain, a linker region and a hemopexin-like domain. A number of MMPs have been identified that degrade aggrecan (Fosang *et al.* 1991). Stromelysins (MMP-3,-10), collagenases (MMP-1,-8,-13), matrilysin (MMP-7) and gelatinases (MMP-2, MMP-9) all cleave aggrecan at multiple sites, but the major site is $\text{Asn}^{341}\text{-}^{342}\text{Phe}$ which lies within the interglobular domain (IGD) of aggrecan (Fig.1.3) (Fosang *et al.* 1991; Fosang *et al.* 1993). Collagenases (MMP-1, MMP-8) cleave at a second site ($\text{Asp}^{441}\text{-}^{442}\text{Leu}$) within the IGD, which is not recognised either by stromelysins or gelatinases (Fosang *et al.* 1993). These cleavages potentially cause major loss of function because large GAG bearing fragments would be released from the tissue reducing the ability of cartilage to resist compression.

ADAMTSs and ADAMs: The N-terminal sequence of the major aggrecan degradation products from bovine cartilage explant cultures stimulated with IL-1α was determined to be [374]ARGXVILXAKPDF, which did not correspond to any known proteinase cleavage at the time (Sandy *et al.* 1991). Furthermore, analysis of the aggrecan fragments from bovine synovial fluid (Ilic *et al.* 1992) and from patients with OA (Lohmander *et al.* 1993) also

revealed cleavage at the Glu^{373}-^{374}Ala bond. The term 'aggrecanase' was coined to describe an unknown enzyme or enzymes that cleave at the Glu^{373}-^{374}Ala bond. Aggrecanase-1 (Tortorella *et al.* 1999) and aggrecanase-2 (Abbaszade *et al.* 1999), purified from IL-1-stimulated bovine nasal cartilage cultures were the first proteinases identified to make the Glu^{373}-^{374}Ala bond cleavage in the IGD domain of aggrecan. The assay for the purification of aggrecanases relied upon an anti-^{374}ARGS monoclonal antibody (Hughes *et al.* 1995), which recognised the newly generated N-terminal ^{374}ARGS of the aggrecan fragments but not the same sequence in intact aggrecan. Peptide sequences obtained from the purification of aggrecanases were used to screen cDNA libraries (Abbaszade *et al.* 1999; Tortorella *et al.* 1999).

The cDNAs encoded for aggrecanase-1 and aggrecanase-2 showed they belonged to a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family of enzymes, and they are now known as ADAMTS-4 and ADAMTS-5 respectively(Kuno *et al.* 1997). ADAMTS is a subset of a disintegrin and metalloproteinase (ADAM) family of proteinases and its first member (ADAMTS-1) was discovered as a novel gene expressed in cachexigenic tumours (Kuno *et al.* 1997). Their structure consists of an N-terminal signal peptide, pro-domain, catalytic domain, disintegrin domain, thrombospondin type I domain, cysteine rich domain, spacer domain and 0-14 additional C-terminal thrombospondin type I domains (Fig. 1.5) ADAMTS proteinases are related to other members of the ADAM family; however unlike the majority of the ADAM family they lack the transmembrane domain in addition to having the thrombospondin motifs. There are currently 32 members of ADAM family in the MEROPS database (Rawlings *et al.* 2004), which show extensive homology with snake metalloproteinases. They are mainly cell bound proteins. The founder members of the ADAM family, ADAM-1 and ADAM-2 were shown to be involved in sperm-egg fusion (Primakoff *et al.* 1987). ADAM-17 also known as TNFα converting enzyme

(TACE) is responsible for the processing and the release of TNFα from the cell surface

membrane (Black *et al.* 1997). This enzyme is also implicated in the shedding of other

regulatory molecules such as transforming growth factor α (TGFα), amyloid precursor

protein (APP), neuronal cell adhesion molecules (NCAM) etc. (Peschon *et al.* 1998;

Kalus *et al.* 2006) However, to date, the functions of majority of ADAM family members

have yet to be elucidated.

ADAMTS domain structure

Fig. 1.5 Schematic representation of the domain structure of ADAMTS members. The conserved HEXXH motif is found in the catalytic domain. Pro = Pro domain, Cat = Catalytic domain, DIS = disintegrin domain, TSP = thrombospondin domain, Spacer = Space domain, CysR = Cysteine rich domain

ADAMTS-4 mRNA is upregulated in isolated chondrocytes and cartilage explants stimulated with IL-1α, TNF-α or transforming growth factor-β (TGF-β) (Moulharat *et al.* 2004). The regulation of ADAMTS-5 mRNA by these cytokines has been unclear. Tortorella *et al.* (2001) and Moulharat *et al.* (2004) reported that ADAMTS-5 mRNA expression was constitutive in human cartilage explants and unaffected by IL-1α, TNF-α or TGF-β. Others have reported that IL-1α upregulates ADAMTS-5 mRNA levels in bovine cartilage explants (Little *et al.* 2002; Arai *et al.* 2004), in a human chondrosarcoma cell line (Koshy *et al.* 2002) and in human articular chondrocytes (Sawaji *et al.* 2008). Much work on characterisation, activation and regulation of ADAMTS-4 has been carried out since it was the first aggrecanase identified. It was a surprise therefore when in 2005 it was revealed that cartilage of mice null for ADAMTS-5, but not for ADAMTS-4 was protected from loss of aggrecan following IL-1 stimulation (Glasson *et al.* 2005; Stanton *et al.* 2005). Similar protection from loss of cartilage aggrecan was also observed in post-surgical OA and antigen-induced (methylated BSA) arthritis in the ADAMTS-5 null animals (Glasson *et al.* 2005; Stanton *et al.* 2005). These studies indicated that ADAMTS-5 is a major mediator of aggrecanolysis in murine arthritis.

Six other ADAMTS enzymes (ADAMTS-1, -8,-9,-15,-16,-18) have been reported which cleave aggrecan at the Glu^{373}-^{374}Ala bond. ADAMTS-1 cleaves mouse aggrecan (Rodriguez-Manzaneque *et al.* 2002) and also the proteoglycan versican VI of human aorta (Sandy 2001). In antigen-induced arthritis, there was no difference in aggrecan degradation between ADAMTS-1-null mice and wild type controls (Little *et al.* 2005). ADAMTS-8 shows weak aggrecanolytic activity despite being strongly homologous to ADAMTS-1, - 4 and -5 (Collins-Racie *et al.* 2004). ADAMTS-9 is widely expressed in embryonic and adult tissues and cleaves bovine aggrecan at the Glu^{373}-^{374}Ala *in vitro* (Somerville *et al.* 2003). Not much is known about ADAMTS-15, -16, -18 apart from their

aggrecanolytic activity at the Glu^{373}-^{374}Ala bond (Zeng *et al.* 2006). The main functions of

ADAMTS family members, where known, are shown in Table 1.

Enzyme	Substrate	Activity
ADAMTS-1	Aggrecan; versican; α$_2$-macroglobulin	Cleaves proteoglycan core proteins, anti-angiogenic
ADAMTS-2	Procollagen I, procollagen II	Processing of N-propeptide of procollagen
ADAMTS-3	Procollagen II	Processing of N-propeptide of procollagen
ADAMTS-4	Aggrecan, versican, brevican	Cleavage of proteoglycan core proteins
ADAMTS-5	Aggrecan	Cleavage of aggrecan core proteins
ADAMTS-8	Aggrecan	Cleaves aggrecan core protein
ADAMTS-9	Aggrecan	
ADAMTS-13	Von willebrand factor	Activity results in thrombotic thrombocytopenia purpura
ADAMTS-14	Procollagen 1	Procollagen N-protease
ADAMTS-15	Aggrecan	Cleaves aggrecan core protein

Table 1. The ADAMTSs, their substrates and biological activity are shown. Adapted from Nagase and Kashiwagi (2003).

TIMPs: TIMPs are endogenous inhibitors of the metalloproteinases. The family

consists of four members in vertebrates, TIMP-1, -2, -3 and -4, which have amino acid

sequence identity of 40-50%. TIMPs range in molecular mass from 21- to 29 kDa and are

widely expressed throughout the body (Brew *et al.* 2000). TIMP-1, -2 and -4 are found in

the body fluids and in tissues, whereas TIMP-3 is associated with the ECM (Pavloff *et al.*

1992).

TIMPs have two domains. The inhibitory activity resides in the N-terminal domain,

which forms a 1:1 molar complex with metalloproteinase (Murphy *et al.* 1991). Each

domain contains three di-sulphide bonds (Williamson *et al.* 1990). The first three

dimensional structure of a TIMP was solved by NMR and was the N-terminal domain of

TIMP-2. It contained two α-helices and a 5-stranded antiparallel β-sheet (Williamson *et al.*

1994). The NMR structure of N-TIMP-2 by itself was not sufficient to understand the

mechanism by which MMPs was inhibited by TIMPs. The crystal structure of TIMP-1 with

MMP-3 revealed that the C-terminal domain includes two adjacent β-sheets, made by two parallel and two anti-parallel β-strands (Gomis-Ruth *et al.* 1997). Approximately 75 % of the total contact that the TIMP-1 has with MMP-3 takes place in a 'binding ridge' that inserts in the MMP-3 binding groove (Gomis-Ruth *et al.* 1997).

TIMPs are high affinity inhibitors and their K_i values are typically in the low nanomolar range. As a general rule TIMPs can inhibit all the MMPs (Nagase 2000). TIMP-3 has a unique property of being able to inhibit ADAMs (e.g. ADAM-10, -12 and -17) (Amour *et al.* 1998; Amour *et al.* 2000) and ADAMTSs (e.g. ADAMTS-1, -4 and -5) (Hashimoto *et al.* 2001; Kashiwagi *et al.* 2001; Rodriguez-Manzaneque *et al.* 2002) enzymes. The K_i values of TIMP-3 indicate that it is a better inhibitor of ADAMs and ADAMTSs than MMPs (Amour *et al.* 1998; Kashiwagi *et al.* 2001). TIMPs are involved in diverse biological functions. TIMP-1 and -2 have erythroid-potentiating activities and cell growth-promoting activities (Gasson *et al.* 1985; Hayakawa *et al.* 1992). Lastly, TIMP-3 is a pro-apoptotic in many different cells, such as melanoma, Hela, colon carcinoma, and rat smooth vascular cells (Bian *et al.* 1996).

Plasma proteinase inhibitors: There are 5 major proteinase inhibitors found in blood plasma: α-2 macroglobulin (α2M), $α_1$ proteinase inhibitor ($α_1$ PI), inter-α-trypsin inhibitor (IαI), $α_1$ cysteine proteinase inhibitor (αCPI) and β1-anticollagenase ($β_1$AC) (also known as TIMP-1) (Travis *et al.* 1983). These inhibitors regulate the enzymatic activity in the blood and tissue fluids. α-2M is a broad-spectrum inhibitor that is active against all major classes of proteinases: serine, cysteine, aspartic acid and metalloproteinase. $α_1$ PI and IαI inhibit only serine proteases, $α_1$ cysteine proteinase inhibitor inhibits only cysteine proteases, and the β1-anticollagenase ($β_1$AC) inhibit collagenolytic activity of metalloproteinase class.

1.6 Regulation of proteinases and ECM by cytokines

A number of factors have been implicated in the homeostasis of the ECM in the cartilage, which may be relevant to the pathogenesis of OA.

Cytokines: OA is not considered to be a severe inflammatory disease in the sense that rheumatoid arthritis is, but episodes of inflammation are common and there is often histological evidence of synovitis. Inflammatory cells and cytokines that are not normally present in normal joints are found in patients with OA. In particular, IL-1 which is the most potent and was the first cartilage resorbing cytokine, has frequently been implicated in the pathogenesis of cartilage matrix degradation in OA.

Fell et al. (1977) first described an *in vitro* model in which they co-cultured normal porcine synovial tissue with porcine cartilage explants. They noted progressive loss of metachromatic staining of proteoglycans. Based on these findings they speculated that synovial tissue was producing a soluble factor, which they named catabolin that stimulated chondrocytes to resorb surrounding cartilage matrix (Fell *et al.* 1977; Dingle *et al.* 1979). Catabolin was later shown to be physicochemically similar to IL-1 (Saklatvala *et al.* 1984). Since these early studies further evidence has accumulated suggesting that IL-1 is present and may play a role in the pathogenesis of OA: This is summarised in Table 2.

Nature of study on IL-1	Reference
Detected in OA synovial fluid	(Wood *et al.* 1983)
Detected in OA synovium	(Myers *et al.* 1990)
Detected in OA cartilage	(Middleton *et al.* 1996)
Immunolocalisation in OA cartilage	(Wood *et al.* 1983; Tetlow *et al.* 2001)
Animal models	
Intra-articular injection stimulates cartilage loss	(Wood *et al.* 1983)
Intra-articular IL-1ra reduces progression of early OA in animal models	(Caron *et al.* 1996; Pelletier *et al.* 1997)

Table.2. Evidence of the role of IL-1 in the pathogenesis of OA. (Adapted from Goldring *et al*. 2004)

Cytokines can be divided into several functional categories based on the different roles they have been reported to play in OA cartilage. With respect to regulation of chondrocyte function, it is possible to classify them: (1) catabolic cytokines that cause matrix degradation; (2) anti-catabolic or inhibitory cytokines that antagonise the catabolic ones; (3) anabolic cytokines and growth factors (4) regulatory cytokines which modulate the activity of other cytokines (Figure 1.4).

The catabolic cytokines implicated in mediating cartilage damage in OA include IL-1 and TNFα. The injection of recombinant IL-1 in rats, mice and rabbits stimulates the destruction of articular cartilage (O'Byrne *et al.* 1990). However, when IL-1 was combined with TNFα and injected simultaneously, there was enhanced cartilage damage that exceeded the effects observed with either cytokine alone (Page Thomas *et al.* 1991). In addition to catabolic effects, IL-1 and TNFα can also adversely affect the synthetic activity of chondrocytes by inhibiting the synthesis of proteoglycans and type II collagen (Saklatvala 1986). These cytokines induce chondrocytes to synthesise MMPs as well as prostaglandins (e.g. PGE_2) and nitric oxide (NO) that also modulates catabolic activities in cartilage. Combining IL-1 with oncostatin M (OSM) increases expression of matrix-degrading proteinases in cartilage compared with IL-1 alone (Barksby *et al.* 2006). Other cytokines thought to play a catabolic role in cartilage include IL-17 and IL-18 (Van den Berg 2002). IL-6 and LIF (leukaemia inhibitory factor) are thought to play a regulatory role similar to OSM. Studies using the IL-1 receptor antagonist (IL-1ra) show that IL-1-mediated catabolic effects can be abrogated by this inhibitor (Pelletier *et al.* 1997).

Cytokines and growth factors in OA

Catabolic	Regulatory	Anti-catabolic	anabolic
IL-1	IL-6	IL-4	IGF-1
TNFα	LIF	IL-10	TGFβ1, 2, 3
IL-17	OSM	IL-13	BMP-2,4,6,7,9,13
IL-18		IL-1ra	FGF-2
		Activin A	CTGF
		FGF-2	

Fig 1.6. Classification of the role of cytokines and growth factors in OA
The role of cytokines that currently believed to have a catabolic role, regulatory and anti-catabolic or anabolic role in OA are shown. Abbreviations: IL- interleukin, OSM – oncostatin M, LIF – leukaemia inhibitory factor, IL-1ra – IL-1 receptor antagonist, IGF-1 – insulin-like growth factor-1, FGF-2 – fibroblast growth factor-2, CTGF – connective tissue growth factor (Adapted from Goldring and Goldring 2004).

1.7   Responses of cartilage to mechanical injury

As discussed previously direct injury to a joint is a major predisposing cause of OA. Various injuries including intra-articular fracture (Furman *et al.* 2006), anterior cruciate ligament (ACL) rupture (Lohmander *et al.* 2004), meniscal damage (Sharma *et al.* 2008) or menisectomy (Englund *et al.* 2004) all increase an individual's risk of developing OA. The mechanisms by which injury to a joint leads to degeneration of cartilage are unknown.

Some years ago, our laboratory noted that dissecting articular cartilage from the joint surface (explantation) immediately activated all three mitogen activated protein kinase (MAPK) pathways (Gruber *et al.* 2004). These were extracellular regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 MAPK. The degradation of IκBα was also observed, implying the possible activation of NFκB. Subsequently, it was shown that IκBα kinase (IKK), the main enzyme that activates NFκB, was activated within seconds of injury (Watt *et al.* 2013). The translocation of the p65 subunit of NFκB from the cytoplasm to the nucleus can be visualised by confocal microscopy 30 min after explantation (A. Didangelos, PhD thesis 2008). How these pathways, typically activated by inflammatory cytokines or microbial products, are activated by injury is under investigation.

Tonia Vincent in our lab was the first to investigate this induction of signalling. She found that upon dissection of porcine cartilage, a soluble factor was released into the medium that activated ERK. This was purified and identified as FGF-2 (Vincent *et al.* 2004). FGF-2 is located in the pericellular matrix where it is bound to the heparan sulphate chains of perlecan. Subsequent studies showed that the ERK activation induced by loading cartilage could be inhibited using an ERK inhibitor (Vincent *et al.* 2004). Therefore cartilage loading may lead to the release of a growth factor that modulates its responses to injury. FGF-2 was not thought to be responsible for the activation of NFκB or JNK.

More recently our lab has shown that protein tyrosine phosphorylation also occurs within seconds of the injury to the cartilage. Some of the proteins tyrosine-phosphorylated were identified by mass spectrometry e.g. focal adhesion kinase, paxillin and cortactin, and found to be src kinase substrates (Watt *et al.* 2013). A src inhibitor prevented the tyrosine phosphorylation. Fiona Watt has also shown that activation of MAPKs, NFκB and src occurs in synovial tissue upon dissection (Fiona Watt, personal communication). This unpublished work strongly suggests that cells of the synovium respond to injury in a fashion very similar to those of cartilage.

In the mouse, our lab has used avulsion of the femoral head epiphysis as a model of injury. This too showed activation of MAPK and NFκB pathways, and of inflammatory response genes (Chong *et al.* 2013). Activation of the same intracellular signalling pathways in different connective tissues upon injury (articular cartilage, synovium and epiphysis) suggests it may be a generic response. The stimulus that activates the intracellular signaling following dissection is not understood. No soluble factors have been found from damaged tissues that activate JNK and NFκB in chondrocytes. Perhaps the cells directly sense and respond to the damage to the ECM.

The mechanism by which cells in general respond to damage is controversial. Damage is thought to release intracellular molecules such as high mobility group box protein (HMGb1) and heat shock protein (HSPs) which act via TLRs to cause inflammatory signalling (Chen *et al.* 2010; Manson *et al.* 2012; Shen *et al.* 2013). In addition ECM components such as hyaluronic acid fragments are reported to activate inflammatory signalling (Scheibner *et al.* 2006). The stimulatory activity of some of these purified molecules has been attributed to contamination with bacterial products (Gao *et al.* 2003; Youn *et al.* 2008) and some of them seem to interact with several receptors, which makes it difficult to be sure of the role of a particular mediator in driving damaged-induced signalling.

The dissection of articular cartilage does not cause much release of the cellular contents as chondrocytes only occupy 2% of the hyaline cartilage volume and the MAPKs and NFκB pathways are activated in seconds. HMGb1 takes much longer to activate signalling pathways (Yang *et al.* 2005). We have also observed that recutting rested cartilage explants does not reactivate the JNK and NFκB pathways (A. Didangelos, PhD thesis 2008). An appealing hypothesis is that the chondrocytes directly sense the damage to the tissue and the sensing mechanism is not reset when the dissected cartilage is maintained in culture. While the injury response of the cartilage has been investigated extensively, the response of the dissected synovium, a cellularly much more complex tissue, has not.

1.8   Aims and objectives.

Our laboratory has been studying cytokines and proteinases involved in the breakdown of cartilage aggrecan. Cartilage or synovium are dissected into culture medium where they are maintained and their behaviour studied. The injury of dissection causes rapid activation of intracellular signalling pathways typically seen when cells are stimulated by inflammatory stimuli such as bacterial lipopolysaccharide (LPS) or the cytokines interleukin-1 (IL-1) and tumour necrosis factor α (TNFα). The consequent changes in gene expression and protein synthesis represent injury responses, which if occurring in vivo, would likely promote local inflammation and tissue repair. The cultured medium of the damaged tissue can be analysed for factors that affect the cartilage metabolism. A product of cultured synovial tissue that caused cartilage to resorb its ECM by stimulating chondrocytes was identified in this way more than 30 years ago and called catabolin (Dingle *et al.* 1979). It was partially purified and characterised as a small (20 kDa) acidic protein (pI 4.5-5.0) (Saklatvala *et al.* 1980). Subsequently, a protein with similar physical and biological characteristics was purified to homogeneity from pig leukocyte culture medium and identified as the recently defined pleiotropic cytokine, IL-1α (Saklatvala *et al.* 1983; Saklatvala *et al.* 1984). Later TNF-α, another product of activated leukocytes, was also shown to induce breakdown and inhibit synthesis of proteoglycan in cartilage (Saklatvala 1986). Its action was similar to and additive with IL-1. While the role of these cytokines in rheumatoid arthritis (RA) is well established, their role, if any, in OA is unclear.

The original aim of my project was to investigate what cytokines were made by the dissected synovium. This was based on the hypothesis that the original synovial catabolin was never shown to be identical with IL-1 α (which it resembled), and in the meantime other cytokines (e.g. IL-17 & IL-18) had been discovered and reported to have catabolic effects on chondrocytes. In early experiments I found that synovium produced aggrecanase activity in

amounts sufficient to interfere with the cartilage assays I needed to use to search for other regulatory cytokines. It was necessary to remove interfering proteinase from the medium if the cytokines present were to be investigated. The aggrecan-degrading enzymes of synovium have never been fully investigated or identified and in arthritis. It is possible that proteinases causing cartilage destruction arise from synovial tissue as well as chondrocytes. Purifying and identifying these synovial aggrecanases became the primary objective of focus of my work.

Chapter 2

Materials and Methods

2.1   Molecular biology methods

Reagents used in Molecular Biology: Materials were purchased from the following sources: TOP 10 Electrocomp^{TM} *E.coli* cells, Luria-Bertani (LB) broth, LB agar were obtained from Invitrogen (Paisley, UK); plasmid maxiprep kit was obtained from Qiagen (Crawley, UK); LB bacto-tryptone and yeast extract from BD biosciences (Oxford, UK); and chloroform, acetic acid, ethylenediaminetetraacetic acid (EDTA), ethidium bromide from VWR international (Leicestershire, UK).

Isolation of plasmid from TOP 10 *E.coli* cells: Bacterial strain (TOP 10) containing pCMV6 entry vector with full length TIMP-3 ORF, C-terminally tagged with FLAG epitope (vector containing full length TIMP-3 ORF was bought from OriGene technologies) was added to 50 μl of TOP10 electrocompetent *E.coli.* The bacteria was spread evenly on the bottom of a cold electroporation cuvette and then pulsed in a BTX Electro Cell Manipulator with 2100 V for 5 milliseconds. Then 500 μl of LB media was added and the cells were incubated at 37 °C. The following day single colonies were chosen to inoculate 2 ml of LB media containing 50 μg/ml kanamycin. To obtain 500 μg of DNA, 100 ml of culture were grown. After 16 hours of growth, the bacteria were pelleted by centrifugation at 3000 g for 30 min at 4 °C. The plasmid was extracted according to manufacturers instructions (Qiagen maxiprep handbook). Briefly, bacteria were lysed under alkaline conditions and RNase treated before binding the plasmid DNA to Qiagen anion exchange resin under low salt and neutral pH conditions by gravity flow. RNA and protein contaminants were removed under medium salt (1.0 M) conditions and plasmid DNA was eluted in high salt (1.6 M) conditions. The plasmid DNA was concentrated and desalted by isopropanol precipitation. The plasmid pellet was redissolved in 10 mM Tris-HCl pH 8.0, 1 mM EDTA (TE buffer). The concentration of the plasmid was determined by measuring the absorbance at A_{260} in a Nanodrop spectrophotometer (Perkin Elmer). The purity of the plasmid was checked by

agarose gel (w/v 1% agarose) electrophoresis running in 0.2 M Tris-HCl pH 7.3, 0.1 M

acetic acid, 5 mM EDTA (0.5 × TAE buffer) containing ethidium bromide and visualised by

a Biorad UV system.

2.2 SDS-PAGE, Western blots, CBB stain and silver stain methods

SDS-PAGE and Western blot reagents: Materials used for western blot and sodium

dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from the

following sources: 30% (w/v) acrylamide from Severn Biotech (Worcestershire, UK);

glycine, bromophenol blue, β-mercaptoethanol (β-ME), Coomassie Brilliant Blue R-250

(CBB), Tween-20, SDS and sodium thiosulphate from Sigma-Aldrich (Dorset, UK); pre-

stained Precision Protein Standards[TM] from BioRad (Hemel Hempstead, UK); 4-12% Tris-

glycine gels and polyvinylidene difluoride (PVDF) membrane from Invitrogen (Paisley,

UK); anti-rabbit horseradish peroxidise (HRP)-linked antibody, anti-mouse (HRP)-linked

antibody, anti-goat (HRP)-linked antibody from Dako (Cambridgeshire, UK);

chemiluminescent reagents from GE Healthcare (Buckinghamshire, UK); silver nitrate from

Fisher scientific (Loughborough, UK); dithiothrietol (DTT) from Alexis Biochemicals (San

Diego, USA); formaldehyde, formic acid, methanol, glycerol phosphate buffered saline

(PBS, 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl pH 7.4) from VWR international

(Leicestershire, UK); sodium carbonate from Calbiochem (Merck Chemicals Ltd,

Nottingham, UK) and Marvel[TM] dry semi-skimmed milk from Premier Foods (St. Albans,

UK).

Monoclonal antibody BC-3 (raised in mouse against the new N-terminus generated

by cleavage of aggrecan core protein at the RNITEGE[373] [374]ARGSVIL site) was purchased

from Abcam (Cambridge, UK) and monoclonal 2-B-6 (raised in mouse against the aggrecan

chondroitanase stubs that remain after deglycosylation with chondroitinase ABC and

keratanase) was a gift from Prof. Bruce Caterson (University of Wales, Cardiff). The anti-

AGEG antibody that recognised the N-terminal neo-epitope [1820]AGEG of aggrecan was affinity purified from rabbit serum after immunising with AGEGPSGGC peptide sequence and kindly provided by Prof. Hideaki Nagase, Kennedy Institute of Rheumatology..

SDS-PAGE: Proteins were resolved by SDS-PAGE with reduction using a modification of the tris-glycine buffer system according to Laemmli (1970). Polyacrylamide gels were made with 6-12% (v/v) total acrylamide depending on the size of the proteins to be separated. Samples were mixed with an appropriate amount of quadruple strength 200 mM Tris-HCl 6.8, 2% (v/v) SDS, 10% (v/v) glycerol and a small amount of bromophenol blue and loaded into the wells of the stacking gel (4% (v/v) acrylamide). The Tris-glycine gels were run at 140 V for 2 hours.

Coomassie Brilliant Blue R-250 staining for protein: SDS-PAGE gels were placed in the staining solution of 0.1% (w/v) CBB, 50% (v/v) methanol and 20% (v/v) acetic acid for 45 min and destained with 30% (v/v) methanol and 1% (v/v) formic acid. The destaining solution was changed every 10 minutes for 1 hour and the gels were further destained for 24 hr at room temperature (~25 $^{\circ}$C). The gels were then dried.

Silver staining for protein: For increased sensitivity of detection, proteins were also visualised in SDS-PAGE gels using a silver stain (Shevchenko *et al.* 1996). The gels were incubated in the fix solution of 50% (v/v) methanol and 5 % (v/v) acetic acid for 30 min. This was followed by a wash with 50% (v/v) methanol for 10 min and two further washes with deionised water (dH$_2$O) for 10 min each. The gels were incubated in a sensitiser solution of 0.02% (w/v) sodium thiosulphate for 1 min, washed twice with dH$_2$O and left with cold silver reagent of 0.1 % (v/v) silver nitrate at 4 $^{\circ}$C for 20 min. After incubation, the gels were washed twice with dH$_2$O for 1 min, before development. Formalin (0.04% (v/v)

was added to the developing solution of 2% (w/v) sodium carbonate immediately before use. Development was stopped with 5% (v/v) acetic acid. The gels were then dried.

Western blots: Proteins separated by SDS-PAGE were transferred onto the PVDF membrane at 30 mA for 90 min in a transfer buffer of 20% (v/v) methanol , 25 mM Tris-HCl pH 7.5, 192 mM glycine, with the apparatus assembled as per manufacturer's instructions (Novex/invitrogen). After transfer, the membrane was blocked using 5% (w/v) dry skimmed milk (Marvel^{TM}) in PBS for 1 hour at room temperature. Membranes were washed for 10 min in 20 mM Tris-HCl pH 7.6, 137 mM NaCl, 1% (v/v) Tween 20, 0.02 % NaN$_3$ (TBS-Tween) before incubation with primary antibody in 5% (w/v) dry skimmed milk in TBS-Tween for 1 hour or overnight at 4 ^{o}C. The membrane was washed three times with TBS-Tween for 5 min before the addition of a 1:1000 dilution of relevant horseradish peroxidise conjugated secondary antibody in 5% (w/v) dry semi-skimmed milk in TBS-Tween for 1 hour at room temperature. The membranes were washed three times with TBS-Tween for 5 min before being incubated with the chemiluminescent reagents for 2 min. The membranes were exposed to films for increasing time points. The films were then developed.

2.3   Tissue preparation and culture

Materials used for cell and tissue culture: Materials were purchased from the following sources: Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, HEPES from Biowhittaker (Berkshire, UK); amphotericin B from Gibco (Paisley, UK); fetal calf serum(FCS) from Labtech international (East sussex, UK); Falcon plates from Beckton-Dickinson (Oxford, UK); Recombinant human interleukin-1α was prepared in-house by Lesley Rawlinson (Kennedy institute, University of Oxford). Porcine metacarpophangeal joints were supplied by Cheale Meats Ltd (Essex, UK).

Cell line culture: Human epithelial kidney cells transformed with the Epstein Barr nuclear antigen (HEK293/EBNA) cells were cultured in DMEM containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10 % (v/v) fetal calf serum (FCS) in a 5 % CO_2 incubator. The cells were obtained from Matrix Biology lab, Kennedy Institute of Rheumatology. The cells were passaged every 3-4 days using trypsin EDTA (sigma). Briefly, cells were allowed to reach 70-80% confluency before aspirating the media in a sterile culture cabinet. The cells were washed twice with 10 ml of sterile 1 X PBS solution. The petri dish was incubated with 4 ml of 0.05 % trypsin/EDTA solution at 37 °C.

Cartilage explant culture: Porcine articular cartilage from metacarpophalangeal joints of 3-9 month old pigs was dissected using a scalpel and cut into small pieces of approximately 3mm long and 3mm wide. Each piece was roughly the same thickness (~2 mm) and weighed approximately 10 mg. After dissection, the live cartilage was allowed to rest for 24 hours in a serum free media at 37 $^{\circ}$C under 5 % (v/v) CO_2 in DMEM containing 100 U/ml penicillin and streptomycin, 2 ug/ml amphotericin B and 25 mM HEPES pH 7.0. If dead cartilage explants were needed, the explants were freeze-thawed 3 times before resting them for 24 hours at 37 ^{0}C under 5 % (v/v) CO_2 in serum-free DMEM containing antibiotics as listed above.

Synovial tissue culture: Synovial tissue from pig metacarpophalangeal joints was dissected with a scalpel and cultured in serum-free DMEM with 100 U/ml penicillin and streptomycin, 2 ug/ml amphotericin B and 25 mM HEPES (1 g/2.5 ml) for 24 hours. The synovial culture medium (SYCM) was harvested, centrifuged (13000 rpm, 10 min) and treated with hyaluronidase from bovine testes [100ug/ml] for 1 hour at 37 ^{o}C to break down the hyaluronan, which makes the synovial medium viscous and interferes with the mobility of proteins on SDS-PAGE. The SYCM was stored at 4 ^{o}C.

2.4 Protein expression, purification and refolding

Protein expression and purification reagents: Materials were purchased from the following sources: Q anion exchange column (HiTrap Q), Ni^{2+} agarose column (HisTrap), Superdex-75 and Superdex-200 pre-packed columns were obtained from GE healthcare (Buckinghamshire, UK); bovine serum albumin, β-mercaptoethanol (β-ME), 2-hydroxy-ethyl-disulphide, cystamine dihydrochloride, imidazole, guanidine hydrochloride (GuHCl), nickel sulphate ($NiSO_4$), sodium azide (NaN_3), sodium Chloride (NaCl), anti-FLAG M2-agarose, 3 X FLAG peptide and anti-FLAG M2 antibody were purchased from Sigma-Aldrich (Dorset, UK); isopropyl β-D-thiogalactopyranoside (IPTG) from Biogene (Cambridge, UK); 1 M calcium chloride solution ($CaCl_2$), 99.9 % (v/v) bis-distilled glycerol was purchased from VWR international (Leicestershire, UK); bacterial proteinase cocktail set II (20 µM AEBSF, 1.7 µM Bestatin, 200 nM E-64, 85 µM EDTA, 2 µM Pepstatin A) from Calbiochem (Merck Chemicals ltd, Nottingham , UK); dithiothreitol (DTT) from Alexis Biochemicals (San Deigo, USA); SpectraPor dialysis membranes from Fisher Scientific (Loughborough, UK); Viva Spin concentrators from Vivascience (Sartorius AG, Surrey, UK). Recombinant proteins: the catalytic domain of MMP-1 (MMP-1ΔC) and N-terminal domain of TIMP-1 (N-TIMP-1) were supplied by Dr. Ngee Han Lim and Dr. Linda Troeberg respectively (The Kennedy Institute of Rheumatology). Full length TIMP-3 was obtained from R & D systems (Abingdon, UK).

Expression, purification and refolding of N-TIMP-3 from bacteria: The BL-21 (DE3) strain of *E.coli* containing pET42b vector with N-TIMP-3 (N-terminal inhibitory domain of N-TIMP) was kindly provided by Prof. Hideaki Nagase, (The Kennedy Institute of Rheumatology). The pET42 vector was constructed using human cDNA encoding the N-Terminal region of mature human TIMP-3, residues Cys1 to Asn121. The sequence was amplified with specific primers that introduced *NdeI* restriction site at the 5´-end and a *Not*I

restriction site at the 3′ end. The PCR products were cloned into pET42 vector using the *Nde*I and *Not*I sites to produce the coding sequence for truncated TIMP-3 (N-TIMP-3) with a His tag attached to the C terminus. An overnight culture of 20 ml of Luria Broth (LB) containing bacteria and kanamycin (33 ug/ml) at 37 ^{o}C was used to seed one litre of LB. The bacteria were grown at 37 ^{o}C until the cell density reached between 0.6-0.7 A_{600} units. The bacteria were induced to make N-TIMP-3-His by the addition of 1 mM IPTG for 4 hours. The bacteria were collected by centrifugation a 3000 g for 30 min.

The bacterial pellet from one litre of bacterial culture was resuspended in a 20 ml of 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% (v/v) NaN_3, 1 mM EDTA (TBS-EDTA). The inclusion bodies were isolated by mechanically lysing bacteria 5 times at 1500 psi pressure using French press and centrifugation of lysate at 24, 000 g for 15 min at 4 ^{o}C. The pelleted inclusion bodies from each litre of bacteria was solubilised in 20 ml of 6 M GuHCl, 20 mM Tris-HCl pH 8.0, 100 mM β-mercaptoethanol supplemented with proteinase inhibitors (20 μM AEBSF, 1.7 μM bestatin, 200 nM E-64, 85 μM EDTA, 2 μM pepstatin A; 1:1000 dilution of proteinase inhibitor cocktail II) by shaking for 2-3 hours at room temperature. The insoluble material was removed by centrifugation at 24,000 g for 30 min at 4 ^{o}C. The supernatant was diluted 6 times with 6 M GuHCl, 20 mM Tris-HCl pH 8.0 to bring the final concentration of β-mercaptoethanol to below 20 mM. This prevented the reduction of nickel ion in the subsequent purification steps.

Prepacked column containing Ni^{2+} charged resin with a typical binding capacity of 40 mg of protein per ml of resin, was equilibrated with 5 column volumes of equilibration buffer (6M GuHCl, 20 mM Tris-HCl pH 8.0). The diluted supernatant was applied to the column at a flow rate of 1 ml per min. The column was washed extensively with washing buffer (6M GuHCl, 20 mM Tris-HCl pH 8.0, 20 mM imidazole) to remove impurities bound to the column. The bound material was eluted with 400 mM imidazole, 6M GuHCl, 20 mM

Tris-HCl pH 8.0 and 2 ml fractions were collected. The column was regenerated by stripping the column with stripping buffer (20 mM Na_3PO_4, 0.5 M NaCl, 50 mM EDTA, pH 7.4) and then charging with 0.5 ml of 0.1 M $NiSO_4$ per 1 ml of resin.

The protein concentration was estimated by taking absorbance at 280 nm using molar extinction coefficient (ε) 15, 450 M^{-1} cm^{-1}. The protein was diluted to a concentration of less than 50 µg/ml in 6M GuHCl, 20 mM Tris-HCl pH 8.0, 20 mM cystamine dihydrochloride, 20 % glycerol and placed into dialysis tubing 34 mm diameter SpectrPor3 membrane, 3.5 kDa molecular weight cut off) and dialysed against 10 volumes of 20 % glycerol , 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$, 5 mM β-mercaptoethanol, 1 mM 2-hydroxy-ethyl- disulphide 4 ^{o}C for 24 hours. The was followed by further three dialysis against 10 volumes of 20 % (v/v) glycerol , 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$ at 4 ^{o}C for 24 hours.

The precipitated N-TIMP-3 protein was removed by centrifugation at 12 000 g for 30 minutes. The supernatant was passed through a cell strainer (70 µm Nylon) to remove the floating N-TIMP-3 precipitate particles from supernatant. The clarified supernatant was applied to Ni^{2+} charged pre-packed column equilibrated with 20% (v/v) glycerol, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$ at 2 ml per min before eluting with the same buffer with 400 mM imidazole. The eluted protein was collected as 2 ml fractions. This procedure concentrated the protein from large volume. The fractions were pooled and dialysed against 50 volumes of 20% (v/v) glycerol, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$ twice at 4 ^{o}C to remove imidazole.

The inhibitory activity of N-TIMP-3 was tested against MMP1ΔC. An absorbance reading at wavelength of 280 nm was used to calculate the concentration of N-TIMP-3 in the preparation. A fixed concentration of 20 nM MMP-1ΔC was titrated with increasing concentration N-TIMP-3. Since N-TIMP-3 and MMP-1ΔC bind in a 1:1 stoichiometric

complex, complete inhibition of MMP-1C gave the active concentration of N-TIMP-3 in the preparation.

Expression and purification of GST-IGD-FLAG: Recombinant aggrecan interglobular domain (IGD) substrate is a ~50 KDa recombinant protein comprising the IGD of aggrecan (Fig.1) and contains an aggrecanase cleavage site (NITEGE^{373}-^{374}ARGS). The cleavage generates a 30 KDa fragment with C-terminal NITEGE^{373} and a 20 KDa fragment with N-terminal ^{374}ARGS. The cleavage can be detected by NITEGE^{373} specific neo-epitope antibody. The pGEX4T1 plasmid containing the GST-IGD-FLAG sequence was kindly provided by Dr Ngee Han Lim (Kennedy Institute of Rheumatology). A starter culture of BL-21 cells transformed with the plasmid sequence in 20 ml LB was grown overnight at 37 ^{o}C. This was used to seed the main culture of 1 L of LB. The main culture was grown at 37 ^{o}C until the cell density (A$_{600}$) reached 0.5. The bacteria was induced to produce GST-IGD-FLAG by stimulation with 100 µM IPTG overnight at room temperature. After induction, the bacteria were spun down at 3000 g for 15 min at 4 ^{o}C. The bacteria were resuspended in TBS-EDTA (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% (v/v) NaN$_3$, 1 mM EDTA) buffer with proteinase inhibitors (1/1000 dilution of proteinase inhibitor cocktail II) and mechanically lysed using a French press 5 times at 1500 psi. The lysed bacteria were centrifuged at 24000 g for 30 min at 4 ^{o}C. The supernatant, containing the GST-IGD-FLAG, was applied to a glutathione-Sepharose 4B column equilibrated with 50 mM Tris-HCl pH 8.0, 150 mM NaCl. After application, the column was washed with 0.5 M NaCl, 50 mM Tris-HCl pH 8.0 and the material bound to the column was eluted with 10 mM reduced glutathione, 50 mM Tris-HCl pH 8.0. The eluted material was dialysed three times against 10 volumes of 50 mM Tris-HCl pH 8.0, 150 mM NaCl. This substrate was concentrated to A$_{280}$ > 2.5 using spin concentrators.

2.5 Enzyme activity assays

Reagents used in activity assays: Reagents were bought from the following sources: (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(3-[4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH$_2$ (Mca-PLAQAV-Dpa-AR) fluorogenic peptide substrate was from R & D systems (Abingdon, UK); anti-NITEGE antibody was obtained from Dr. Ngee Han Lim (Kenndey Institute of Rheumatology).

MMP-1ΔC activity assay: The MMP-1ΔC activity assay was carried out in a 96-well polypropylene plate. A 20nM MMP-1ΔCwas preincubated with different concentrations of the inhibitor (0-1 μM) in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10mM CaCl$_2$, 0.05% Brij-35, 0.02 % NaN$_3$ at 37 °C for 1 hour. A small volume (10% final volume) of internally quenched fluorescent substrate (Mca-PLGL-Dap(Dnp)-AR)was added to bring the final concentration of substrate to 2 μM. The reaction was incubated at 37 °C for a further 1 hour. The resulting amount of cleavage was determined by measuring the fluorescence at 420 nm after excitation at 320 nm in a Spectramax fluorometer, Molecular Devices, California.

N-TIMP-3 activity assay: N-TIMP-3 inhibits MMP-1ΔC by binding reversibly to form tight complexes with a 1:1 stiochiometry. The active concentration of N-TIMP-3 can therefore be determined by titration against an MMP-1ΔC solution of known concentration. The figure 2.1 shows the theoretical inhibition curves at different initial enzyme concentration (E_o) relative to the inhibition constant (K_i) of the enzyme. In order to obtain complete inhibition of the enzyme at 1:1 ratio of initial inhibitor concentration to the enzyme concentration ($[I_0]/[E_0] = 1$), ideally, the concentration of starting enzyme should be 1000 times the K_i (curve 1). However, for TIMPs, 1000 times the K_i would be micromolar quantities, which would require large amounts of enzyme and inhibitor. For titration purposes, 20 nM MMP1ΔC was used which represents approximately 30 times excess of enzyme concentration compared to the K_i (0.66 nM) of N-TIMP-3 (Kashiwagi *et al.* 2001).

This would yield a theoretical inhibition curve between curves 2 and 3 and deemed suitable

for titrating unknown concentration of N-TIMP-3.

Curve	1	2	3	4	5	6
$[E_0]/K_i$	1000	100	10	1	0.1	0.01
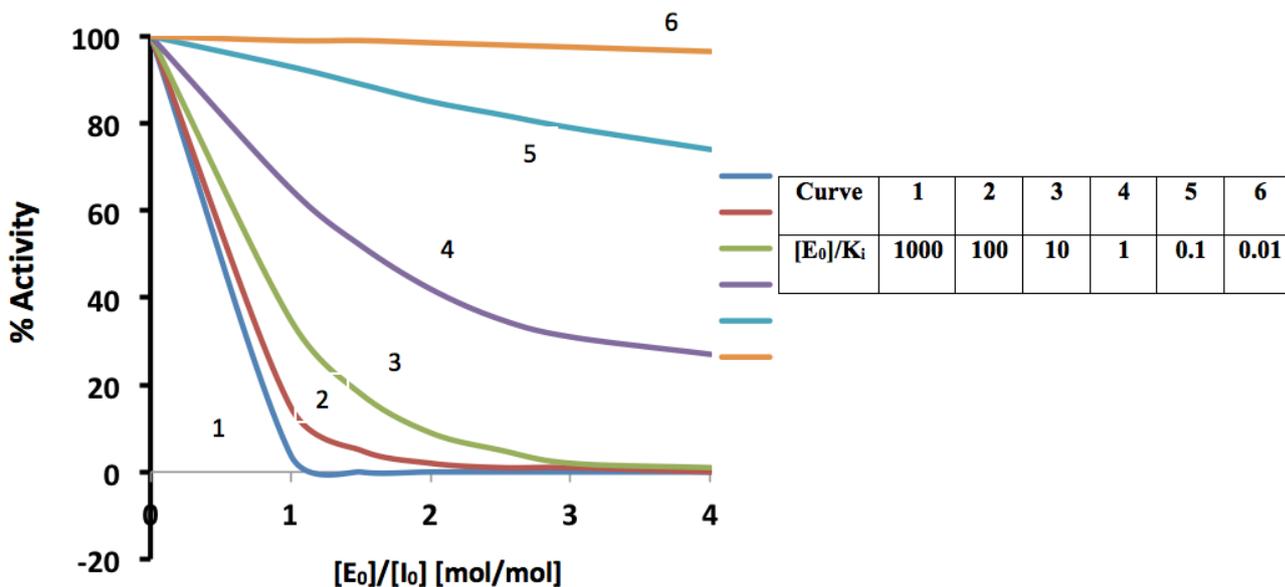
Fig. 2.1 Theoretical inhibition curves showing the % activity of enzyme as a function of molar ratios of enzyme/inhibitor concentrations ($[E_0]/[I_0]$) for variable $[E_0]/K_i$. The figure was adapted from (Bieth 1995).

For unknown quantities of N-TIMP-3, 20 nM MMP1ΔC (3.6 ul of 1 µM stock) was pre-

incubated with increasing concentration of N-TIMP-3 (0-200nM; concentration based on

A_{280}) for 1 hour at 37 °C in 200 µl reaction volume. The amount of uninhibited MMP1ΔC

was then measured by the addition of 20 ul of fluorescent substrate. From the graph of

fluorescence against estimated inhibitor concentration, the final active concentration of N-

TIMP-3 can be determined.

Aggrecanase activity assay using GST-IGD-FLAG substrate: GST-IGD-FLAG (7.5 µM) was incubated with 10 ul SYCM in a 20 µl volume of TNC buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM CaCl$_2$, 0.02 % (v/v) NaN$_3$) for 24 hours. The enzyme activity was stopped by the addition of 10 µl of 2 × SDS-sample buffer (100 mM Tris pH 6.8, 1 % (v/v) SDS, 5% (v/v) glycerol, 0.1 % (v/v) bromophenol blue). The samples were electrophoresed on a 12% polyacrylamide gel and proteins transferred onto PVDF membrane by western blot. The membrane was immunoblotted with anti-NITEGE antibody (1: 1000 dilution) and then developed.

Aggrecanase activity on bovine aggrecan: SYCM (1:5, 1:10 or 1:20) was incubated with bovine aggrecan (50 μg) for 24 hours at 37 °C. The digest was deglycosylated with chondroitinase ABC and keratanase enzymes at a final concentration of 0.0125 U, in double strength buffer of 200 mM sodium acetate, 50 mM Tris-HCl, pH 6.8 to remove the glycosaminoglycan (GAG) chains. The samples were precipitated with 1 ml ice-cold acetone by incubating at -20 $^{\circ}$C for 15 min and then spun at 13 000 g in a microcentrifuge for 10 minutes. The supernatants were removed, the pellet dried and 50 µl of 1 × reducing sample buffer added. The samples were boiled for 5 minutes at 95 $^{\circ}$C and subjected to SDS-PAGE.

Cartilage aggrecan degradation assay: The live or dead cartilage explants were washed three times with serum-free DMEM before stimulation with IL-1α [10 ng/ml] or synovial culture medium (SYCM; 300 µl aliquot) for 24 hours. The cartilage conditioned medium was harvested and a 150 µl aliquot was deglycosylated by adding chondroitinase ABC and keratanase enzymes at a final concentration of 0.0125 U, in double strength buffer of 200 mM sodium acetate, 50 mM Tris-HCl, pH 6.8 to remove the glycosaminoglycan (GAG) chains. The samples were incubated for 24 hours at 37 $^{\circ}$C and precipitated with 1 ml ice-cold acetone, incubated at -20 $^{\circ}$C for 15 min and then spun at 13 000 g in a microcentrifuge for 10

minutes. The supernatants were removed, the pellet dried and 50 ul of $1 \times$ reducing sample buffer added. The samples were boiled for 5 minutes at 95 $^{\circ}$C and subjected to SDS-PAGE.

Aggrecanase activity ELISA: SYCM and fractions obtained from chromatography (at appropriate dilution e.g. 1:5 or 1:10 in the reaction buffer supplied with the ELISA kit) were incubated with aggrecan interglobular domain substrate (aggrecan IGD) (100 nM) in a 1 mM MES pH 6.0 buffer containing proteinase inhibitors (0.4 mM AEBSF, 0.1 nM pepstatin, 0.1 nM leupeptin) at 37 $^{\circ}$C for 15 min on a orbital shaker with a rotating speed of 1000 rpm. The proteolytic reactions were stopped with 1 mM EDTA solution before adding the digests to wells pre-coated with anti-ARGS neo-epitope antibody in the microtitre plate for 1.5 hours. The wells were then washed three times with the wash buffer. The wells were incubated with peroxidase labelled secondary antibody (1:100 dilution as supplied by the manufacturer) for another 1.5 hours. The wells are washed 5 times with wash buffer before adding 100 μl of 3,3,5,5-tetramethlbenzidine (TMB) solution. The reaction was quenched by the addition of an equal amount of 1 M sulphuric acid. The plate was read immediately at a wavelength of 450 nm using spectrophotometric plate reader with absorbance at 620 nm (A_{620}) used as a reference filter.

2.6   Purification of synovial aggrecanase

Cation exchange chromatography: A 60 ml aliquot of SYCM was centrifuged at 13,000 g. The supernatant was collected. The clarified material was dialysed against 2 litres of buffer (20 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$) for 8 hours. The dialysate was centrifuged to remove debris and applied to HiTrap S column (1 ml) in 10 ml batches equilibrated with buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$) at a flow rate of 0.5 ml/min using AKTA HPLC system (GE Healthcare UK). The proteins were eluted from the column in the same buffer with increasing salt gradient over 20 column volumes. 1 ml fractions were collected and assayed for activity on bovine aggrecan and aggrecanase ELISA.

Gel filtration chromatography: Active fractions from cation exchange chromatography were pooled and concentrated to about 5 ml using Viva Spin concentrators with a molecular weight cut off of 3 kDa. The concentrate was centrifuged at 13000 g. The clarified concentrate was applied to Sephacryl S200 (120 ml), equilibrated with buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$), at a flow rate of 0.5 ml/min. Fractions (5ml) were collected in the same buffer and assayed on bovine aggrecan and aggrecanase ELISA.

Anion exchange chromatography: Active fractions were pooled (25 ml) and dialysed against 2 litres of Tris buffer without NaCl (20 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$). The dialysate was applied to SMART mono Q column (100 μl) at a flowrate of 0.1 ml/min. The column was eluted with tris buffer with increasing salt over 40 column volumes. The fractions (100 μl) were collected and assayed on for activity on bovine aggrecan and aggrecanase ELISA.

Affinity chromatography: A 15 μl aliquot of Ni^{2+} agarose beads was washed twice with dH_2O followed by further two washes of Tris buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$). A 100 μl aliquot of N-TIMP-3 (2 μg active protein) was incubated with washed Ni^{2+} agarose beads for 30 min. The beads were then washed twice with tris buffer as above. The active fraction from mono Q chromatography was applied to N-TIMP-3 bound Ni^{2+} agarose beads (15 μl) for 1 hour. The beads were then washed 3 times with tris buffer. Sample buffer (50 μl) containing 50 mM DDT was added to the beads. The material was then heated for 10 min at 100 °C before electrophoresing it on 4-12 % (v/v) NuPage gradient gel.

2.7 Mass spectrometry

Protein bands from gel electrophoresis were identified by mass spectrometry in collaboration with Dr. Benedikt Kessler, Henry Wellcome building for molecular physiology, University of Oxford. Silver stained protein bands were manually cut out of the gel for trypsin digestion and reductively alkylated as previously described (Adam et. al. 2011).

Peptides were separated on an Acquity nano UPLC system (Waters) supplemented with a 25 cm C18 column, 1.7 µm particle size (Waters) using a linear gradient from 3% buffer A (0.1% (v/v) formic acid in water) to 40% buffer B (0.1% (v/v) formic acid in acetonitrile) at a flow rate of 250 nl/min (approx. 7000 psi) from 0 to 90 min. Peptides were ionized and introduced to an LTQ Orbitrap Velos tandem mass spectrometer (Thermo Scientific) using an electrospray ionization (ESI) source. Collision induced dissociation (CID) was induced on the twenty most abundant ions per full MS scan. Raw data was converted to Mascot generic files using msconvert (Kessner et al., 2008). Searches were performed using the SwissProt database (06/2011) with MASCOT (Perkins et al., 1999) and CPFP 1.3.0 (Trudgian et al., 2010) with the following settings: Variable modifications: 2-succinyl (cysteine, +116.01 Da), pyridylethyl (cysteine, +105.06 Da), oxidation (methionine, +15.00 Da), peptide tolerance: ± 10 ppm, fragment tolerance: ± 0.5 Da.

Results

Chapter 3

Characterising aggrecanolytic activity made by injured synovial tissue

3.1   Introduction

Our laboratory is using a surgical model of OA in the mouse (Clements *et al.* 2003; Glasson *et al.* 2005) to elucidate the molecular mechanisms of disease. This model involves sectioning the anterior attachment of the medial meniscus, a fibrocartilagenous tissue which creates a concave surface for articulation with the medial femoral condyle and distributes load. The medial meniscus is displaced medially creating an abnormal contact between the femoral and tibial articular surfaces. Loss of aggrecan and superficial fibrillations are apparent at the site of abnormal contact within 2 weeks of surgery and the cartilage surfaces gradually degenerate over about 12 weeks. There is debate about whether OA is a simple wear and tear process leading to loss of cartilage substance by mechanical attrition, or an active process involving for example production of proteinases which degrade cartilage matrix. There is evidence of up regulation of MMP-1 and MMP-13 transcripts in late osteoarthritic cartilage (Aigner *et al.* 2001), along with reports of collagenase (Billinghurst *et al.* 1997) and aggrecanase activity in osteoarthritic cartilage (Malfait *et al.* 2002). In 2005, it was revealed that ADAMTS-5, but not ADAMTS-4 knockout mice were protected from loss of cartilage aggrecan following IL-1 stimulation of cartilage explants (Glasson *et al.* 2005; Stanton *et al.* 2005). Similar protection from loss of cartilage aggrecan was also observed in post-surgical OA and antigen-induced (methylated BSA) arthritis in the ADAMTS-5 null animals (Glasson *et al.* 2005; Stanton *et al.* 2005). Recent but unpublished work in our lab has shown that MyD88$^{-/-}$ knockout mice are strongly protected from surgically-induced OA. MyD88 is an adaptor protein which is essential for the formation of signalling complexes of the IL-1 and Toll-like receptor family. The protection shows that murine OA is an active process and depends on intracellular signalling pathways which typically control inflammatory response genes. The cellular processes involved could occur in the damaged meniscus, in the adjacent synovium or in the cartilage. Cells of any or all of these tissues could be a source of

degradative proteinases. There is uncertainty to what extent the proteinases involved are produced in response to cytokines or other biochemical mediators or to mechanical stresses. Thus, the current consensus is that the balance between anabolism and catabolism of ECM is tipped towards catabolism and this is due to increase in catabolism rather than a decrease in anabolism.

The role of cytokines in OA is unclear. The two best known cytokines that activate chondrocytes to resorb their proteoglycan matrix (and inhibit the resynthesis) are IL-1 and tumour necrosis factor-alpha (TNFα) (Saklatvala 1986) and ADAMTS-5 is strongly implicated in this response (Glasson *et al.* 2005). Prior to the purification of IL-1α and IL-1β as cartilage resorbing cytokines made by leukocytes (Saklatvala *et al.* 1983; Saklatvala *et al.* 1984), a molecule with similar activity had been identified in SYCM and called catabolin (Dingle *et al.* 1979; Saklatvala 1981). This was physicochemically similar to IL-1α (pI ~ 4.5 -5.0), but was never purified to homogeneity and never proven immunologically to be IL-1. Moreover, the early attempts at purification of synovial catabolin (and of leukocyte IL-1) employed a relatively insensitive assay for GAG release from cartilage explants, based on reactivity with the metachromatic dye dimethylmethylene blue (DMMB), and depended on low resolution chromatography. Furthermore, NH_4SO_4 fractionation was used as an initial procedure which resulted in 70%-80% loss in the cartilage-catabolic activity of the porcine synovial culture medium (Saklatvala 1981). Thus damaged synovial tissue may produce IL-1 or IL-1-like cytokines which could stimulate proteinase production by synovium itself and by chondrocytes.

There is also evidence that synovial tissue produces aggrecan degrading proteinases. Fell *at al.* (1977) showed that co-culture of synovial tissue with dead cartilage caused aggrecan loss from the cartilage. The culture of bovine synovial tissue generates a soluble proteoglycan-degrading activity and the N-terminal sequence of the major proteoglycan fragments in the

culture was determined to be ^{374}AXGSVIL which confirmed cleavage at the Glu^{373}-^{374}Ala bond (Vankemmelbeke *et al.* 1999). Later, Buttle and his colleagues probed the bovine synovial culture medium with an antibody to ADAMTS-5 which detected multiple protein bands: an intense band of ~60 kDa and two minor bands of 61 kDa and 63 kDa. But, it was not shown which bands were ADAMTS-5. Immunoprecipitation of SYCM with this antibody removed only 20% of the total aggrecan degrading activity as assessed on bovine aggrecan-entrapped in polyacrylamide (Vankemmelbeke *et al.* 2001). The incubation of synovium-conditioned medium with bovine aggrecan generated more aggrecan fragments than recombinant ADAMTS5 which further indicated that there could be other proteinases present in the synovial culture medium. Moreover, the agents leading to increased aggrecanolytic activity in cartilage explants, IL-1 and retinoic acid, didn't upregulate the expression of synovial ADAMTS-5 mRNA, nor did they increase the amount of aggrecanase activity generated by the synovium (Vankemmelbeke *et al.* 2001).

Thus neither the cytokines nor the possible aggrecanases made by synovium in culture have been identified. I set out to identify these proteins since they may be involved in cartilage degradation *in vivo.*

3.2   Aggrecan degrading activity from synovial tissue

Synovial tissue (~ 1g) from porcine metacarpophalangeal joints was cultured in serum free DMEM (2.5 ml) for 24 hours and SYCM was harvested as outlined in Materials and Methods. The medium was applied to live porcine cartilage explants for 24 hours and aggrecanolysis was assessed by measuring release of neo-epitope bearing fragments into the culture medium with anti-ARGS and anti-AGEG antibodies. Fig. 3.1A shows that ARGS- and AGEG- containing fragments were present after incubation of cartilage with SYCM compared to incubation with DMEM alone (Fig. 3.1A, lane 1 vs. lane 4). IL-1 also caused the release of large (~280 kDa) ARGS-bearing fragment and two smaller fragments representing further cleavages. The release of these neo-epitope bearing fragments was prevented if IL-1 receptor antagonist protein (IRAP) was added along with IL-1 (Fig. 3.1A, lane 2 vs. lane 3). The effect of synovial culture medium was slightly suppressed by the inclusion of IRAP suggesting the presence of IL-1 in SYCM (Fig. 3.1A, lane 4 vs lane 5). Similar suppression of aggrecanolytic activity was also observed when SYCM was applied to cycloheximide treated live explants suggesting the presence of chondrocyte activating mediators in SYCM (Fig. 3.1B, lane 3 vs. lane 4).

It was important to check that the aggrecan fragments apparently generated by SYCM from the cartilage did not originate from the synovial culture. Fig. 3.1B lane 1 shows the amount of material present in SYCM that reacted with neo-epitope antibodies before applying it to the cartilage, while fig. 3.1B lane 4 shows the neo-epitope bearing fragments present after the culture with live cartilage. This established that the aggrecan fragments were arising from the cartilage. The fragments could have been produced both as a result of factors such as cytokines from synovium stimulating the chondrocytes to produce proteinases and by proteinases made by synovial tissue which had accumulated in the SYCM. Fig. 3.1B shows that incubating SYCM with either freeze-thawed cartilage explants
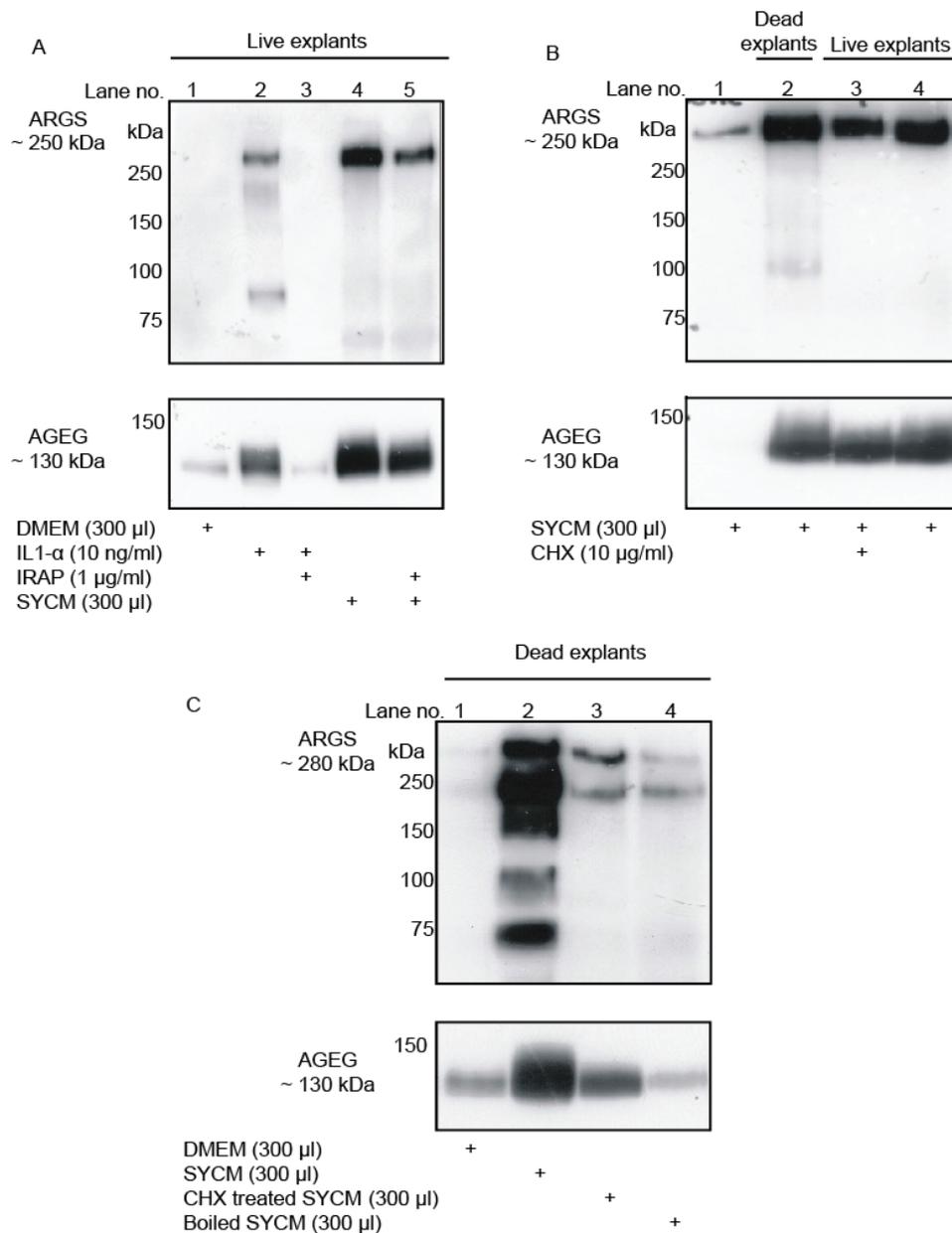
Fig 3.1 The effect of synovial culture medium on (a) live or (b & c) freeze-thawed cartilage explants. (A) Synovial culture medium (300 μl) in the presence and absence of interleukin receptor antagonist protein (IRAP) was applied to live cartilage explants (3mm³, 3 pieces/point) for 24 h. A 150 μl aliquot of harvested medium was deglycosylated and 50 μl of this digest was electrophoresed and transferred onto PVDF membrane. The membranes were immunoblotted with anti-ARGS and anti-AGEG neo-epitope antibodies. **(B)** The synovial culture medium (300 μl) was applied to freeze-thawed or cycloheximide-treated live explants (10 μg/ml) and western blotted with antibodies as described in (a) **(C)** Boiled synovial culture medium (100 °C for 10 min) or medium obtained from synovial tissue cultured with cycloheximide (10 μg/ml) was applied to freeze-thawed cartilage explants and western blotting was carried out with antibodies as described for (a).

(lane 2) or cycloheximide-treated live explants (lane 3) caused release of ARGS and AGEG fragments suggesting the presence of aggrecanolytic proteinases in the SYCM. This activity was abolished by boiling SYCM (Fig. 3.1C, lane 2 vs. lane 4). If the synovial tissue was cultured with cycloheximide (10 μg/ml), no active material was produced (Fig. 3.1C, lane 2 vs. lane 3). This suggests that protein synthesis was necessary for the synovial tissue to produce the proteinases responsible for the aggrecan degradation, and that the enzymes were not pre- existent in the tissue and passively released into the medium. The experiments also indicated that the proteinases would need to be removed from synovial culture medium before the presence of any cytokines affecting aggrecan metabolism could be studied. The aggrecanolytic enzyme activity in SYCM was very interesting since synovium may be a source of matrix-degrading enzymes in disease and, as described earlier, little is known about its aggrecan degrading enzymes and their regulation.

The aggrecan core protein peptides appearing in IL-1 and SYCM treated cartilage explant were analysed with an anti-stubs antibody. This antibody detects peptides with oligosaccharide stubs that remain after deglycosylation of the aggrecan and its fragments with chondroitinase ABC and keratanase. Six major proteoglycan bands were observed when cartilage was cultured dead or live (Fig. 3.2, lane 1 and lane 5). When live cartilage was cultured with IL-1 (lane 2) there was an increase in band 3 and 6 and the presence of new band, labelled 7. Band 3 (~ 280 kDa) and band 6 (130 kDa) were likely to be the large ARGS and the smaller AGEG bearing fragments respectively, which was seen upon stimulation with IL-1α as shown in Fig. 3.1A. Band 1 in Fig. 3.2 was likely to be intact aggrecan core protein. When SYCM was applied to dead cartilage it amplified the proteoglycan fragments 3 to 7 (Fig. 3.2, lane 4 vs. lane 5). This suggests that SYCM may contain more than one aggrecanolytic enzyme or the enzyme was very efficient at cleaving aggrecan at multiple sites. Moreover SYCM on dead cartilage explants and IL-1 on live tissue appeared to generate

fragments with similar molecular weights. Therefore, the enzyme(s) in SYCM might belong to the same family of proteinases as generated upon IL-1 stimulation that cleave at characteristic sites in the core protein of aggrecan.
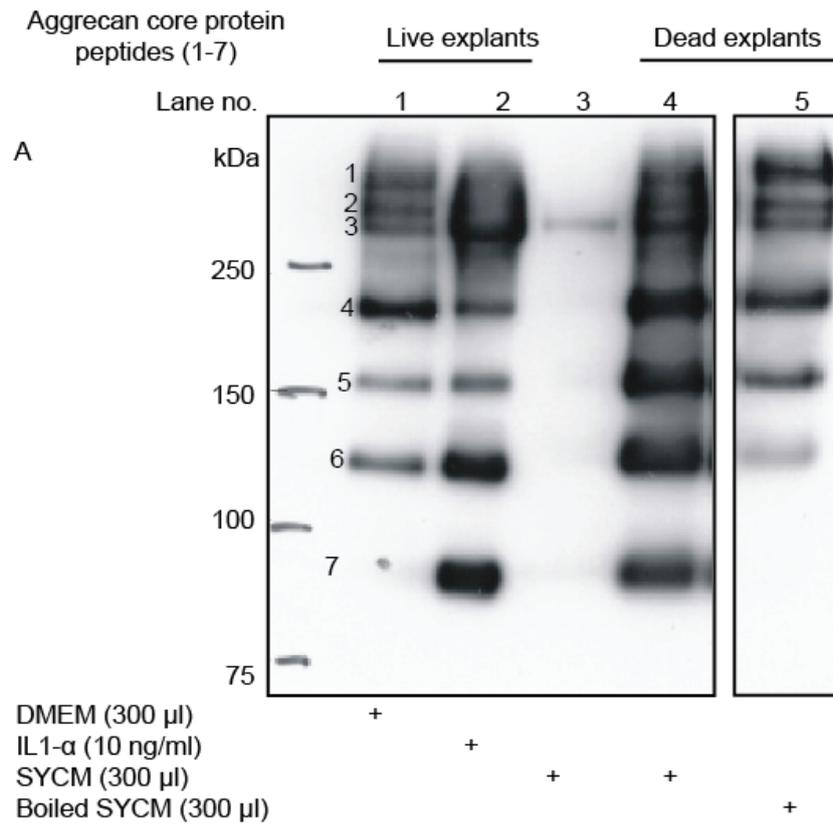
Fig. 3.2 Analysing aggrecan core protein peptides following IL-1 and SYCM stimulation of cartilage explants (A) Aggrecan core protein peptides appearing in the medium of IL-1 and SYCM stimulated live and dead cartilage explants respectively were analysed using stubs antibody. A 10 µl aliquot of samples was electrophoresed and immunoblotted with 2-B-6 anti-stub antibody (1: 10,000) as described in Materials and Methods. Lane 5 was from the same blot but was cropped to align with lanes 1 to 4.

3.3   Characterising substrates for analysing synovial aggrecanase

The aggrecanolytic activity of SYCM was also analysed by incubating it with purified bovine aggrecan, and the release of ARGS- and AGEG- containing fragments was again measured using neo-epitope antibodies. Fig. 3.3A shows that live synovial tissue culture (SYCM) produced aggrecanolytic enzymes generating ARGS (~ 250 kDa) and AGEG (~130 kDa) bearing protein fragments whereas freeze-thawed synovium (F/T SYCM) was unable to generate the activity (lane 1 & 2 vs. lane 3). The results replicated the generation of neo-epitope bearing fragments by SYCM when applied to freeze-thawed porcine cartilage explants (Fig. 3.1B lane 2). Two additional protein bands were also observed with molecular weights 37 kDa and 23 kDa (Fig. 3.3A, lane 1 to 3). The bovine aggrecan and SYCM preparations were probed with anti-ARGS antibody to determine whether the anti-ARGS reactive protein bands were the cleavage products generated by synovial aggrecanase(s) or antigens present in either the aggrecan or SYCM preparations. Fig. 3.3B shows the presence of 37 kDa and 23 kDa protein bands in SYCM (lane 2), which were absent from the bovine aggrecan preparation (lane 1). Therefore the smaller protein bands were non-specifically stained material present in SYCM detected by anti-ARGS antibody. A relatively small amount of ARGS bearing fragment (~250 kDa) was also observed in SYCM preparation that was absent in freeze-thawed synovial tissue culture (Fig. 3.3B lane 2). The origin of this fragment is unclear but may represent the product of synovial aggrecanolytic enzyme(s) on aggrecan present in the synovial culture.

The aggrecan degrading activity was also assessed on 50 kDa recombinant interglobular aggrecan domain (rIGD) substrate containing the aggrecanase cleavage site (NITEGE^{373}-^{374}ARGS). The cleavage at this site was detected by NITEGE^{373} specific neo-epitope antibody. Nine preparations of SYCM, generated over an 8 month period were analysed for aggrecanase activity. Only 4 samples showed NITEGE generating activity with

rIGD substrate (Fig. 3.3C). Although not shown, the SYCM preparations were all capable of degrading the dead cartilage aggrecan. All the subsequent experiments were carried out using one of the 9 batches of SYCM. Due to variability of results with SYCM preparations on rIGD, the freeze-thawed cartilage explants and bovine aggrecan became the preferred choices of substrate for analysing aggrecan degradation. However in order to ensure reproducibility of the results the quantifiable bovine aggrecan preparation was favoured.
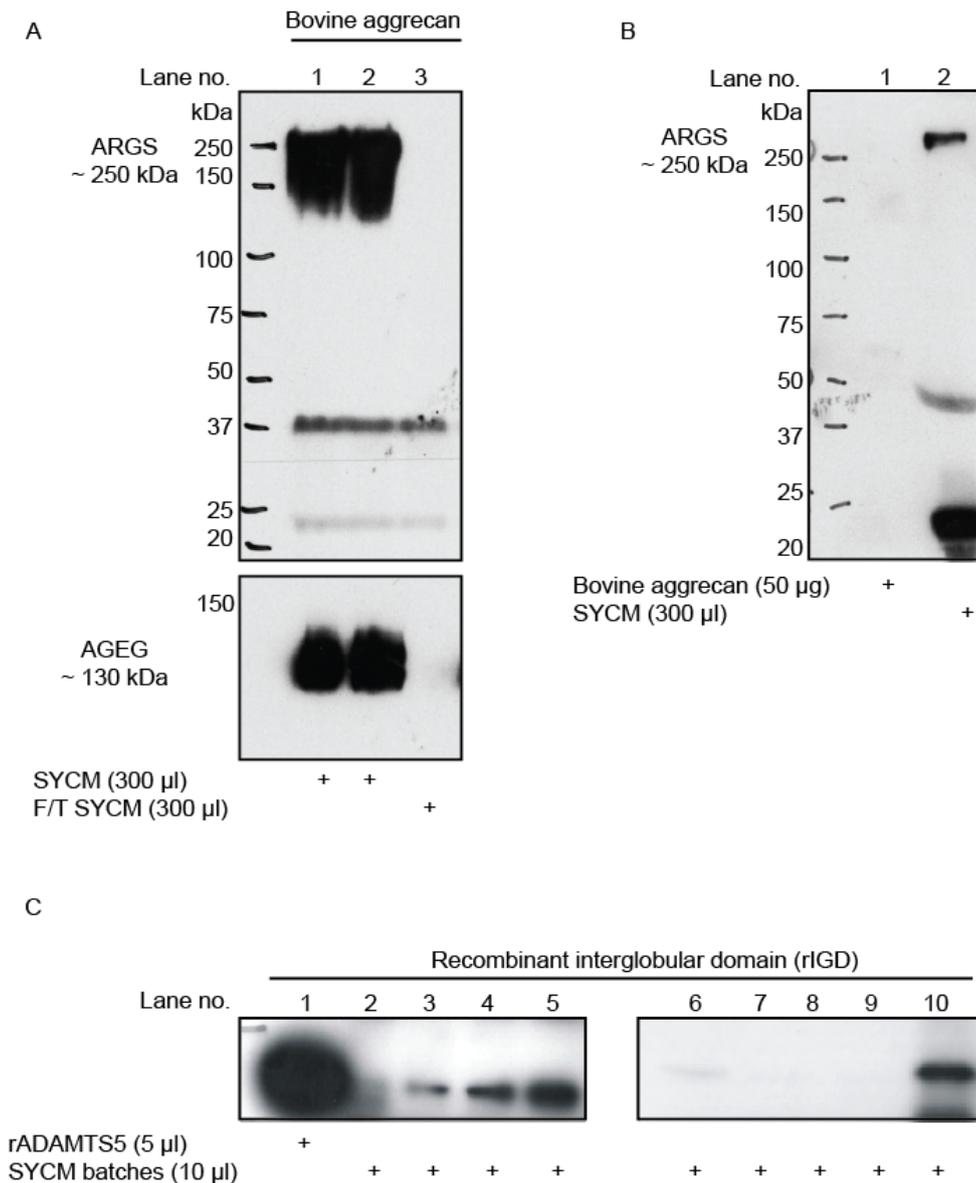
Fig 3.3 Assaying SYCM using bovine aggrecan and recombinant interglobular domain substrate (rIGD). (A) SYCM (300 μl) and freeze-thawed SYCM (F/T, SYCM; 300 μl) was incubated with bovine aggrecan (50 μg) for 24 hours at 37 °C and electrophoresed on a 4-12% NuPage bis-tris gradient gel. The proteins were transferred to PVDF membrane and immunoblotted with anti-ARGS and anti-AGEG antibodies as described in Materials and Methods. **(B)** SYCM (300 μl) and bovine aggrecan (50 μg) were electrophoresed on a 4-12% NuPage bis-tris gradient gel and proteins were transferred to PVDF membrane. The PVDF membrane was immunoblotted with anti- ARGS and anti-AGEG antibodies as described in Materials and Methods. **(C)** A 10 μl aliquot of SYCM samples were analysed for aggrecanolytic activity by incubating them with rIGD substrate (17 μM) for 24 hours at 37 °C. The cleavage at NITEGE^{373}-^{374}ARGS site was probed with anti-NITEGE antibody as described in Materials and Methods. A 5 μl aliquot of crude culture medium of HEK93 cells expressing recombinant ADAMTS5 (rADAMTS5) was also incubated with the rIGD as a positive control.

A commercially available enzyme-linked immunosorbant assay (ELISA) for the release of ARGS neo-epitope was also tested against synovial aggrecan degrading enzyme(s) for obtaining a quick readout for activity. A standard curve for the assay was obtained by using recombinant ADAMTS-4 (0-1.5 nM) to obtain its linear range. Recombinant ADAMTS4 (0-1.5 nM) was incubated with aggrecan IGD (100 nM) in a 1 mM MES pH 6.0 buffer containing proteinase inhibitors (0.4 mM AEBSF, 0.1 nM pepstatin, 0.1 nM leupeptin) at $37^{o}C$ for 15 min. The proteolytic reactions were stopped with 1 mM EDTA solution before adding the digests to wells pre-coated with anti-ARGS neo-epitope antibody in the microtitre plate. The bound ARGS peptide from the proteolytic digests was detected with peroxidase labelled antibody. The amount of peroxidase bound to different wells was determined by taking absorbance at 450 nm (A_{240}) with absorbance at 620 nm (A_{620}) used as a reference filter. The ratio of A_{450}/A_{620} was expressed as unit of activity. The standard curve was linear up to A_{450}/A_{620} average reading of 1.230 units obtained with 0.188 nM of ADAMTS-4 (Fig. 3.4A). The lowest concentration of rADAMTS4 used was 24 pM giving an average A_{450} of 0.158 units and therefore, the values of A_{450} above 0.158 were considered significant (Fig. 3.4B).

SYCM tested at 1:5 dilution gave A_{450}/A_{620} value of approximately 0.80 units, which was within the linear range of the assay and represented a ~ 20 fold increase over the buffer control (~0.045 units) (Fig 3.4B). The enzymatic activity of SYCM was abolished when SYCM was either boiled or synovial tissue freeze-thawed (F/T SYCM) before cultured in the medium. However cycloheximide treated synovial tissue culture medium (CHX SYCM) showed ~ 3 fold higher enzymatic activity compared to boiled SYCM or freeze-thawed synovial tissue culture medium (F/T SYCM). The increase in aggrecanolytic activity was also observed when CHX SYCM was applied to freeze-thawed porcine cartilage explants (Fig. 3.1C lane 3 vs. lane 4). As synovial tissue was dissected into culture medium containing

cycloheximide, it may have taken time for the protein synthesis inhibitor to penetrate into the cells of the synovial tissue and inhibit the production of injury induced aggrecanolytic enzyme(s). Nevertheless the aggrecanolytic activity in CHX medium was still 4 fold less than that of live SYCM (Fig. 3.4B).

A   **Standard curve for aggrecanase ELISA**

ADAMTS-4 [nM]	A_{450}/A_{620}	
0.000	0.044	0.044
0.024	0.141	0.174
0.047	0.278	0.358
0.094	0.468	0.574
0.188	1.180	1.280
0.375	2.205	2.350
0.750	2.902	2.996
1.500	3.023	3.170

B
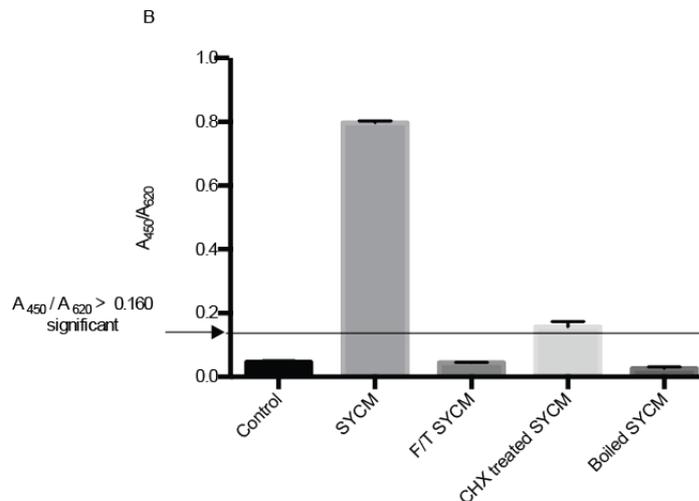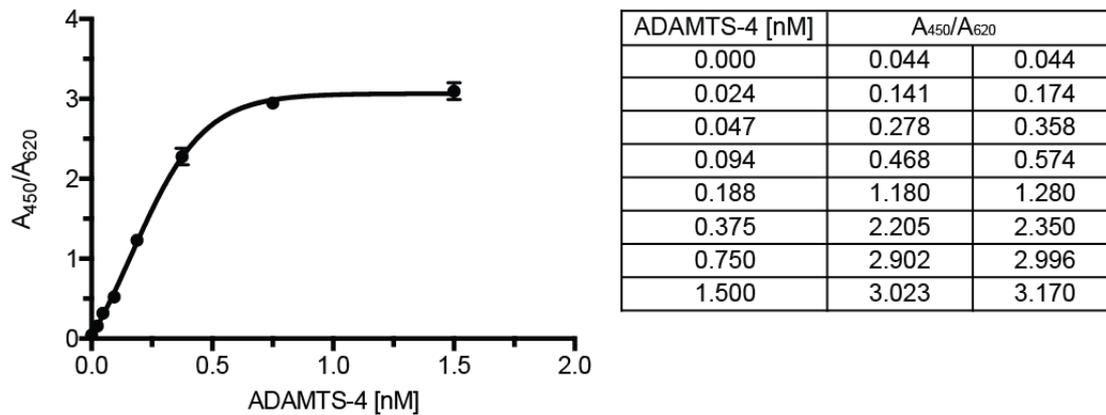
Fig 3.4 Testing aggrecanase ELISA for analysing aggrecan degrading enzyme(s) made by synovial tissue. **(A)** Recombinant ADAMTS4 (0-1.5 nM) was incubated with aggrecan IGD (100 nM) in a 1 mM MES pH 6.0 buffer containing proteinase inhibitors (0.4 mM AEBSF, 0.1 nM pepstatin, 0.1 nM leupeptin) at 37°C for 15 min. The proteolytic reactions were stopped with 1 mM EDTA solution before adding the digests to wells precoated with anti-ARGS neoepitope antibody in the microtitre plate. The bound ARGS peptide from the proteolytic digests was detected with peroxidase labelled antibody. The amount of peroxidase bound to different wells was determined by taking absorbance at 450 nm (A_{240}) with absorbance at 620 nm (A_{620}) used as a reference filter **(B)** The medium obtained from live synovial tissue culture (SYCM), freeze-thawed synovial tissue culture (F/T SYCM), cycloheximide (10ug/ml) treated synovial tissue culture (CHX treated SYCM) and boiled SYCM were tested for aggrecanolytic activity at 1:5 dilution using ELISA as described above. A_{450}/A_{620} values greater than 0.160 were considered significant. n = 1, error bars represent standard deviation of the three replicates for the one experiment.

3.4   Inhibition of aggrecan-degrading activity by TIMP-3

The sensitivity of the aggrecan-degrading enzyme to TIMP-3 and TIMP-1 was determined to characterise it further. These early experiments were carried out on freeze-thawed cartilage explants. Fig. 3.5 shows that the ARGS-generating activity of SYCM was completely inhibited by TIMP-3 (100 nM) but was unaffected by TIMP-1 (Fig. 3.5a, upper panel). The AGEG-generating activity in this experiment was reduced by TIMP-3 but was also unaffected by TIMP-1 (Fig. 3.5A, lower panel). It became important to determine the linear increase of the aggrecan degradation in this assay in order to account for the apparently less efficient inhibition of the production of AGEG fragment by TIMP-3. An approximate linear increase of the aggrecan-degrading activity was determined by incubating freeze-thawed cartilage explants with 300 μl of SYCM for different periods of time up to 31 hours at 37 $^{\circ}$C. Fig. 3.5B shows that the cleavage at TAQE^{1819} ^{1820}AGEG site generating ^{1829}AGEG fragment was linear up to 24 hours when 5 μl of sample was electrophoresed and PVDF membrane exposed to x-ray films for 30 s. Within the linear range of the activity assay and using the same pool of freeze-thawed cartilage explants, TIMP-3 (100 nM) and general metalloproteinase inhibitor GM6001 (10 μM), fully inhibited the release of AGEG and ARGS bearing fragments (Fig. 3.5C, lane 3 & 4 vs. lane 1).
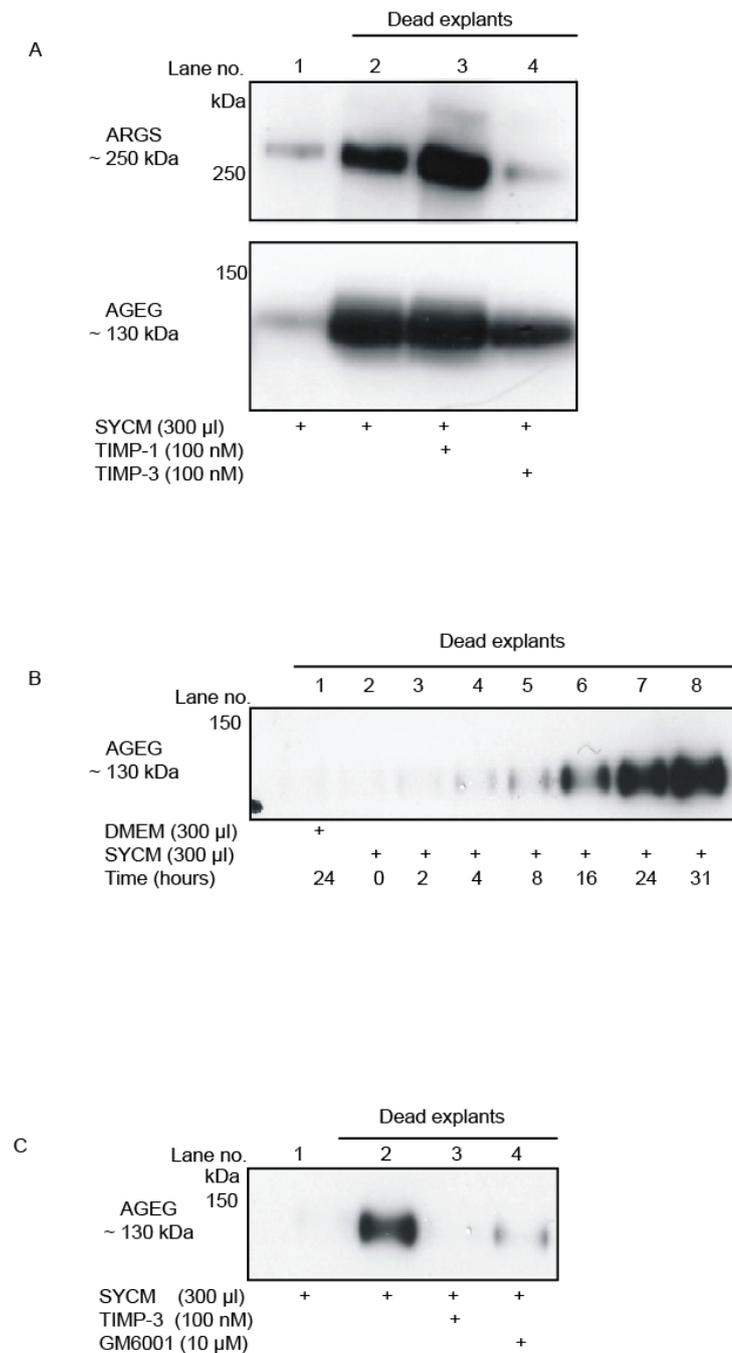
Fig 3.5 TIMP-3 but not TIMP-1 inhibits synovial tissue derived aggrecan-degrading enzyme. (A) SYCM (300 µl) was pre-incubated with TIMP-3 (100 nM) and TIMP-1 (100 nM) for 1 hour and samples were added to freeze-thawed (dead) cartilage explants for 24 hours. The aggrecan fragments released into the cartilage conditioned SYCM were detected by western blotting using ARGS and AGEG neo-epitope antibodies as described in materials and methods section. **(B)** The dead cartilage explants were incubated with SYCM (300 µl) for increasing time periods up to 31 hours. The AGEG-bearing fragment was detected by western blotting as described in Materials and Methods **(C)** SYCM (300 µl) was pre-incubated with TIMP-3 (100 nM) and GM6001 (10 µM) for 1 hour and samples were added to freeze-thawed (dead) cartilage explants for 24 hours. A 5 µl aliquot of cartilage conditioned SYCM was electrophoresed and western blotted with anti-ARGS and anti-AGEG neo-epitope antibodies with 30 second exposure to X-ray film.

The synovial aggrecanolytic activity was titrated with increasing concentration (20,40

and 60 nM) of N-terminal inhibitory domain of TIMP-3 (N-TIMP-3). The aggrecan degrading

activity was completely abolished when SYCM containing 40 nM N-TIMP-3 was applied to

freeze-thawed cartilage explants (Fig. 3.6A, lane 5 vs. lane 3). The lack of aggrecanolysis in

the presence of N-TIMP-3 (40 nM & 100 nM) was also observed using bovine aggrecan (Fig.

3.6B, lane 2 & 3) and recombinant aggrecan-IGD substrate of aggrecanase ELISA (Fig. 3.6C).

As seen on freeze-thawed cartilage explants, the inclusion of TIMP-1 to SYCM failed to

suppress the generation of ARGS- and AGEG- bearing fragments from bovine aggrecan (Fig.

3.6B lane 6) and ARGS bearing peptide fragment from aggrecan-IGD (Fig. 3.6C). The ARGS

generating activity was also completely inhibited by EDTA (1 mM) and GM6001 (10 μM)

but unlike EDTA, GM6001 was not able to completely abolish the AGEG generating activity

on bovine aggrecan (Fig. 3.6B, lane 5). The reduced inhibition by GM6001 can be attributed

to lower dose of GM6001 used relative to EDTA (10 μM vs. 1 mM). These results are

consistent with synovial tissue producing aggrecan-degrading enzyme of ADAM/ADAMTS

type.
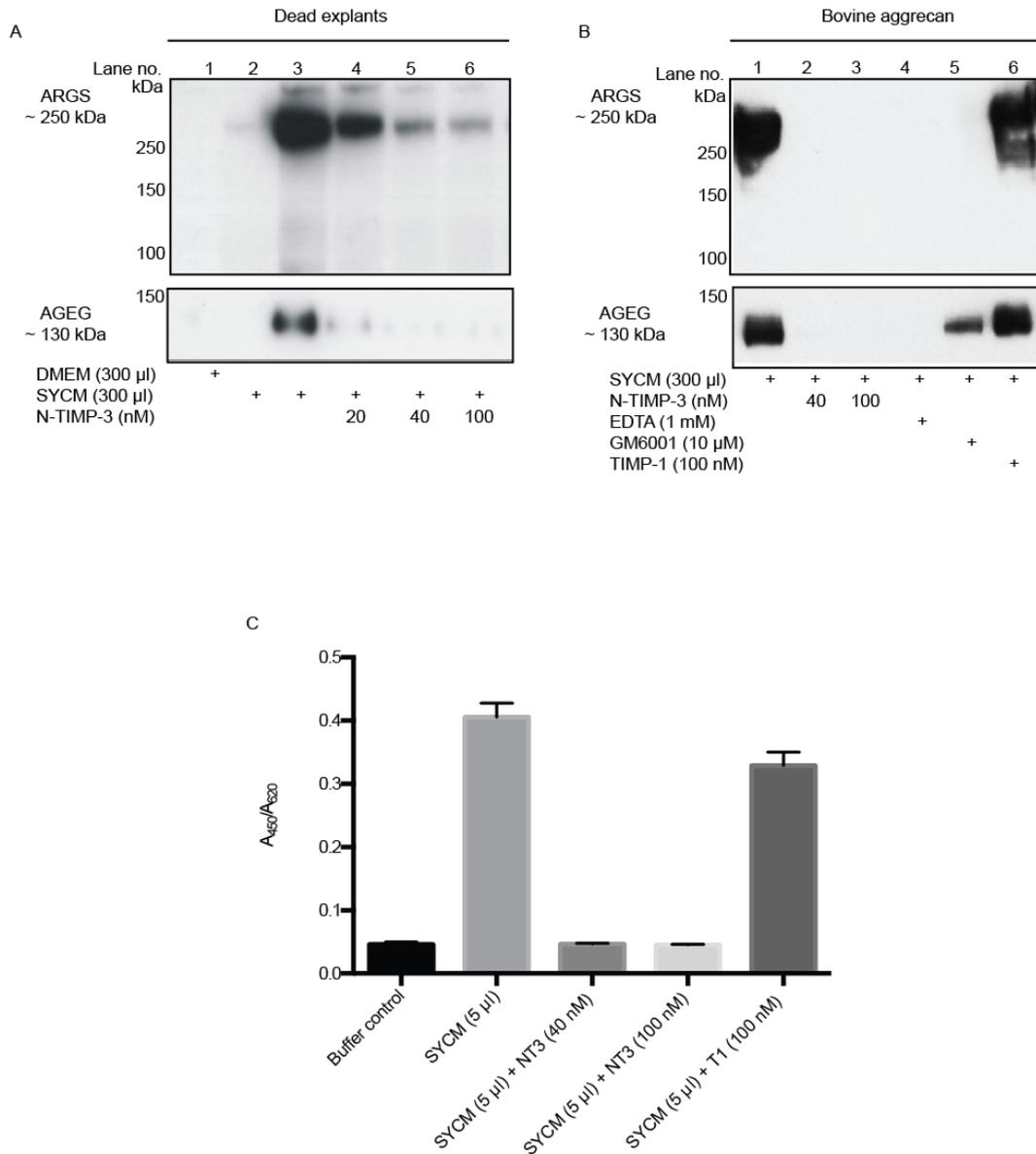
Fig.3.6. Testing different substrates against synovial aggrecan degrading activity (A) & (B) SYCM (300 μl) was pre-incubated with increasing concentrations (20 to 100 nM) of N-terminal inhibitory domain of TIMP-3 (N-TIMP-3), EDTA (1mM), GM6001 (10 μM) and TIMP-1 (100 nM) for 1 hour before applying the samples to dead cartilage explants or bovine aggrecan for 24 hours. The cartilage-conditioned medium was immunoblotted with ARGS and AGEG neo-epitope antibodies as described in Material and Methods. **(C)** SYCM (300 μl) was pre-incubated with N-TIMP-3 (40 & 100 nM) and TIMP-1 (100 nM) for 1 hour at 37 ^{o}C. The aggrecanolytic activity of the medium was assessed at 1 in 10 dilution using aggrecanase ELISA as described in Materials and Methods. n = 5, the error bars represent the standard deviation of the values obtained from the five experiments.

3.5 Discussion

My experiments show that injured synovial tissue generates an active form of aggrecan-degrading enzyme, which is in agreement with other reports (Fell *et al.* 1977; Vankemmelbeke *et al.* 1999; Vankemmelbeke *et al.* 2001). Here, I describe an assay for the activity of this proteoglycan-degrading enzyme using freeze-thawed (dead) cartilage explants as substrate. The proximity of the synovial tissue to the cartilage makes the assay possibly physiologically significant since the enzymes are acting on aggrecan within the real ECM. The cartilage substrate is a readily available substrate when the synovial tissue is being dissected and the sensitivity and reproducibility achieved with neo-epitope antibodies makes the assay suitable for aggrecan degradation studies. While all samples of synovial culture medium were active on the dead cartilage explants, only about half showed activity on rIGD. On further analysis, I found that anti-ARGS antibody couldn't detect even the ARGS bearing epitope upon cleavage of IGD substrate with either ADAMTS5 or ADAMTS4. It is not clear why rIGD showed variability in the results. It is probable that bacterial purification of this substrate gives truncated forms of protein, which are less susceptible to cleavage by enzyme. Moreover, the rIGD is an artificial system that can only be used to assay one neo-epitope but the dead cartilage substrate can be used for ARGS, AGEG or any other neo-epitope, as well as for aggrecan cleavage in general which can be detected with the stubs antibody. The cartilage and bovine aggrecan substrates are therefore more versatile for analysing enzymes in the SYCM. The bovine aggrecan preparation gives control over the quantity being used for each experiment and therefore ensures reproducibility of the conditions. However the use of aggrecan or cartilage substrate to analyse aggrecan degradation is time consuming, taking 3-4 days to complete the whole process whereas the aggrecanase ELISA takes only 3 hours and provides reproducible numerical values for different batches of SYCM. The aggrecan degrading activity observed on aggrecanase ELISA was also completely abolished with

TIMP-3 but not TIMP-1 thus mimicking the results obtained with physiological substrates i.e. bovine aggrecan and dead cartilage explants. Hence aggrecanase ELISA provides a relatively quick readout for the synovial aggrecanolytic activity and a valuable tool for purifying the enzyme from SYCM.

Vankemmelbeke and his colleagues used bovine aggrecan trapped in polyacrylamide gel as a substrate for observing aggrecanolytic activity in synovial tissue explant cultures (Vankemmelbeke *et al.* 2001). The amount of aggrecan release from the substrate was quantified using DMMB dye assay, which measures the amount of sulphated GAGs in the medium by taking absorbance at 525 nm. I also tested DMMB dye assay to assess the aggrecanolysis mediated by proteinases in the SYCM. However, I observed a very high level of absorbance at 525 nm in SYCM alone suggesting the presence of GAGs in the culture medium. On incubating the SYCM with either bovine aggrecan or freeze-thawed cartilage explants, no significant release was observed relative to control (SYCM only). DMMB is a generic dye, which is likely to bind any negatively charged sulphated molecules. The synovial explant cultures are very heterogeneous and likely to contain components of synovial tissue ECM that could interfere with assay. Similar high background levels are also a problem when analysing the culture medium of cartilage explants using DMMB dye assay. Saklatvala *et al.* 1979 also highlights this problem where 1 in 4 of the experiments analysing the cartilage degradation upon IL-1 stimulation failed due to high background release of GAGs from unstimulated cartilage explants (Saklatvala 1981). Given the problems with DMMB assay when analysing SYCM, the method was not used to assess cartilage degradation.

The sensitivity of synovium-derived aggrecanolytic enzyme to TIMP-3 and GM6001 indicate that it could belong to ADAMTS/ADAM family. A number of ADAMTS enzymes (ADAMTS 4,-5,-1, -8, -9,-15, -16) have been reported to cleave aggrecan but to date only ADAMTS-4 and ADAMTS-5 has been reported to be TIMP-3 sensitive (Kashiwagi *et al.*

2001). It is possible that the enzyme in SYCM is ADAMTS-4 or -5. The TIMP-3 sensitivity of the enzyme can be exploited to purify the aggrecan-degrading enzyme by affinity chromatography. A TIMP-3 matrix could also be used to deplete the neo-epitope generating enzyme in SYCM and the proteinase depleted medium could then be analysed for cytokines that regulate chondrocyte catabolic functions.

Chapter 4

Purification of synovial aggrecanase

4.1 Introduction

Early results have shown that damaged synovial tissue is a source of aggrecan degrading enzyme(s). The SYCM may consist of one or more aggrecan degrading enzyme(s). One way to identify these enzymes is to use ADAMTS antibodies. Our laboratories at Kennedy Institute (Prof. Jeremy Saklatvala & Prof. Hideaki Nagase) have made several antibodies to ADAMTS-4 and ADAMTS-5 and tested a number of other commercial antibodies over the years but none has reliably and convincingly detected enzymes in the culture medium. The ADAMTS antibodies work well with recombinant form of enzymes. But with tissue culture medium such as IL-1 stimulated cartilage cultures multiple bands were observed and no IL-1 inducible bands were seen consistently. An alternative approach was to use chromatography to purify the enzyme(s) and identify them by mass spectrometry. Different chromatographic techniques that work on different principles to separate molecules in a mixture were used: ion exchange chromatography separate proteins on charge whereas gel filtration on the basis of size. The separated proteins in different fractions would be tested for aggrecanolytic activity using the assays available for aggrecan degradation e.g. freeze-thawed cartilage explant assay, bovine aggrecan assay and aggrecanase activity ELISA. The fractions containing the aggrecanolytic activity would be pooled for further chromatographic separation. Initially the SYCM will be chromatographed on low-resolution columns to enrich and purify the enzymes from the bulk of other proteins which will followed by microscale purification using high resolution columns. An affinity chromatography step could also conceivably be used, probably as a final step to isolate the enzyme from a relatively small number of other proteins. Mass spectrometry would be used to identify the candidate proteins bands obtained after purification.

4.2 Anion exchange chromatography of synovial culture medium

Anion exchange chromatography was carried out on SYCM to separate and purify the enzyme or enzymes. A HighTrap Q anion exchange column was used and eluted with a salt gradient. Fractions were collected and analysed for their activity at 1 in 10 dilution on freeze-thawed cartilage explants. Figure 4.1A shows the elution profile of proteins as an absorbance trace at 280 nm as the salt gradient was developed. A peak of AGEG-generating enzyme was seen in fractions 8 and 9 (Fig. 4.1B, lower panel). However, no ARGS band was generated by fractions 8 and 9 (Fig. 4.1B, upper panel). The intensity of the AGEG band generated by fractions 5 and 6 was similar to that observed when the synovial culture medium was diluted 4-fold (Fig. 4.1B, lower panel). At this dilution no ARGS band was observed. Assaying a larger amount of the fractions might have generated the ARGS band. It was calculated from the gradient elution that fractions 8 & 9 contained aproximately 0.4-0.5 M NaCl. Salt concentrations higher than 0.2 M have an inhibitory effect on aggrecan degradation (data not shown). So fractions 7,8,9 & 10 were dialysed to remove salt and pooled. The pooled fractions when applied to freeze-thawed explants generated both ARGS and AGEG bearing fragments (Fig. 4.2A). The SYCM had been diluted 5-fold before applying it to the column as shown in Fig. 4.1A and the flow through (FT) fraction was therefore concentrated 5-fold by ultrafiltration before applying it to dead explants. No ARGS or AGEG bands were produced by the FT fraction. SYCM, flowthrough (FT) and fractions (4-12) were also analysed for aggrecanolytic activity using aggrecanase ELISA. The peak activity was observed in fractions 7 to 10 (Fig. 4.1C). The absorbance values of A_{450}/A_{620} were taken as arbitrary units of aggrecan degrading activity. An estimated 180 units of activity were present in 5 ml of SYCM and a total of 116 units were recovered in fractions 7 to 10 after anion exchange chromatography. It was calculated that approximately 65% of aggrecanolytic activity was recovered from the HiTrap Q column. However the
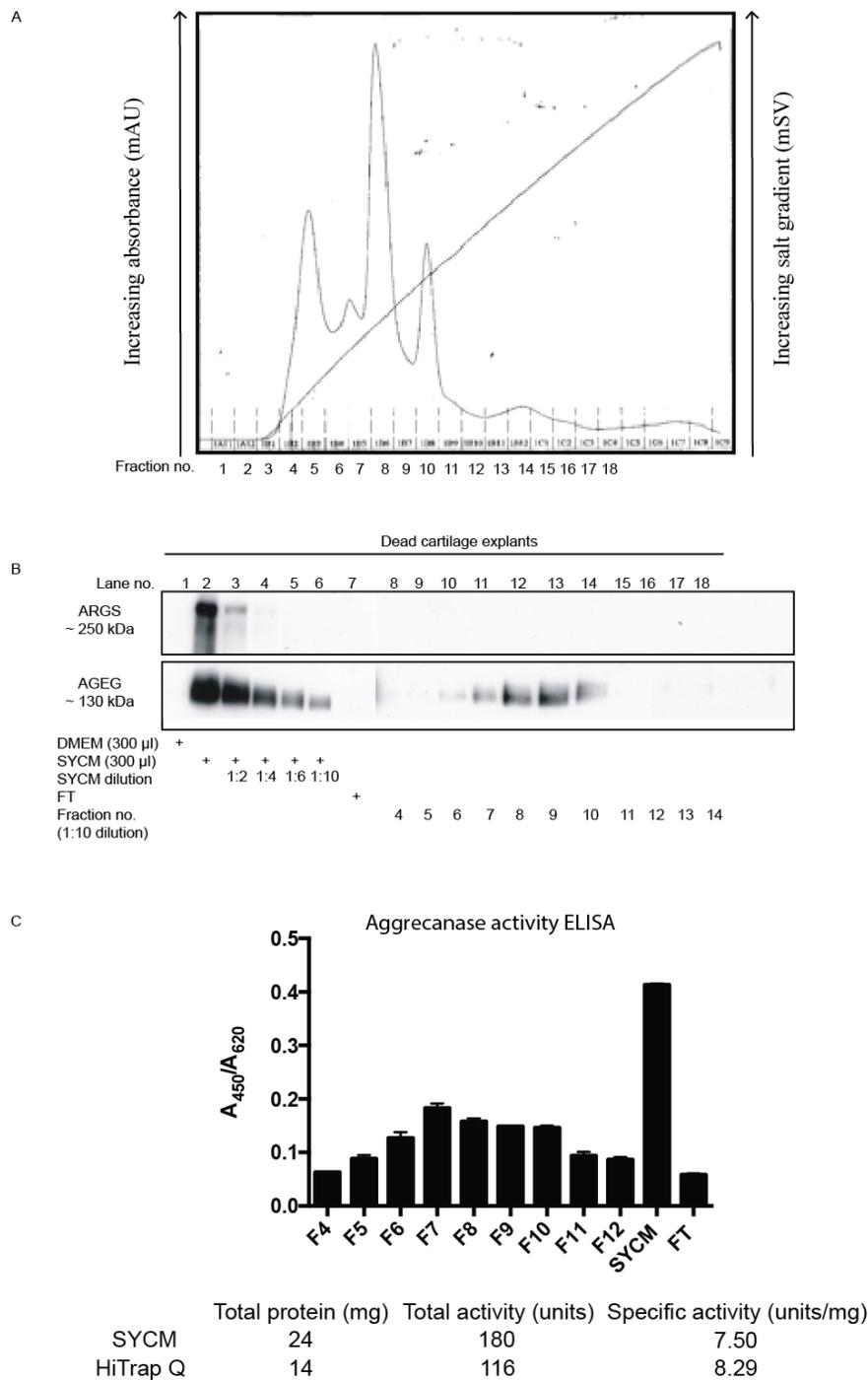
Fig 4.1 Anion exchange chromatography of synovial culture medium on agarose Q column. A 1ml HighTrap Q column was equilibrated with 20 mM Tris-HCl, pH 8.0. A 5 ml aliquot of SYCM was diluted 5-fold in 20 mM Tris-HCl, pH 8.0 and applied to the column. The unabsorbed material was collected as flow through (FT) fraction and concentrated 25-fold using Vivaspin ultrafilters (MW cut off 2KDa). The column was eluted with a gradient of NaCl (0 - 1 M over 20 ml) and fractions (1ml) were collected (A) A_{280} of the eluate (B) Fractions were diluted 1:10 using buffer A (25 mM HEPES, 10 mM $CaCl_2$) and applied to dead cartilage explants for 24 h at 37 °C. Western blots for ARGS and AGEG fragments was carried as described in Material and methods (C) SYCM, FT and fractions (1:10 dilutions) were incubated with aggrecan-IGD (100 nM) for 15 min at 37 °C. The reactions were quenched with 1 mM EDTA and ARGS bearing peptide in proteolytic digests was quantified using ELISA module as described in Materials and Methods.

specific activity (units of activity per 1 mg of protein) of pooled fractions (7 to 10) was similar to SYCM suggesting that purification of enzyme(s) using anion exchange chromatography was poor (Fig. 4.1C lower panel).

Aliquots (25 μl) of SYCM, FT and pooled medium of fractions 7 to 10 were electrophoresed and proteins visualised by staining with silver. The silver stained gel showed that majority of proteins in SYCM were also present in the pooled anion exchange fractions and hence contributing to low specific activity observed (Fig. 4.2B lane 4 vs. lane 2). A number of plasma proteins were abundant in SYCM e.g albumin (60 kDa), heavy and light chains of IgG (50 kDa & 25 kDa respectively) and transferrin (75 kDa) (Fig. 4.3A lane 2). The identity of the plasma proteins in SYCM or fractions was determined by comparing the molecular weights of known plasma protein in serum and SYCM. The presence of these proteins was not surprising as damage to synovial tissue would release the contents of capillaries into the culture medium alongside aggrecanolytic enzyme(s) made by injured synovium. The proteins in fractions 7, 8 and 9 were stained with Coomassie Brilliant Blue, which showed an abundant protein band of albumin (60 kDa) in all three fractions (Fig. 4.2C). Given the abundance of albumin in fractions containing aggrecanolytic activity, it was decided to use commercially available kits to remove albumin which would improve the purification on the anion exchange step and further chromatography.

A high affinity albumin and IgG removal kit designed for the removal of albumin and IgG proteins from human serum was tested against SYCM. The kit comprised recombinantly expressed peptide ligand coupled to agarose matrix with high affinity for albumin and IgG. A 50 μl aliquot of human serum was used to test the efficiency of the matrix. It removed about 95% of the albumin from the human serum, but the removal of IgG was much less successful (Fig. 4.3A lane 4 vs. lane 3). However the kit only removed about 5 % of porcine albumin from SYCM (Fig.4.3B lane 4 vs. 2) and the majority of the putative albumin was in the flow through

(lane 3). The differences in pig and human albumin protein sequence might explain the inability of the kit to remove albumin and IgG from SYCM. A different albumin and IgG removal matrix utilising a generic dye-based affinity ligand for albumin (proprietary Cibacron Blue matrix) and protein G agarose for IgG was deployed for the removal of albumin and IgG from SYCM. Fig. 4.3C shows that the unabsorbed material obtained from the mixture of protein G and Cibrecan Blue agarose matrices was devoid of albumin and IgG (lane 5 vs. lane 3). However the analysis of proteins bound to the matrix shows that besides albumin and IgG, numerous other proteins had bound to the agarose beads (lane 5). It was important to determine whether the aggrecanolytic activity was present in the FT. The FT was applied to bovine aggrecan and showed no aggrecan degrading activity (Fig 4.4D lane 3 & 4). This suggested that the enzyme(s) had non-specifically adhered to the agarose matix and was likely to be present in the eluted material. The elution of matrix bound proteins was carried out using SDS sample buffer with strong heating (10 min at 100 °C). Hence it was not possible to determine the aggrecan degrading activity of eluted material. As both of the albumin removal procedures didn't show promising results, the idea of removing albumin followed by anion exchange chromatography was abandoned. It was decided to try a strong cation exchange matrix (HiTrap S) for the purification of synovial aggrecanase(s).
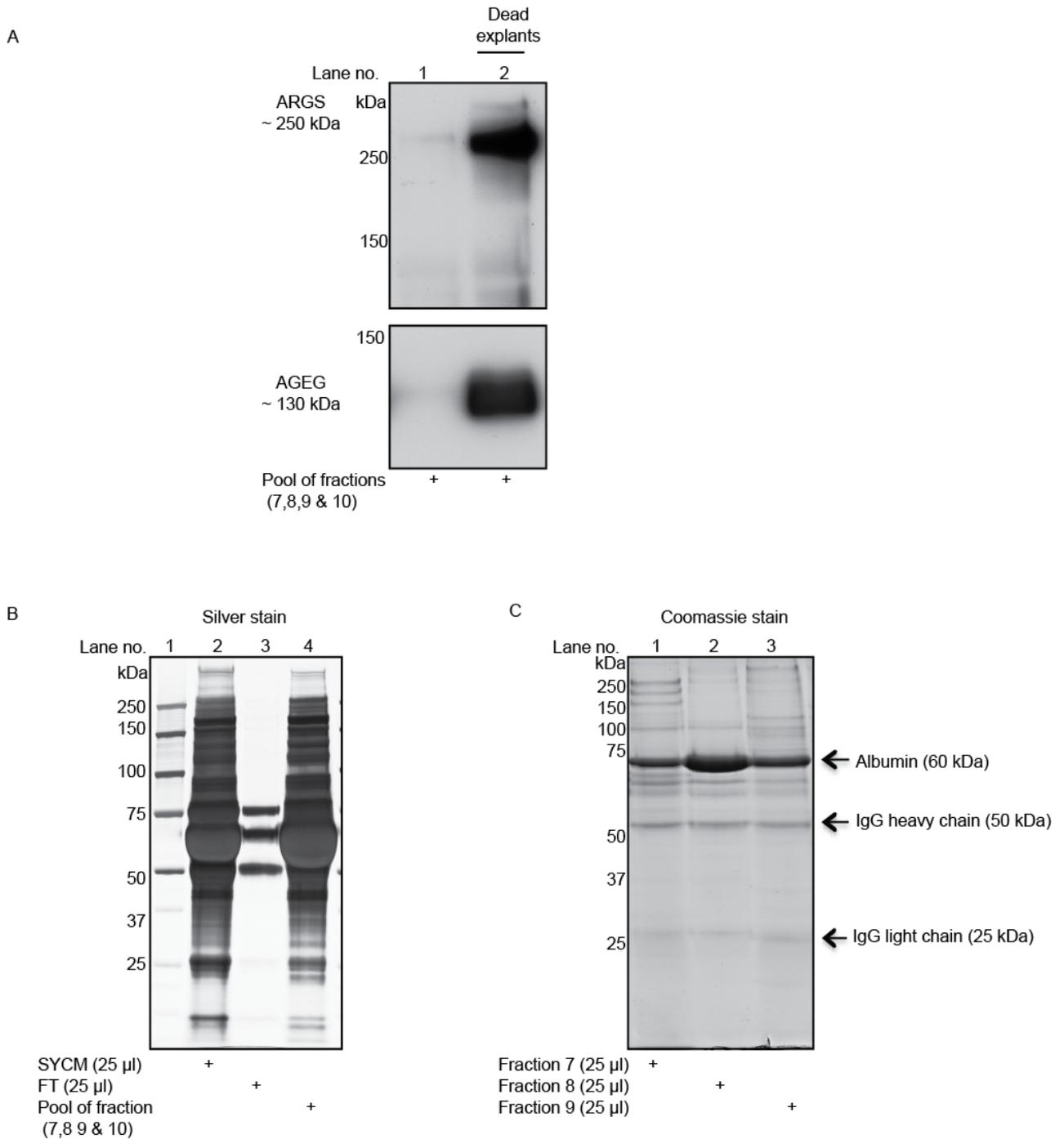
Fig. 4.2 Analysis of aggrecanolytic activity and protein content of fractions from anion exchange chromatography of SYCM on agarose Q column (A) The fractions (7 to 10) showing aggrecanolytic activity in fig. 4.1A were pooled and dialysed against 20 mM Tris pH 8.0, 150 mM NaCl and 5 mM CaCl$_2$ for 4 hours. The dialysate was applied to dead cartilage explants and aggrecanolysis observed using ARGS and AGEG neo-epitope antibodies as described in Material and methods. **(B)** A 25 μl aliquot of dialysate was electrophoresed on a 4-12 % gradient gel and the proteins visualized with silver stain as described in Material and Methods. **(C)** A 25 μl aliquots of fractions 7,8 & 9 were electrophoresed and the gel was stained with Coomassie Brilliant Blue as outlined in Materials and Methods.
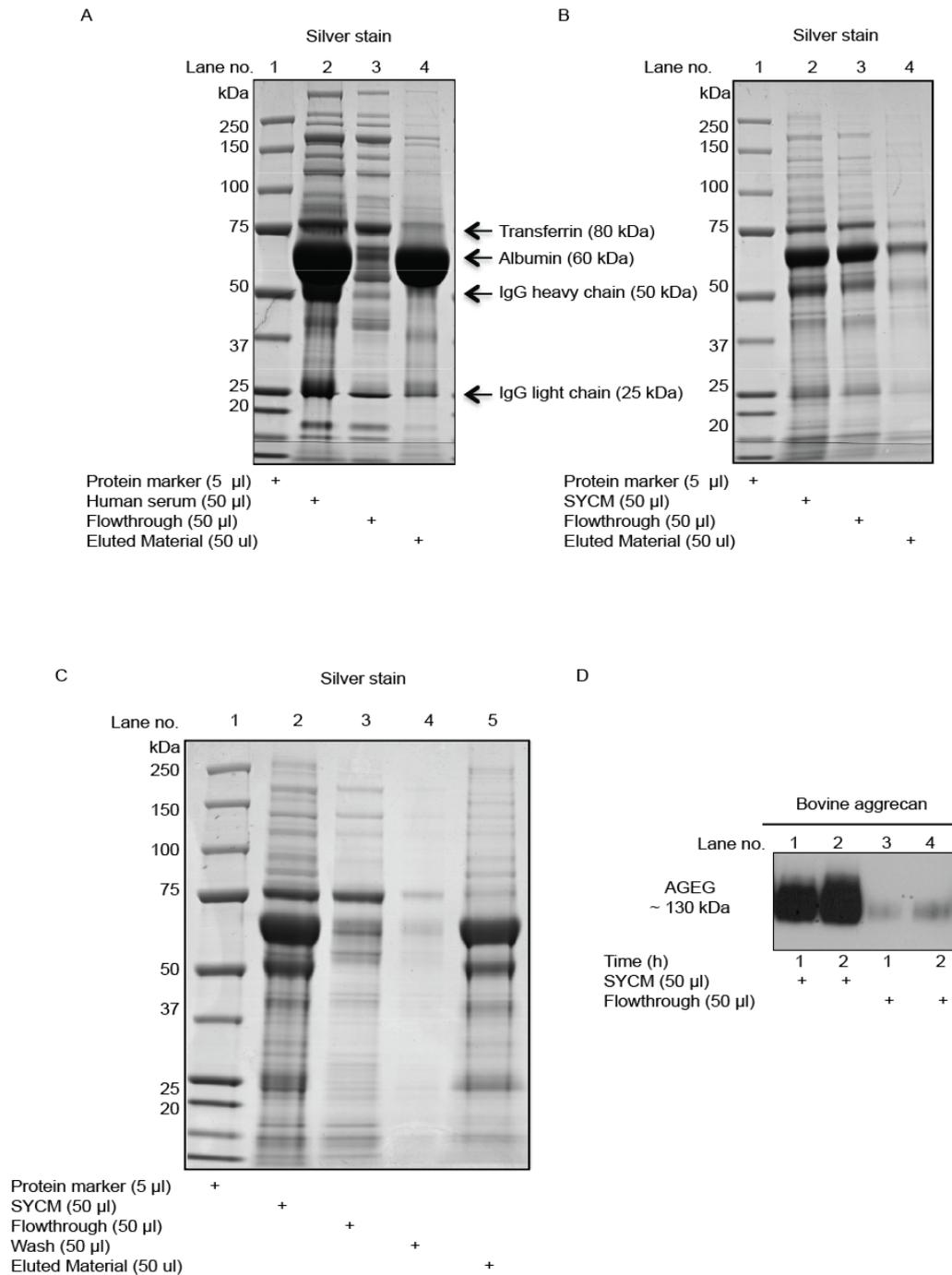
Fig. 4.3 Testing matrices for albumin and IgG removal on SYCM. Aliquots (50 μl) of human serum **(A)** and SYCM **(B)** were applied to a column packed with commercial agarose beads coupled high affinity proprietary peptide ligands to albumin and IgG. The unabsorbed material was collected as flow through (FT). The beads were eluted with SDS and heating at 100 °C for 10 min. Aliquots (50 μl) of human serum, FT and eluted material were electrophoresed on a gradient gel. The silver staining procedure was used to visualize proteins as described in Material and Methods. **(C)** A 50 μl aliquot of SYCM was applied to a mixture of agarose beads (protein G agarose and Cibacron Blue beads) in a mini column. The beads were spun in a centrifuge to collect the FT. The beads were washed with proprietary wash buffer supplied with the kit and the fluid collected. The material bound to the beads was eluted as described above. A 50 μl aliquots of SYCM , FT and eluted material was electrophoresed on a gradient gel and gel stained with silver as described in Material and Methods.

4.3   Cation exchange chromatography of SYCM

Cation exchange chromatography was carried out on SYCM using a HighTrap S cation exchange column. A 50 ml aliquot of SYCM was applied to the column and the unabsorbed material was collected as FT. The column was eluted with a salt gradient (Fig. 4.4A). Fractions were collected and analysed for their activity at 1 in 10 dilution on bovine aggrecan preparation. An ARGS and AGEG-generating enzyme was seen in fractions 9 to 12 (Fig. 4.4B, lanes 5 to 10). No ARGS bearing fragments were observed in FT (Fig. 4.4B, lane 2). However a faint AGEG generating activity was seen in FT (lane 2, lower panel). Figure 4.4A shows the elution profile of proteins as an absorbance trace at 280 nm as the salt gradient was developed. The enzyme(s) eluted from the column at relatively high salt concentration (0.4-0.5 M NaCl) and were present at the tail end of the absorbance peak observed in the trace. Majority of the eluted proteins concentrated in fractions 3 to 5. Although not shown in fig. 4.4B, fractions 3 to 5 didn't show aggrecan-degrading activity on bovine aggrecan. The presence of aggrecanolytic enzyme(s) in fractions 9 to 12 was also observed with the aggrecanase activity ELISA, which showed the peak of activity in fraction 11 (Fig. 4.5A). It was calculated that approximately 74% of the aggrecanolytic activity in SYCM was recovered in the pooled fractions 8 to 13 (Fig. 4.5A, lower panel) and the specific activity of the pooled medium was 8 fold higher than SYCM. The degree of purification achieved was confirmed when 25 μl aliquots of SYCM, FT and pool of fractions (9,10,11 and 12) were electrophoresed and stained with silver. The abundant proteins of the SYCM were not bound by the cation exchanger and were found in the FT (Fig. 4.5A lane 3). Fig. 4.5B lane 4 shows that far less proteins were present in the pooled fractions which was ideal for further purification of the synovial aggrecanase(s).

A

B

Fig 4.4 Cation exchange chromatography of SYCM using agarose S column (HiTrap S) PART I A 10 ml aliquot of SYCM was applied to 1 ml HiTrap S column. The unabsorbed material was collected as FT. The bound proteins were eluted with increasing salt gradient (0 to 1 M NaCl over 20 ml) and 1 ml fractions were collected. (A) A_{280} of the eluate (B) SYCM, FT and Fractions 6 to 15 were diluted 1:10 using 25 mM Tris-HCl pH 8.0, 10 mM $CaCl_2$ and applied to bovine aggrecan for 24 h at 37 °C. Western blots for ARGS and AGEG fragments was carried as described in Material and methods

A

Aggrecanase activity ELISA

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
SYCM (10 ml)	50	360	7.20
HiTrap S (6 ml)	4.5	265	58.89

B

Silver stain

Protein marker (5 µl)	+
SYCM (25 µl)	+
FT (25 µl)	+
Pool of fraction (25 µl) (9,10, 11 & 12)	+

Fig 4.5 Cation exchange chromatography of SYCM on agarose S column (HiTrap S)
Part II (A) A 10 ml aliquot of SYCM was applied to HiTrap S column. The proteins bound to the agarose matrix were eluted with salt gradient (0 to 1 M NaCl) and 1 ml fractions were collected. The fractions 7 to 15 were incubated with aggrecan-IGD (100 mM) for 15 min at 37 °C. The proteolytic reactions were quenched with 1 mM EDTA. The ARGS bearing peptide in the digests was measured using the ELISA procedure as outlined in the Materials and Methods. The protein concentration of SYCM and pooled medium (fraction 7 to 13) was determined using lowry protein assay (B) A 25 µl aliquots of SYCM, FT and pool of fractions (9,10,11 & 12) wee electrophoresed on a 4-12 % gradient gel. The silver staining procedure was used to visualise the proteins as described in Materials and Methods.

4.4   Gel filtration chromatography of SYCM on Sephacryl S-200

A large volume of synovial culture medium (60 ml) was ion-exchanged on a HiTrap S column (1 ml) in 10 ml batches. The fractions (1 ml) from each batch were analysed for aggrecan degrading activity on bovine aggrecan as shown in Fig. 4.4B. The active fractions from six runs were pooled (25 ml) and concentrated to 5 ml using a 3.5 kDa cut-off spin concentrator and applied to Sephacryl S-200 column equilibrated and eluted with buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 10 mM $CaCl_2$). The gel filtration column was calibrated with goat IgG (160 kDa), conalbumin (75 kDa), ovalbumin (45 kDa), ribonuclease (13.5 kDa) and aprotonin (6 kDa) kDa) and run under the same conditions to yield a calibration curve of the fractionation range of the matrix (250-5 kDa) (Fig. 4.6A). The void volume (V_o) of the column was determined using dextran (2000 kDa). The aggrecanolytic assay on bovine aggrecan showed that there were two peaks of ARGS and AGEG generating activity. One in the void volume and other within the fractionation range of the column. The ARGS generating activity of this included peak was spread equally in fractions 7 to 10, whereas AGEG generating activity peaked in fractions 12 to 13 (Fig. 4.6B lane 7 to 10). The aggrecan degrading activity was also measured using the aggrecanase ELISA. This confirmed the presence of aggrecan degrading activity in fractions 11 to 14 (Fig. 4.6C) with highest amount of activity apparently in fractions 12 and 13. The calibration curve was compared with the elution profile of sample to estimate the molecular weight of the aggrecan degrading species present in fractions 12 and 13. The elution volumes (V_e) were found at maximum peak height of each respective protein using the calibration curve of the standard proteins. The K_{av} values of the proteins was calculated using the equation $K_{av} = (V_e - V_o) / (V_c - V_o)$ where V_o = void volume of column, V_e = elution volume and V_c = geometric column volume. A graph of K_{av} versus log molecular weight was drawn and a curve was fitted around the points (Fig.4.7).
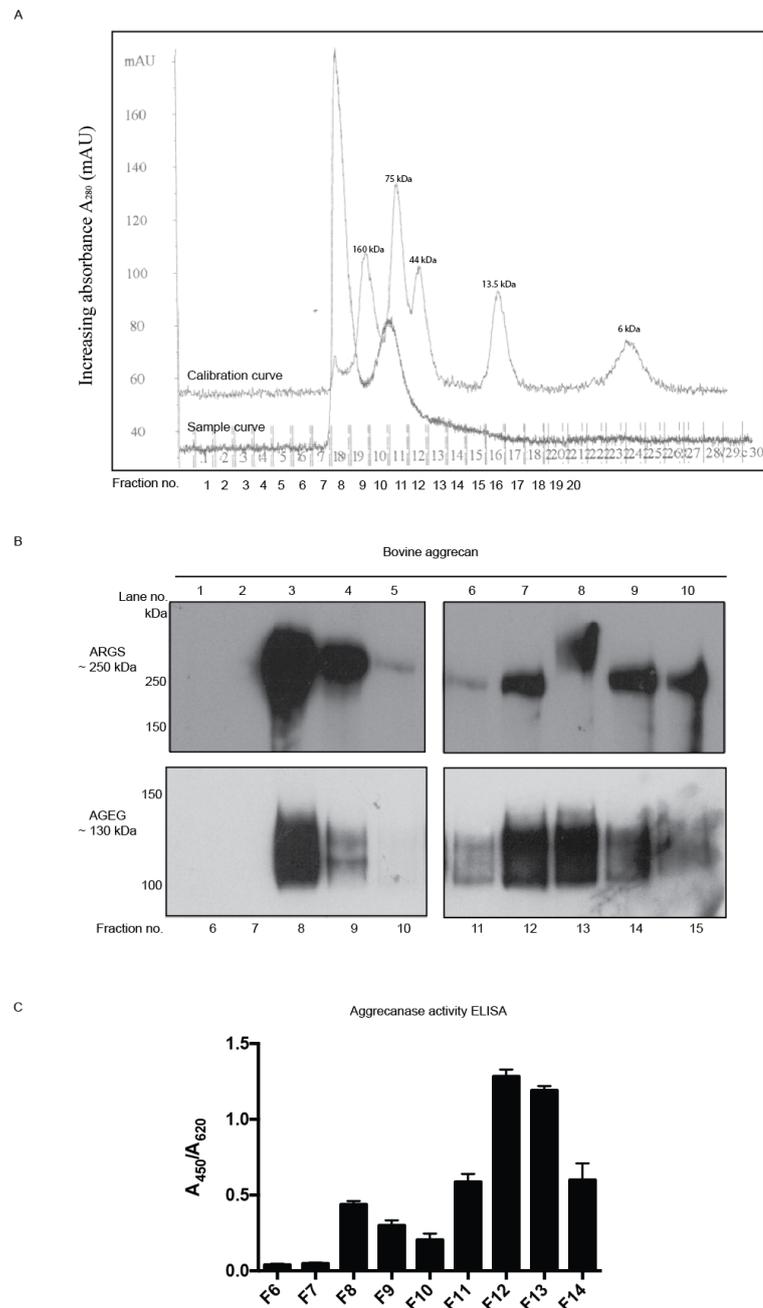
Fig 4.6 Chromatographic separation of proteins on Sephacryl S200 gel filtration column following HiTrap S chromatography. A 60 ml aliquot of SYCM was partially purified using 1 ml HiTrap S column. The fractions showing aggrecan degrading activity were pooled and concentrated to 5 ml using 3.5 kDa spin concentrator and applied to a Sephacryl S-200 gel filtration column (16 X 600 mm) that was equilibrated with 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$. The fractionation range of the gel filtration column was calibrated with goat IgG (160 kDa), conalbumin (75 kDa), ovalbumin (45 kDa), ribonuclease (13.5 kDa) and aprotonin (6 kDa). The column was run at 0.5 ml/min and eluted material was collected as 5 ml fractions. (A) The elution profile of pooled medium is drawn as an absorbance at 280 nm on vertical axis and fractions collected on the horizontal axis is compared against the elution profile of the calibration curve. (B) The fractions were incubated with bovine aggrecan (50 μg) for 24 hours at 37 °C and western blotted for ARGS and AGEG fragments as described in materials and methods. (C) The aggrecanolytic activity of the fractions was analaysed at 1 in 10 dilution using aggrecanase activity ELISA as described in Materials and Methods.

The elution volume of aggrecan degrading enzyme was taken as 60 ml (midway fraction 12 and 13) and the molecular weight was estimated as approximately 36-37 Da (Fig. 4.7). The degree of purification achieved with the gel filtration step was assessed by electrophoresing a 100 μl aliquot of the pooled fractions 12 & 13. The silver stain of the proteins showed that gel filtration step had fewer proteins relative to cation exchange step with majority of them present in the region of 60 to 30 kDa (Fig. 4.7B lane 4 vs. 3).

The first peak of ARGS and AGEG generating activity, which was observed using bovine aggrecan substrate in fractions 8 & 9 corresponded to the void volume of the gel filtration column and the molecular weight of species greater than 200 kDa. This aggrecan-degrading enzyme may form a part of a multimeric complex, or be bound to a high molecular weight material. The aggrecanolytic activity on bovine aggrecan observed in the void volume appeared to be as strong as that seen in the later fractions, but the ELISA showed barely detectable activity in fractions 8 & 9. On calculations made using the activity observed with aggrecanase ELISA, 35 units of activity were observed in the excluded fractions 8 & 9 while 115 units of activity were observed in fraction 12 & 13. Hence included peak of aggrecanolytic activity contained 3 times as much activity as excluded peak. This was puzzling: perhaps the recombinant form of aggrecan IGD used in aggrecanase ELISA was sterically hindered and not accessible to the high molecular weight form of the aggrecan-degrading enzyme.

A

Graph of K_{av} vs Log Mr

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

V_e = elution volume of peak of interest
V_o = void volume (37.5 ml)
V_t = total column volume (120)

V_e of enzyme is 60 ml (fraction 12-13)

K_{av} = (60-37.5)/(120-37.5) = 0.273

Aprotonin – 6 kDa
Ribonuclease – 13.5 kDa
Ovalbumin – 44 kDa
Conalbumin – 75 kDa
IgG – 160 kDa

Peak activity 12-13 has K_{av} = 0.272

K_{av}

Log Mr

36.3 kDa MW of aggrecanolytic species

B

Silver stain

Lane no. 1 2 3   4

SYCM (50 µl)   +
CIEX (50 µl)   +
GF (100 µl)   +
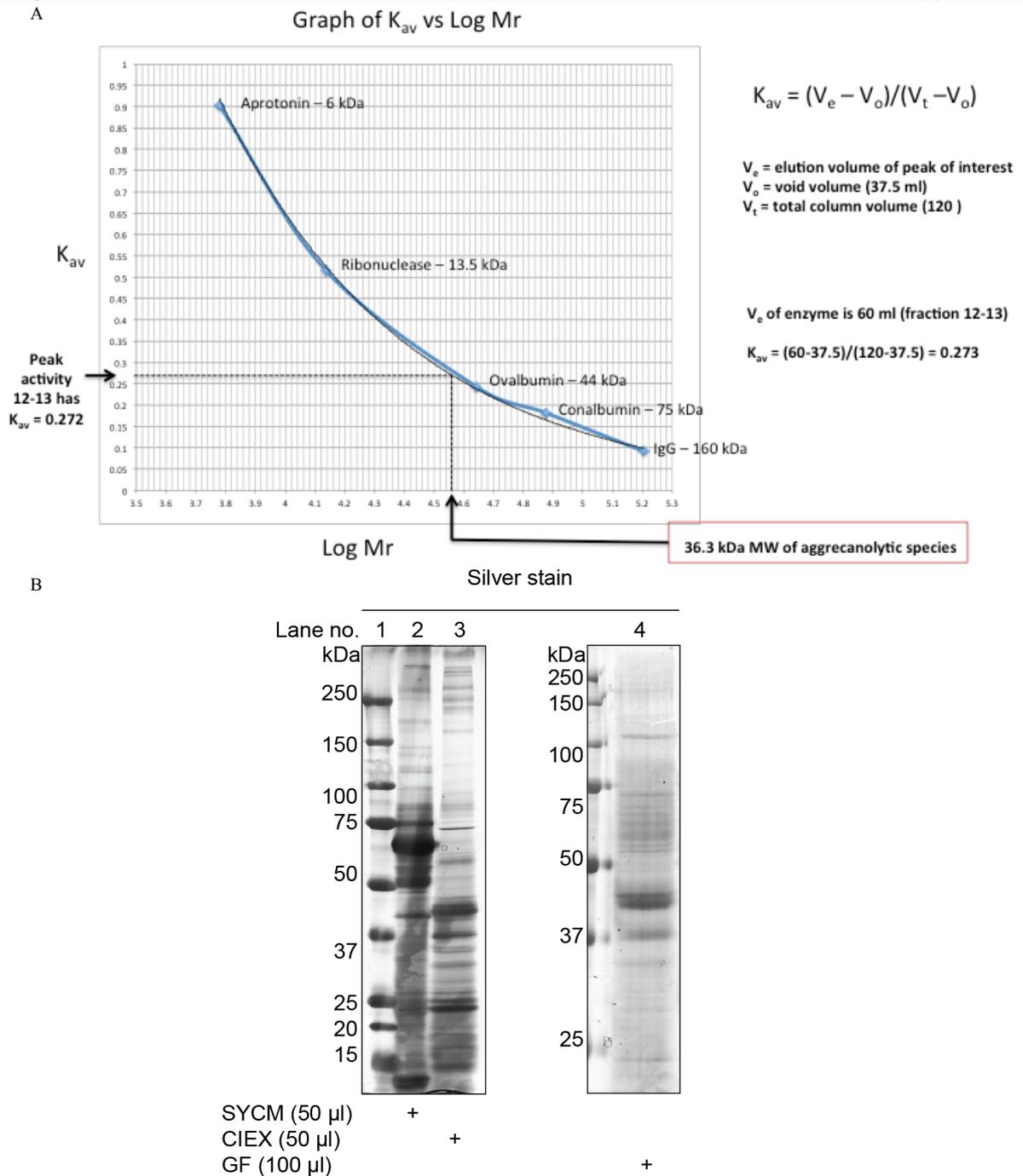
Fig 4.7. (A) Molecular weight determination of the included aggrecanase using Sephacryl S-200 chromatography. (B) Analysis by PAGE. A 2 ml protein mix of standard proteins (goat IgG (160 kDa), conalbumin (75 kDa), ovalbumin (45 kDa), ribonuclease (13.5 kDa) and aprotonin (6 kDa) was applied to Sephacryl S-200 equilibrated with 20 mM Tris-HCl, 150 mM NaCl and 10 mM CaCl$_2$ at a flow rate of 0.5 ml/min. The elution volumes (V_e) were found at maximum peak height of each respective protein using the calibration curve. The K_{av} values of the proteins was calculated using the equation $K_{av} = (V_e - V_o)/ (V_c - V_o)$ where V_0 = void volume of column, V_e = elution volume and V_c = geometric column volume. A calibration curve of K_{av} versus log molecular weight was drawn and a curve was fitted around the points. The elution volume of aggrecan degrading enzyme was taken 60 ml (midway fraction 12 and 13) and molecular weight was estimated as approximately 36 kDa. Aliquots of SYCM, pooled medium after cation exchange chromatography (CIEX) and pooled fractions 12-15 after gel filtration (GF) were electrophoresed on a 4-12 % gradient gel and the gel was silver stained as described in Materials and Methods.

4.5   Gel filtration chromatography of SYCM on Superose-6 column 1

The high molecular weight form of aggrecanolytic activity in the void volume of Sephacryl S-200 may comprise of one or more species of aggrecan-degrading enzyme. A Superose-6 column with a wide separation range (5000-5 kDa) was therefore chosen to separate the enzyme(s) in the pooled fractions after cation exchange chromatography. Figure 4.8A shows the absorbance trace observed when concentrated after cation exchange fractions (500 μl) was chromatographed on a Superose-6 column. The column was calibrated with dextran (2000 kDa), thyroglobulin (690 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (66 kDa) and ribonuclease (13.5 kDa) and run under the same conditions to yield a calibration curve (Fig. 4.8A). The aggrecanolytic assay using the bovine aggrecan showed an ARGS and AGEG generating activity in fractions 18 and 19 (Fig. 4.8B lane 14 & 15). It was estimated from the calibration curve that the aggrecanolytic species in these fractions had a molecular weight between 50 to 30 kDa. Moreover, the aggrecanase activity ELISA detected strong aggrecan-degrading activity only in fractions 18 and 19 and weak activity around the void volume (Fig. 4.9). Approximately 6 units of activity were observed in the included fractions 8 & 9 while 0.25 units of activity were observed in fraction 8 & 9. Hence included peak of aggrecanolytic activity contained 24 times as much activity as excluded peak. This suggests that the enzymatic entity in fractions 18 and 19 represented the ~37 kDa species observed in the fractionation range of the Sephacryl S-200 column. No other significant ARGS generating activity was observed in any of the fractions obtained from Superose-6 chromatography. However an AGEG generating activity was seen in the void volume (fraction 8 & 9) of the Superose-6 column indicating the presence of aggrecan-degrading enzyme of molecular weight greater than 2000 kDa (Fig. 4.9A). But it was surprising that the fractions 8 or 9 were not able to show significant ARGS generating activity. The activity may have become visible in fraction 8 & 9 if they were assayed at a higher concentration or if a higher concentration of bovine aggrecan was used in the assay.
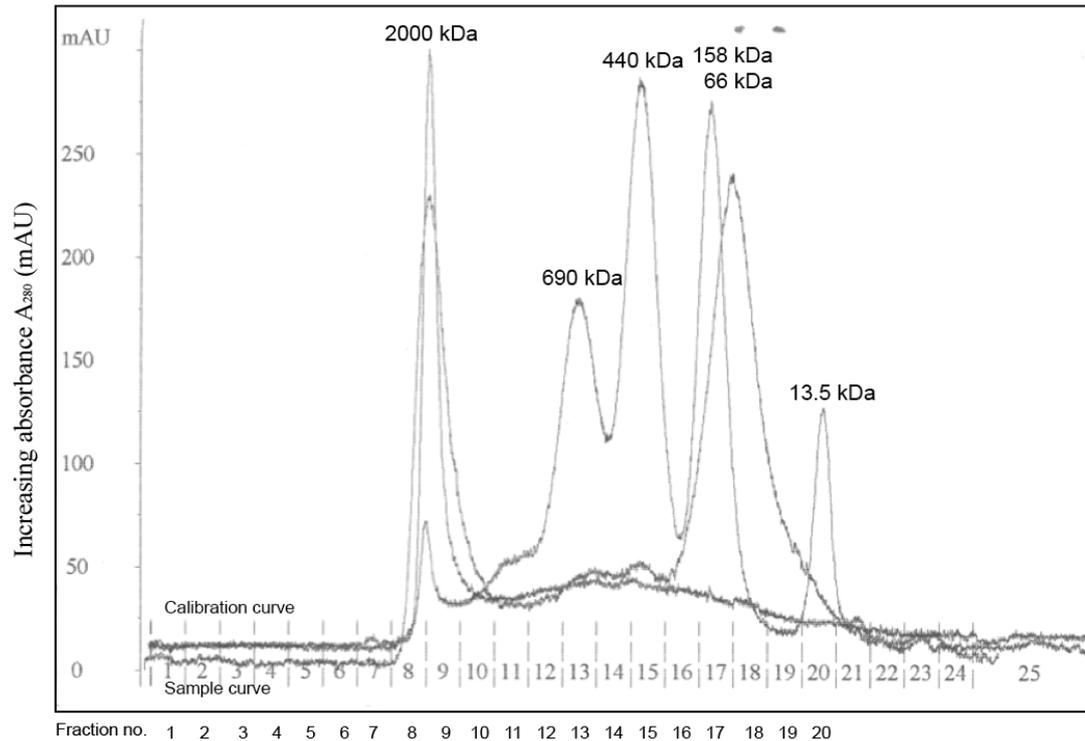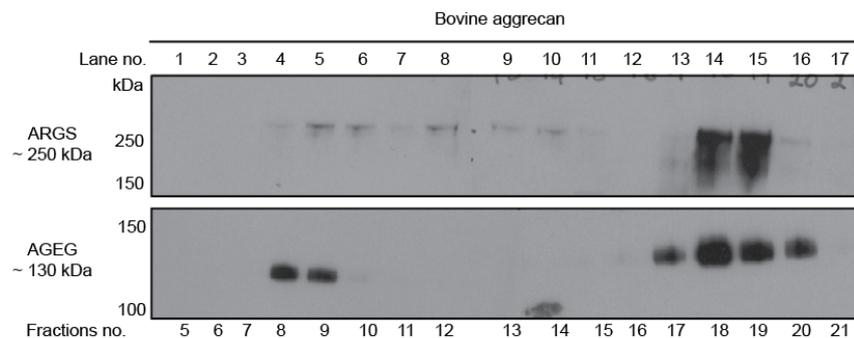
Fig 4.8 Chromatographic separation of proteins on Superose-6 gel filtration column following HiTrap S chromatography. A 30 ml aliquot of SYCM was partially purified using 1 ml HiTrap S column. The fractions showing aggrecan-degrading activity were pooled and concentrated to 0.5 ml using 3.5 kDa spin concentrator and applied to a superose 6 gel filtration column (10 × 300 mm) that was equilibrated with 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM CaCl$_2$. The fractionation range of the gel filtration column was calibrated with dextran (2000 kDa), thyroglobulin (690 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (66 kDa) and ribonuclease (13.5 kDa). The column was run at 0.5 ml/min and eluted material was collected as 1 ml fractions. (A) The elution profile of pooled medium is drawn as an absorbance trace at 280 nm on vertical axis and fractions collected on the horizontal axis were compared against the elution profile of the calibration curve. (B) The fractions at 1 in 5 dilution were incubated with bovine aggrecan (50 μg) for 24 hours at 37 °C and western blotted for ARGS and AGEG fragments as described in materials and methods.

A

Aggrecanase activity ELISA
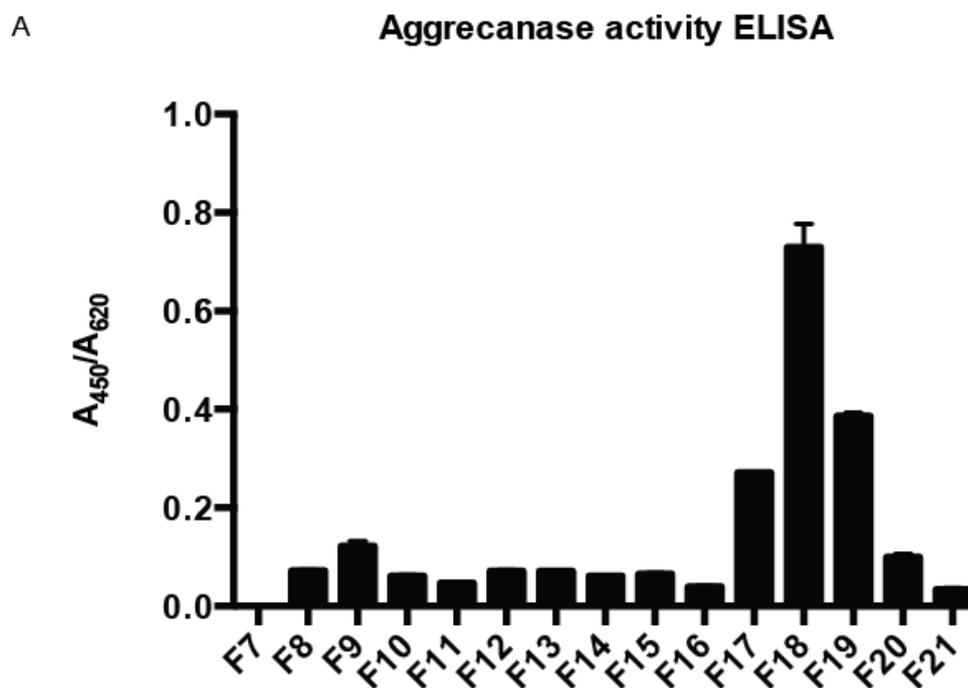
Fig 4.9 Measuring aggrecan-degrading activity of fractions obtained after Superose-6 chromatography (A) The 1 ml fractions obtained after Superose-6 chromatography were incubated with aggrecan-IGD at 1: 5 dilution for 15 min at 37 °C. The ARGS bearing peptide was quantified as described in Materials and Methods.

4.6   Gel filtration chromatography of SYCM on superose 6 column 2

The chromatographic separation of aggrecan-degrading enzymes on Superose-6 and Sephacryl S-200 suggested a low molecular weight form of enzyme around 40 kDa and a high molecular weight form, perhaps in a complex. Recently Yamamoto *et al* have shown that ADAMTS-5, a major aggrecanase in murine models of arthritis, binds to low-density lipoprotein receptor-related protein 1 (LRP1), a 600-kDa cell surface protein by which it gets endocytosed. This modulates the extracellular activity of ADAMTS-5. It is also known that proteinases cleave LRP1 near the membrane to release a 550-kDa extracelluar subunit (sLRP1) into the medium that is capable of binding wide array of molecules (> 40 ligands have been identified). Given the large molecular weight of sLRP1 (~ 550 kDa) and the likelihood of synovial aggrecanase being an ADAMTS, it was possible that the void volume fractions of Sephacryl S-200 and Superose-6 contained a complex of LRP1 and synovial aggrecanase.

In order to investigate the release of sLRP1 into the medium, damaged porcine synovial tissue was cultured for increasing length of time and the medium was probed with an anti-LRP1 antibody that detects epitopes in the extracellular subunit of LRP1. Fig 4.10A shows that a protein of approximate molecular weight 550 kDa accumulates in the culture medium after 2 hours (lane 2) but was rapidly degraded if synovial tissue was left up to 8 hours (lane 4). The loss of 550 kDa protein band could be due to proteolytic processing of sLRP1 by proteinases made by damaged synovial tissue. The release of sLRP1 after 2 and 24 hours was inhibited if synovial tissue was cultured in the presence of cylcoheximide suggesting that the accumulation of sLRP1 was an active process (Fig. 4.10B lane 2). Another anti-LRP1 reactive protein band was observed at a MW of 200 kDa at all time points which was unaffected by the
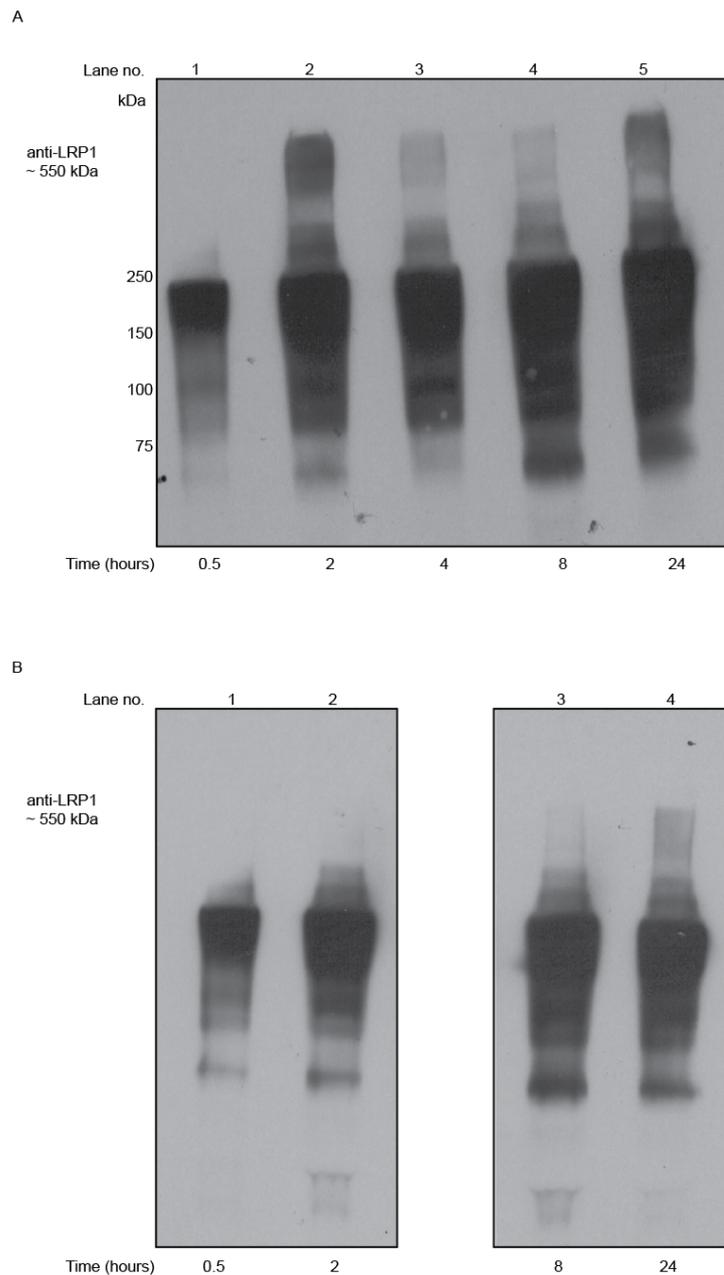
Fig 4.10. sLRP1 is released into the medium of synovial cultures.
(A) Synovial tissue (~ 1g) was cultured for increasing length of time (0.5 to 24 hours) in 2.5 ml of serum free DMEM. A 500 μl aliquot of SYCM was precipitated with tricholoroacetic acid and the pellet was dissolved in SDS containing sample buffer. The solubilised material was electrophoresed on a 4-12 % gradient gel under non-reducing conditions. The proteins were transferred to PVDF membrane and the membrane was immunoblotted using anti-LRP1 antibody at 1:1000. (B) Synovial tissue (~ 1g) was cultured for increasing length of time (0.5 to 24 hours) in the presence of cycloheximide (10 μg/ml) and the medium was probed with anti-LRP1 antibody as described above.

presence of cycloheximide (Fig 4.10A and B). The anti-LRP1 reactive material was either present in synovial fluid that accumulated alongside proteins made by synovial tissue or passively leached from synovial tissue with the passage of time.

The fractions obtained from Sephacryl S-200 chromatography (Fig. 4.6) were probed with anti-LRP1 antibody. The truncated forms of LRP1 were spread in fraction 9 to 11 (Fig. 4.11A lane 3 to 5) but were absent from fractions 12 and 13, which contained the major aggrecanolytic activity (Fig. 4.6B lane 7 to 8). However the long form of sLRP1 was seen in fraction 8 corresponding to the void volume of the gel filtration column (Fig. 4.11A lane 2), which also contained the high molecular weight form of aggrecanolytic enzyme (Fig. 4.6B, lane 3). The presence of sLRP1 and the aggrecanolytic activity in the same fraction may be a coincidence or may represent an interaction between LRP1 and the synovial aggrecanase. In order to investigate this possibility further the fractions obtained after Superose-6 chromatography were probed with anti-LRP1 antibody. The truncated forms of sLRP1 were observed in fractions 15 to 19 (Fig. 4.11B lane 11 to 15) whereas the low molecular weight form of aggrecan-degrading activity was present in fractions 18 and 19 (Fig. 4.8B lane 11 to 15). The lack of co-migration suggests that the two species do not interact with each other. Some long form of the sLRP1 chromatographed in fractions 11 to 13 with an apparent molecular weight of ~ 800 kDa but no aggrecanolytic activity was observed in these fractions (Fig. 4.8B lanes 7 to 9). However some long form of sLRP1 was also observed in the void volume (fraction 8) of Superose-6 chromatography (Fig. 4.11B, lane 4). The AGEG-generating activity was also observed in this same fraction and in fraction 9 in an equal amount (Fig. 4.8B lower panel). One possible explanation could be that a portion LRP1 was bound to some aggrecanolytic enzyme, along with many other high molecular weight proteins in the culture medium. There was a possibility that some of the high molecular weight aggrecanase was complexed with sLRP1.
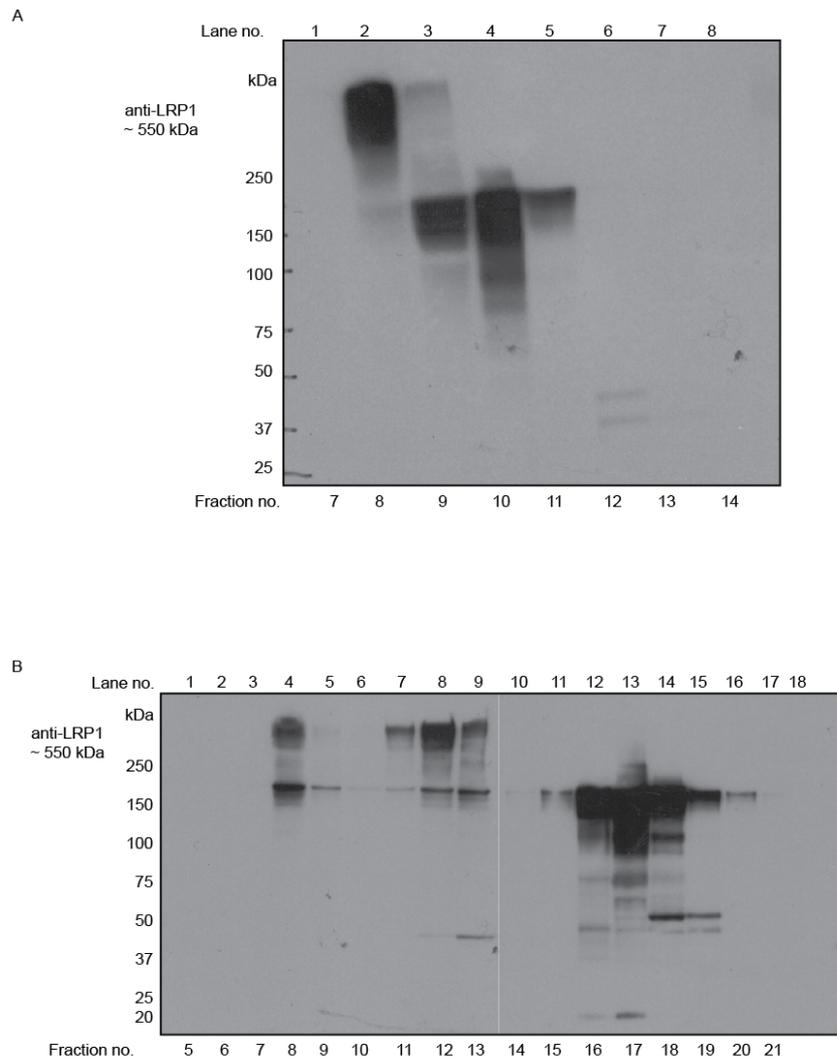
Fig 4.11 Immunoblotting fractions obtained from Sephacryl S-200 and Superose 6 chromatography with anti-LRP1 antibody.
(A) A fresh 60 ml aliquot of SYCM was purified using 1 ml HiTrap S column. The fractions showing aggrecan-degrading activity were pooled and concentrated to 5 ml using 3.5 kDa spin concentrator and applied to Sephacryl S-200 gel filtration column (10 × 300 mm) that was equilibrated with TNC buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM CaCl$_2$). The column was run at 0.5 ml/min and eluted material was collected as 5 ml fractions. A 500 µl aliquot of fractions was mixed with 1.5 ml of ice-cold 100% acetone for 30 min at -20 °C. The precipitate was mixed with SDS sample buffer and electrophoresed on a 4-12% gradient gel under non-reducing conditions. The proteins were transferred onto PVDF membrane and the membrane was immunoblotted with anti-LRP1 antibody at 1 in 1000 dilution as described in Materials and Methods. (B) A 30 ml aliquot of SYCM was chromatographed on a 1 ml HiTrap S column and the fractions showing aggrecan-degrading activity were pooled and concentrated to 0.5 ml using 3.5 kDa spin concentrator. The concentrate was applied to a superose 6 gel filtration column that was equilibrated with TNC buffer. The column was run at 0.5 ml/min and eluted material was collected as 1 ml fractions. A 500 µl aliquot of fractions was probed with anti-LRP1 antibody using the procedure described above in (a).

4.7   Anion exchange chromatography of enzymatically active fractions obtained from gel filtration step

Because the majority of the synovial aggrecanase detectable by assaying either with ELISA or bovine aggrecan was present in the smaller gel filtration peak, it was decided to focus on identifying this species. To purify and concentrate the low molecular weight form of the aggrecan-degrading enzyme, the fractions containing the aggrecan-degrading activity after the gel filtration step were pooled and applied to a microprecision mono Q anion exchange column. The fractions obtained from Sephacryl S-200 were selected because of the higher resolution achieved for the aggrecanolytic enzyme (~ 40 kDa) in the fractionation range of this column. Figure 4.12A shows the absorbance trace of the eluted material from the anion exchange column as the salt gradient was developed. The analysis of the aggrecan-degrading activity on bovine aggrecan showed a strong ARGS generating activity in fraction 8 (Fig. 4.12B lane 4). The aggrecanase activity ELISA further supported this observation by registering strong activity for fraction 8 (Fig. 4.13). However the western blot showing AGEG generating activity didn't work for this experiment (data not shown). But the results obtained for ARGS activity on bovine aggrecan and aggrecanase ELISA were definitive and it was deemed not necessary to repeat the AGEG activity assay. It was decided to keep fraction 8 material for later chromatography steps.
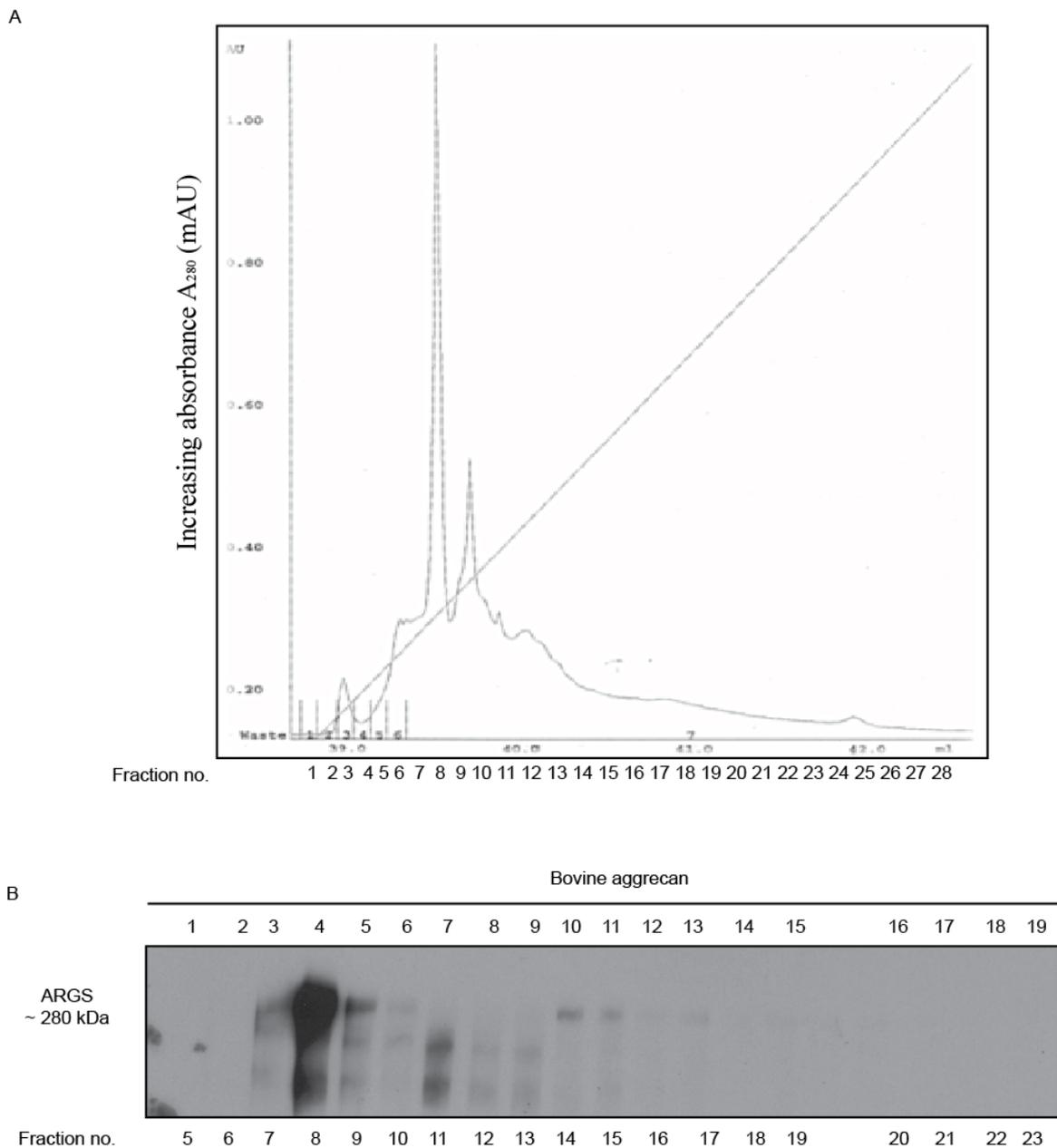
Fig 4.12 Chromatographic separation of proteins on mono Q ion exchange column. A 60 ml aliquot of SYCM was partially purified using 1 ml HiTrap S column. The fractions showing aggrecan-degrading activity were pooled and concentrated to 5 ml using 3.5 kDa spin concentrator and applied to Sephacryl S-200 column (10 × 300 mm) that was equilibrated with TNC buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM CaCl$_2$). The enzymatically active fractions for low molecular species (~ 40 kDa) were pooled. A 25 ml aliquot was applied to mono Q column (100 μl) equilibrated with 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM CaCl$_2$ and the unabsorbed material was collected as flow through. The bound material was eluted with salt gradient over 40 column volumes and 100 μl fractions were collected. (A) The elution profile was drawn as an absorbance trace at 280 nm on vertical axis and fractions collected on the horizontal axis (B) The fractions at 1 in 5 dilution were incubated with bovine aggrecan (50 μg) for 24 hours at 37 °C and the medium western blotted for ARGS and AGEG fragments as described in materials and methods.
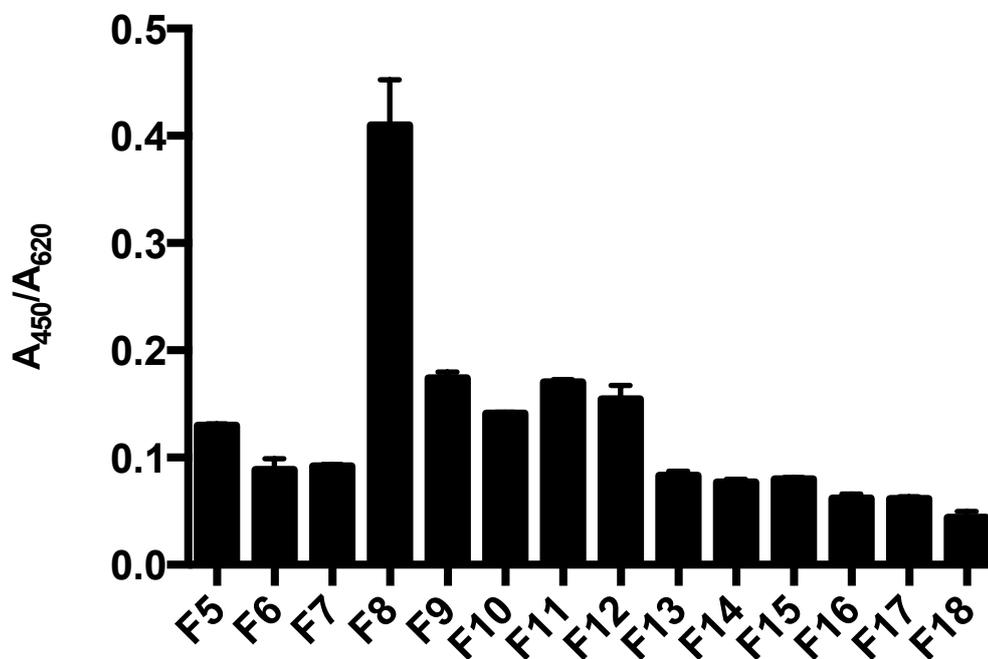
Fig 4.13 Measuring aggrecan-degrading activity of fractions obtained after Mono S chromatography (A) The 100 µl fractions obtained after Mono S chromatography were incubated with aggrecan-IGD at 1: 200 dilution for 15 min at 37 °C. The ARGS bearing peptide was quantified as described in Materials and Methods.

4.8 Discussion

The cation exchange chromatography on HiTrap S columns proved to be a good initial purification step, which lead to 8-fold increase in the specific activity of the synovial aggrecanolytic enzyme(s). Abbaszade *et al*. reported a 20-fold increase in the specific activity of aggrecan-degrading enzymes when purified from IL-1 stimulated bovine cartilage cultures using cation exchange chromatography (Abbaszade *et al.* 1999). Such a large increase in the specific activity was attributed to the increase in the total activity of the medium collected after cation exchange chromatography. This was surprising, as any chromatography step would cause 10-20% decrease of the target species. This increase was explained by the loss of inhibitory activity. Such an increase in the activity was not observed in the initial chromatography step used for the purification of synovial aggrecanase.

The cation exchange chromatography step failed to separate the high and low molecular weight forms of the enzyme(s). However, size-exclusion chromatography (Sephacryl S-200) successfully separated two forms of enzyme(s) present in the pooled fractions after the initial chromatography. Abbaszade *et al*. also used Sephacryl S200 as a purification step but didn't report the high or low molecular weight forms of the enzymes present in the medium (Abbaszade *et al.* 1999). The high MW form of aggrecan-degrading enzyme was difficult to chromatograph in the included volume of the available gel filtration columns. The results have also suggested that synovial aggrecanase(s) were unlikely to interact with LRP1. It may be that LRP1 plays a physiologically different role in cartilage compared to synovial tissue. It is also possible that synovial aggrecanolytic enzyme in SYCM has truncated form of domain structure which prevents its interaction with LRP1. The lack of interaction with LRP1 made it harder to justify the use of LRP1 immunoprecipitation to purify the enzyme in the void volume of gel filtration column. However, the low MW form of aggrecan-degrading enzyme (~ 40 kDa) was chromatographed as the major enzyme in the included volume of Sephacryl S-

200. The anion exchange chromatography sharply concentrated and purified the included peak from Sephacryl S-200 making the fractions ideal for affinity chromatography. An affinity chromatography step using the inhibitor of synovial aggrecanase, TIMP-3, could be used to pull down the low MW form of aggrecan-degrading enzyme. But this requires the successful purification of recombinant TIMP-3 and the application of protein as a tool for affinity purification, which will discussed in the following chapter.

Chapter 5

Purification and immobilisation of N-TIMP-3

5.1 Introduction

In order to affinity purify synovial aggrecanase, recombinant TIMP-3 needed to be expressed in sufficient amount (milligrams) for coupling to an agarose matrix. An expression system producing full length active TIMP-3 would be the ideal system for this purpose. A bacterial expression system expressing full length TIMP-3 has not been successful at Kennedy Institute because of difficulties associated with refolding the denatured TIMP-3 extracted from the inclusion bodies. A mammalian expression system for full-length TIMP-3 using transient transfection of HEK293 cells has been used at the Kennedy but does not give such high yields of protein as bacterial system (Linda Troeberg, personal communication). The truncated form of TIMP-3, the N-terminal inhibitory domain (N-TIMP-3) has been successfully expressed in milligram quantities in a bacterial system, but using N-TIMP-3 rather than full-length protein might be disadvantageous because there might be more steric hindrance to its interaction with enzyme when coupled to agarose matrix (Wisniewska *et al*. 2008). However the majority of lysine residues likely to be involved in the covalent coupling of N-TIMP-3 do not lie in the region involved in its inhibitory properties of TIMP-3 as determined from the 3-D structure of N-TIMP-3-MMP-1C complex. Therefore N-TIMP-3 might work as efficiently as full-length TIMP-3 for purifying the synovial enzyme(s).

5.2   Mammalian expression system for producing full length TIMP-3

I first tried setting up a mammalian expression system for full length TIMP-3 using a commercially available expression vector (OriGene technologies, USA). Full length TIMP-3 was expressed by transient transfection of HEK293 cells the vector (OriGene technologies, USA) containing full length sequence of TIMP-3 with a FLAG tag attached to the C-terminus. HEK293 cells were transfected in the presence and absence of serum and the lysates were probed with anti-FLAG antibody. FLAG-tagged TIMP-3 was found to be intracellularly localised (Fig. 5.1A, lane 5 & 6). Intracellular FLAG tagged TIMP-3 was purified by immunoabsorption chromatography on an anti-FLAG antibody coupled to agarose beads (Fig. 5.1B). From 50 ml of HEK293 lysates (obtained from 50×10 cm dishes) about 20μg of TIMP-3 was obtained. The yield of TIMP-3 from this expression system was too low for it to be used for affinity purification of aggrecanases. Moreover, the TIMP-3 fractions obtained from anti-FLAG chromatography were impure due to non-specific interaction of the agarose beads with proteins in the lysates (Fig. 5.1C). The TIMP-3 containing fractions were ion-exchanged to remove contaminating proteins but this procedure also resulted in 50% loss of TIMP-3 (Data not shown). The purified TIMP-3 fractions showed weak inhibitory activity against MMP-1ΔC (Fig. 5.1D). The inhibitory line formed a curve rather than a steep line to the x-axis, which is characteristic of tight binding inhibitors like TIMP-3. Due to poor yield, impurities in purified fractions and weak inhibitory activity against MMP-1ΔC, the mammalian system for TIMP-3 expression was abandoned.
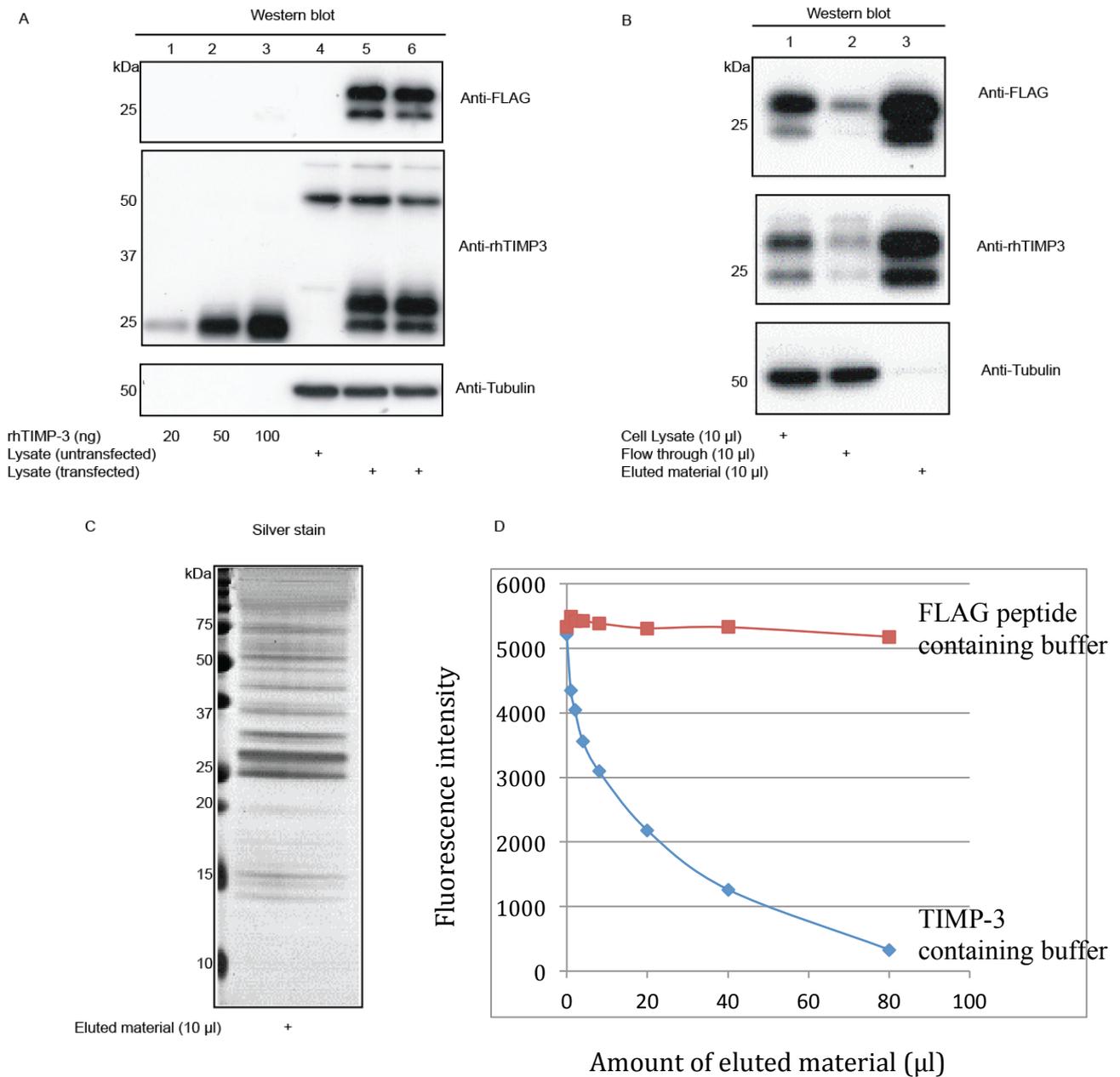
Fig. 5.1. Mammalian expression system for expression of full-length TIMP-3.
(A) Confluent (70-80%) HEK293 cells were transfected with Turbofectin 8.0 in a solution containing 1 μg of DNA. The cells were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0). Aliquots (10 μl) of cell lysates were electrophoresed and probed with anti-FLAG, anti-TIMP-3 and anti-tubulin antibodies as described in the Material and Methods. (B) The cell lysates (1 ml) were incubated with 50 μl aliquot of FLAG agarose beads for 24 hours. The beads were gently pelleted and the supernatant collected as flow through. The beads were eluted with 250 μl of 3 × FLAG peptide. The aliquots (10 μl) of cell lysate, flow through and eluted material were electrophoresed and probed with anti-FLAG, anti-TIMP-3 and anti-tubulin antibodies as described in the Materials and Methods (C) A 10 μl aliquot of eluted material was electrophoresed and silver stained as described in Materials and Methods. (D) MMP1ΔC (20 nM) in a 200 μl reaction volume was titrated with increasing volume of eluted material and FLAG containing buffer. The residual MMP1ΔC activity was measured with fluorescent substrate as described in materials and methods.

5.3 Expression, purification and folding of N-TIMP-3 from *E.coli*

E.coli expressing N-terminal inhibitory domain of TIMP-3 (N-TIMP-3) with His tag attached to the C-terminus to facilitate purification were used. The details of N-TIMP-3 expression, purification and folding procedures are described in the Materials and Methods section. The purification procedure of N-TIMP-3 from 3 litres of bacterial culture is summarised in Table 3. The extract of inclusion bodies from 3 litres of bacterial culture was applied to a 5 ml column packed with Ni^{2+} agarose beads (Fig. 5.2A, lane 6). The column was washed with buffer containing 20 mM imidazole to remove the majority of the non-specific proteins (Fig. 5.2A, lane 8 to 12). The eluted material was collected as 2 ml fractions (Fig. 5.2A, lane 13 to 25). Since all fractions contained sufficiently pure N-TIMP-3 protein, they were pooled for its *in vitro* refolding by dialysis. Approximately 4.5 mg of soluble N-TIMP-3 was obtained after *in vitro* refolding and Ni^{2+} chromatography of 62 mg of unfolded N-TIMP-3 (Table 3). The worst loss of protein occurred at this step (about 5.2 % recovery of the original material) because the majority of the protein formed an insoluble precipitate upon dialysis. The N-TIMP-3 was seen on electrophoresis as a monomer (15 kDa), dimer (30 kDa) and trimer (45 kDa) (Fig. 5.2A, lane 13 to 35). Because monomeric N-TIMP-3 is thought to be the active form (Kashiwagi *et al.* 2001), a Sephacryl S-200 column was used to purify this species. Fig. 5.2C shows the chromatographic separation of the monomeric N-TIMP-3 from the other multimers. Monomeric form of N-TIMP-3 was present in fractions 16 to 19, whereas the probable dimer was in fractions 14 to 17 (Fig. 5.2C). Fractions 16 and 17 showed significant overlap between the dimeric and monomeric forms, but fractions 18 and 19 contained predominantly the 15 kDa monomeric N-TIMP-3 (Fig. 5.2C). Therefore fractions 18 and 19 were pooled and titrated with 20 nM MMP-1ΔC to estimate the concentration of active N-TIMP-3, assuming N-TIMP-3 to MMP1ΔC stoichiometric ratio of 1:1. A 10 µl aliquot of N-TIMP-3 preparation fully neutralised the activity of 20 nM MMP1ΔC (Fig. 5.3).

It was calculated that the active N-TIMP-3 concentration was 360 nM (Fig. 5.3). This corresponded to 55 µg of active N-TIMP-3 protein (MW: 15230 g/mol) in 200 µg of pooled fractions. Hence, only about 28 % of refolded N-TIMP-3 was active against MMP1ΔC.

Purification step	Total protein (mg)	Recovery of protein (%)
Inclusion body extract	87	100.0
Unfolded N-TIMP-3 recovered after Ni^{2+} affinity chromatography	62	72.0
Soluble N-TIMP-3 obtained after *in vitro* refolding and Ni^{2+} affinity chromatography	4.5	5.2
Purified N-TIMP-3 recovered after gel filtration chromatography (fractions 16 to 19)	0.4	0.4

Table 3. Purification of N-TIMP-3 from bacterial culture. Inclusion bodies from 3 litres of bacterial culture were extracted and unfolded N-TIMP-3 was purified by Ni^{2+} affinity chromatography. The protein was refolded by slowly removing the denaturant by dialysis. Insoluble precipitates of N-TIMP-3 were removed by centrifugation and soluble N-TIMP-3 was purified from the supernatant by Ni^{2+} affinity chromatography. Chemical assay (Bradford protein assay) was used to quantify the amount of protein in inclusion body extract, fractions obtained after Ni^{2+} affinity chromatography and from *in vitro* refolding.

Fig. 5.2. Purification, refolding and separation of monomeric N-TIMP-3 (A) Bovine serum albumin (BSA) in the range of 1 to 12 µg (lane 1 to 5), a 20 µl aliquot of inclusion body extract (lane 6) and 5 µl of 2 ml fractions (lane 13 to 35) were electrophoresed on a 15 % polyacrylamide gel and Coomassie Brilliant Blue stained to visualise the proteins as described in Materials and Methods. **(B)** A 20 ml aliquot of refolded N-TIMP-3 was applied to Sephacryl S-200 equilibrated and eluted with TNC buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM CaCl$_2$). The eluted material was collected as 5 ml fractions. A 50 µl aliquot of each fraction was electrophoresed on a gradient gel and proteins visualised with silver stain.

Titrating 20 nM MMP1 wth N-TIMP-3

Calculations to determine the active N-TIMP-3 concentration

1. **10 μl of N-TIMP-3 completely inhibits 20 nM MMP1ΔC in 200 μl.**
2. **20 nM MMP1 in 200 μl assay volume equates to 4×10^{-3} nmoles of MMP1.**
3. **Assuming 1:1 ratio of MMP1ΔC to N-TIMP-3, 4×10^{-3} nmoles of N-TIMP-3 are present in 10 μl of N-TIMP-3.**
4. **Therefore the [N-TIMP-3] ~ 360 nM.**

Fig 5.3. Titrating 20 nM MMP1ΔC with increasing volume of N-TIMP-3.
MMP1ΔC (20 nM) in a 200 μl reaction volume was titrated with increasing volume of purified N-TIMP-3. The residual MMP1ΔC activity was measured with fluorescent substrate as described in materials and methods. The line was extrapolated on to horizontal axis to show the volume of N-TIMP-3 inhibiting 20 nM MMP1ΔC. The information was used to determine the concentration of active N-TIMP-3.

5.4   Covalent coupling of refolded N-TIMP-3 protein

In order to isolate the synovial aggrecanolytic enzyme by an affinity step, the N-TIMP-3 protein needed to be coupled to a solid support. Cyanogen bromide (CNBr) and N-hydroxysuccinimide (NHS) activated agarose beads are commonly used matrices to covalently couple proteins. The N-TIMP-3 protein was purified in buffer containing 20 % (v/v) glycerol. The glycerol concentration was high to prevent the aggregation of N-TIMP-3. In order to immobilise N-TIMP-3, the Tris present in the purification buffer was removed because it contains primary amino groups that can interact with the activated agarose beads. The N-TIMP-3 preparation (300 μg/ml) was dialysed into bicarbonate buffer pH 8.0 containing 20% (v/v) glycerol. The dialysed N-TIMP-3 was incubated with 1 ml of CNBr-activated agarose beads for 6 hours and absorbance at 280 nm (A_{280}) was measured to estimate the concentration of protein left in the supernatant. Less than 10 % of N-TIMP-3 had coupled to CNBr-activated agarose matrix after 2 hours (Fig. 5.4). The covalent coupling of N-TIMP-3 to agarose beads is a time limited reaction, which should be complete within an hour. Therefore increasing the incubation time of CNBr activated agarose beads with N-TIMP-3 didn't increase coupling (data not shown). There are many factors that can reduce the coupling efficiency of N-TIMP-3 namely pH, coupling medium etc. The pH was kept at 8.0 to maintain the amino groups in unprotonated state and thus aiding the formation of covalent bonds with the agarose beads. However, the viscous 20% (v/v) glycerol might hinder the coupling. In order to investigate this possibility, BSA was dissolved in the bicarbonate buffer at 300 μg/ml, either with or without 20% glycerol. The BSA preparations were then coupled to 1 ml of activated CNBr agarose beads and A_{280} was taken at regular intervals to estimate the concentration of BSA left in the supernatant (Fig. 5.5A). Approximately 80 % of BSA was coupled to agarose beads when no glycerol was present in the buffer compared to only 15 % in the presence of 20% glycerol. Hence the presence of 20 % glycerol led to significant impairment of coupling.

N-TIMP-3 coupling to CNBr beads
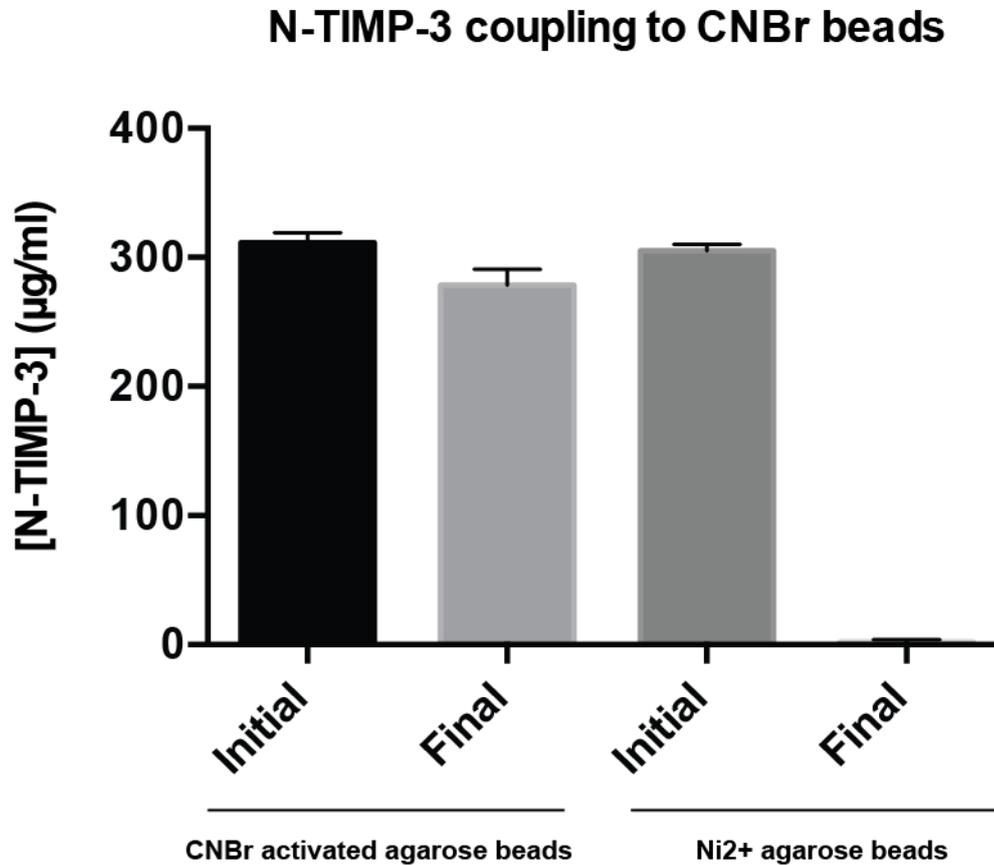
Fig 5.4. Covalent coupling of N-TIMP-3 to CNBr activated agarose beads. N-TIMP-3 (~300 μg/ml) was dialysed into bicarbonate buffer containing 20% glycerol and incubated with 1 ml of CNBr activated beads for 2 hours. Another preparation of N-TIMP-3 (~300 μg/ml) in bicarbonate buffer was incubated with Ni^{2+} agarose beads for 2 hours. The agarose beads were pelleted with gentle centrifugation and the supernatant collected from both incubations of N-TIMP-3. The concentration of N-TIMP-3 was determined by DC protein assay before and after incubation with agarose beads. n = 1, the error bars represent the standard deviation for the triplicates of one experiment.

A

B
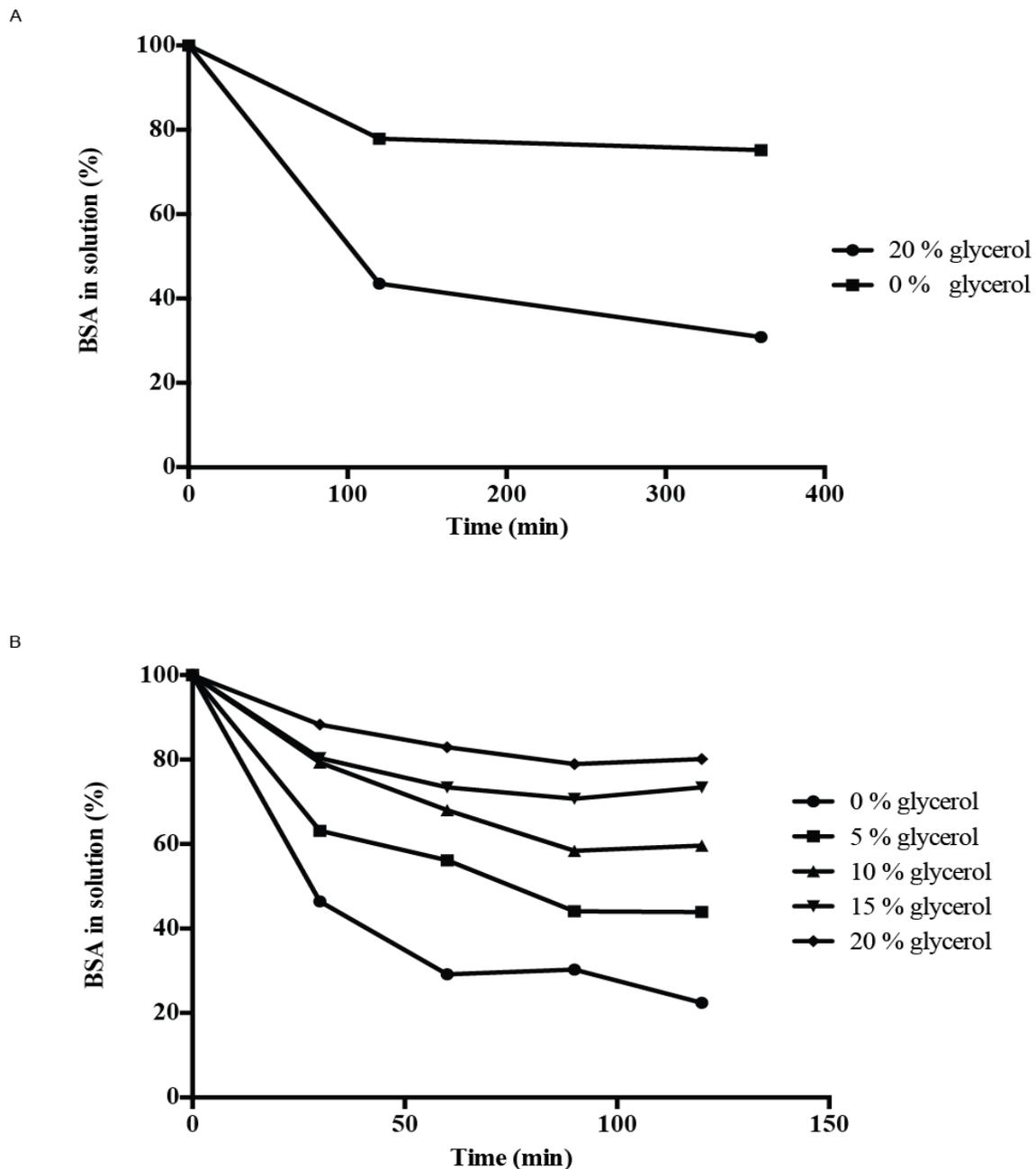
Fig 5.5. Covalent coupling of BSA to CNBr activated beads. (A) BSA (300 μg) was prepared in bicarbonate buffer pH 8.0 containing 20 % and 0 % glycerol. Both preparations were incubated with 1 ml of washed CNBr activated beads. Concentration of BSA remaining in the solution was determined at three time points (0, 2 and 6 hours) using *DC* protein assay. The percentage decrease in BSA concentration was plotted on y-axis against time (min). (B) Five different solutions of BSA (300 μg) in bicarbonate buffer pH 8.0 each containing different glycerol concentration (0%, 5%, 10%, 20%) were prepared. Both preparations were incubated with 1 ml of washed CNBr activated beads. Concentration of BSA remaining in the solution was determined at regular time points (0, 0.5, 1.0, 1.5 and 2.0 hours) using *DC* protein assay. The percentage decrease in BSA concentration was plotted on y-axis against time (min).

It was also not possible to completely remove the glycerol as this caused ~ 90% of the N-TIMP-3 to precipitate. It was decided to investigate whether or not there was a minimum glycerol concentration that keeps N-TIMP-3 in solution but does not hinder its immobilization. The coupling of BSA to CNBr was assessed at increasing glycerol concentrations up to 20% glycerol. Fig 5.5B shows that the coupling of BSA to agarose beads decreased with increasing glycerol concentrations. Even 5% glycerol impaired coupling of BSA to agarose beads. Unfortunately reducing N-TIMP-3 concentration below 20 % resulted in more or less complete N-TIMP-3 precipitation. Similar attempts were made to couple N-TIMP-3 to NHS-activated beads but these were similarly unsuccessful (data not shown). Therefore the covalent coupling of N-TIMP-3 was unsuccessful with activated agarose beads and other ways to immobilize N-TIMP-3 were considered.

5.5 Coupling N-TIMP-3 to Ni^{2+} agarose beads

Given the poor covalent coupling of N-TIMP-3 to either CNBr or NHS activated agarose beads, other approaches to tether N-TIMP-3 to a solid support were considered. The N-TIMP-3 protein was purified from *E.coli* lysate by exploiting the electrostatic attraction between the histidine tag of N-TIMP-3 and Ni^{2+} agarose beads. Therefore it was decided to exploit the affinity of the His tag for Ni^{2+} beads to create an affinity reagent for the isolation of the aggrecanolytic enzyme. It was important to see whether Ni^{2+} N-TIMP-3 beads interacted with proteinase and could be used to isolate the aggrecan-degrading enzyme. The beads were therefore tested for their ability to bind MMP1ΔC. A 50 μl aliquot of N-TIMP-3 (15 μg) was immobilized on agarose beads (Fig 5.6, lane 1). No leakage of N-TIMP-3 was observed upon washing the beads with Tris buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM CaCl$_2$) (Fig. 5.5, lane 2 to 5). MMP1ΔC (5 μg) was incubated with Ni^{2+} N-TIMP-3 agarose beads for 2 hours at 25 °C. Minor leakage of N-TIMP-3 was observed upon washing the agarose beads. However no other protein band was observed. The washed beads were then eluted with a buffer containing 0.5 M imidazole. Two prominent proteins bands of molecular weight 15 kDa (N-TIMP-3) and 20 kDa (MMP1ΔC) were observed (Fig. 5.6, lane 10). The elution of MMP1ΔC along with N-TIMP-3 suggests that N-TIMP-3 coupled Ni^{2+} agarose beads can be used successfully for the affinity purification of MMP1ΔC. The enzyme-inhibitor ratio used in this experiment was 3:1 and a similar procedure could also be used to isolate the aggrecan-degrading enzyme from the SYCM. However MMP1ΔC does not exactly mimic the aggrecanolytic enzyme because its molecular weight is half that of the predicted molecular weight of synovial aggrecanase. Therefore the N-TIMP-3 Ni^{2+} agarose beads were tested for their ability to purify recombinant catalytic domain of ADAMTS-5 (~ 75 kDa). A 1.5 μg quantity of ADAMTS-5 was incubated with the N-TIMP-3-agarose beads. The beads were eluted with a buffer containing 0.5 M imidazole. The eluted material contained a strong

protein band of MW 75 kDa indicating that affinity pull down of ADAMTS-5 was successful (Fig. 5.7 lane 6) Monomeric and dimeric forms of N-TIMP-3 were also observed in the eluted material. A dimeric form of N-TIMP-3 (~30 kDa) was observed because partially purified N-TIMP-3 (refolded N-TIMP-3 without the separation of multimers by gel filtration) was used for the purpose of affinity pull down. Impurities around 60 kDa were also observed which could possibly be present either in Ni^{2+} agarose beads or ADAMTS-5 preparation. Given the successful purification of MMP1ΔC and ADAMTS-5, it was decided to use the same coupling and affinity purification procedure to isolate the synovial aggrecanase.

Silver stain

Legend

1 -   N-TIMP-3 (50 μl)

2-5   Washes   (1 ml)

6   N-TIMP-3 (50 μl) + MMP1ΔC (5 μg)

7   Flow through

8-9   Washes (1 ml)   N-TIMP-3
                                        agarose beads

10   Eluted material (200 μl)

11-   Flow through

12-13 Washes (1 ml)   agarose beads

14   Eluted material (200 μl)

15   MMP1ΔC (5 μg)
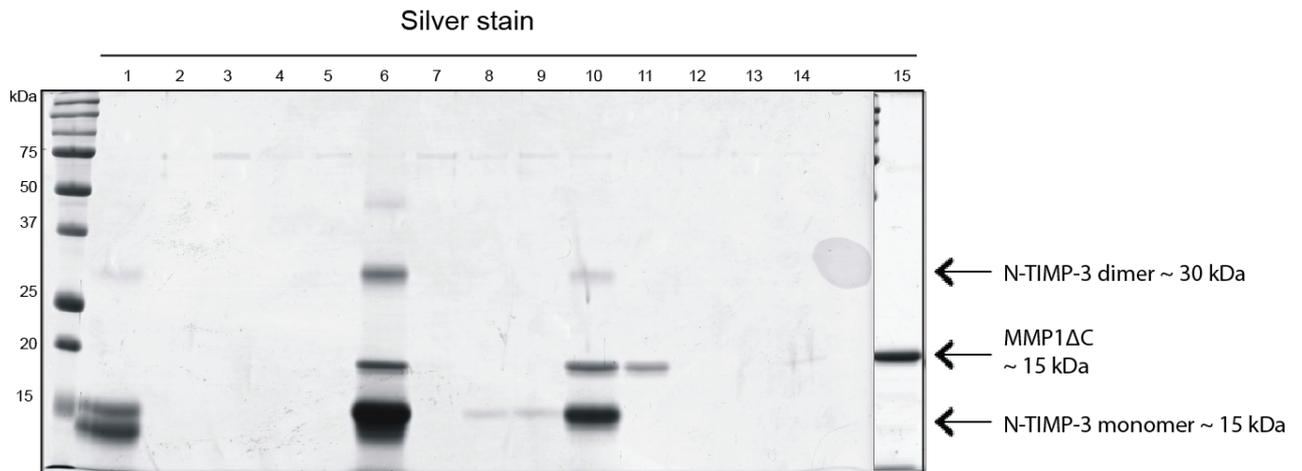
Fig 5.6. Validating N-TIMP-3 agarose beads as a tool for affinity purification.
A 50 μl aliquot of N-TIMP-3 (300 μg/ml, lane 1) was added to 50 μl Ni^{2+} agarose beads and left on gentle shaking for 30 min at 25 $^{\circ}$C. The agarose beads were washed three times with 1 ml of TNC buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM CaCl$_2$) (lane 2-5). A 5 μg quantity of MMP1ΔC (lane 15) was added to N-TIMP-3-agarose beads for 2 hours at 25 $^{\circ}$C. The beads were pelleted on gentle centrifugation (1000 g, 30 s) and supernatant collected (lane 7). The agarose beads were further washed twice with TNC buffer (lane 8 & 9) and eluted with TNC buffer containing 0.5 M imidazole (lane 10). A 5 μg quantity of MMP1ΔC was added also added to Ni^{2+}agarose beads without N-TIMP-3 for 2 hours at 25 $^{\circ}$C. The beads were pelleted on gentle centrifugation (1000 g, 30 s) and supernatant collected (lane 11). The agarose beads were further washed twice with TNC buffer (lane 12 & 13) and eluted with TNC buffer containing 0.5 M imidazole (lane 14) A mixture of two proteins, 5 μg of MMP1ΔC and N-TIMP-3 (50 μl), was run as a positive control (lane 6).

Silver stain
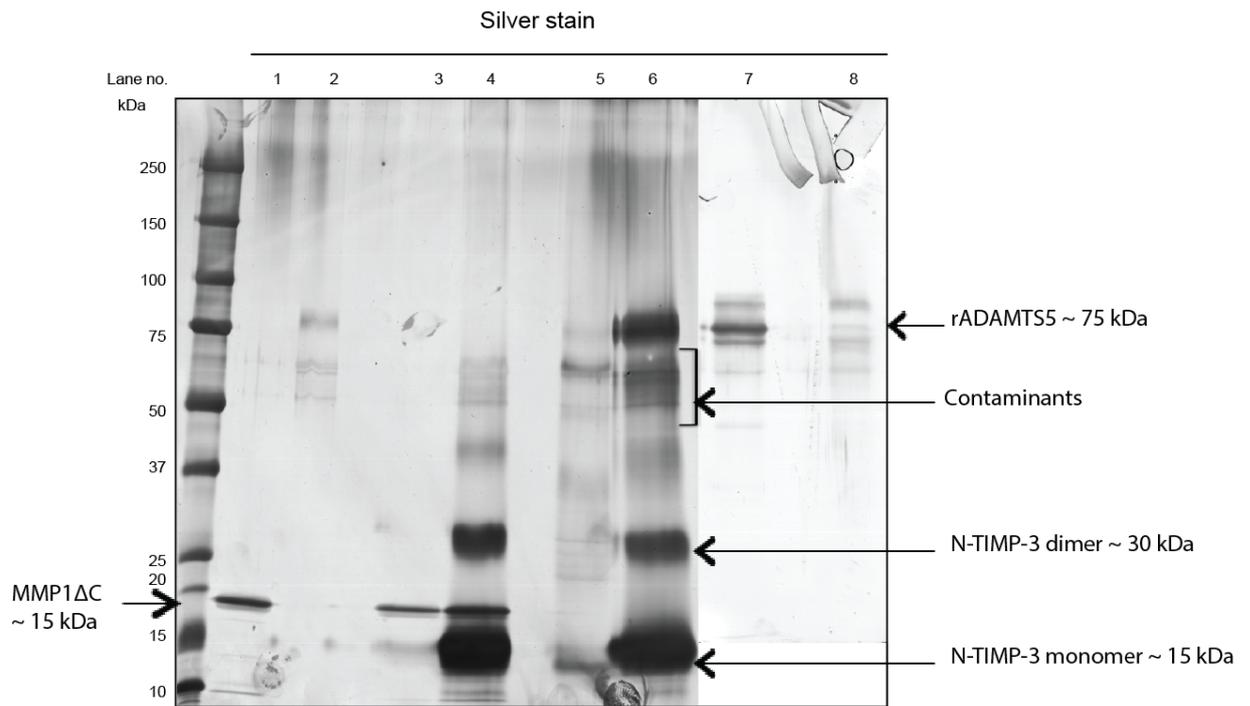
Legend

1-   MMP1ΔC (5 μl)

2-   ADAMTS-5 (2 μl)

3-   MMP1ΔC (2 μl)

4-   N-TIMP-3 (50 μl) + MMP1ΔC (5 μg)

5-   Flow through   } N-TIMP-3 agarose beads

6-   Eluted material

7-   Flowthrough   } agarose beads

8   Eluted material

Fig 5.7. Testing N-TIMP-3 agarose beads for purification of ADAMTS-5.
A 50 μl aliquot of N-TIMP-3 (300 μg/ml) was added to 50 μl Ni^{2+} agarose beads and left on gentle shaking for 30 min at 25 ^{o}C. The agarose beads were washed three times with 1 ml of TNC buffer. A 25 μl aliquot of ADAMTS-5 (480 nM) was incubated with N-TIMP-3-agarose beads for 2 hours at 25 ^{o}C. The agarose beads were pelleted with gentle centrifugation (1000 g, 30 sec) and supernatant collected (lane 5). The agarose beads were eluted with TNC buffer containing 0.5 M imidazole (lane 6). A 25 μl quantity of MMP1ΔC was also added to Ni^{2+}agarose beads without N-TIMP-3 for 2 hours at 25 ^{o}C. The beads were pelleted on gentle centrifugation (1000 g, 30 s) and supernatant collected (lane 7). The agarose beads were eluted with TNC buffer containing 0.5 M imidazole (lane 8). A mixture of two proteins, 5 μg of MMP1ΔC and N-TIMP-3 (50 μl), was run as a positive control in lane 4.

Chapter 6

Affinity purification of synovial aggrecanase

6.1 Introduction

The plan was to adapt the procedure used to purify MMP1ΔC and ADAMTS-5 in Chapter 5 to isolate the synovial aggrecanase. A three-step purification scheme for the low molecular weight form of the enzyme was worked out in Chapter 4. The aim was to use the active fraction from the third step (SMART mono Q anion exchange chromatography) and affinity purify the enzyme on N-TIMP-3 bound Ni^{2+} agarose beads. The proteins bound would then be eluted with imidazole and electrophoresed. The proteins bound specifically to N-TIMP-3 would be selected for identification by mass spectrometry.

6.2   Analysing fraction 8 of mono Q chromatography

The lower molecular weight aggrecanase from SYCM was purified by successive steps as worked out in Chapter 4: firstly chromatography on cation exchange column, then gel filtration on Sephacryl S-200 and thirdly anion exchange chromatography. This third step on a high resolution SMART column concentrated the 40 kDa aggrecanolytic species in a 100 μl fraction (Fig. 6.1B fraction 8). More than 90% of the ARGS generating activity was found in fraction 8 when measured in either the assay on bovine aggrecan or the aggrecanase ELISA (Fig. 6.1B; Fig.6.1A and 6.2B are duplication of Fig.4.12). Fraction 8 was titrated with N-TIMP-3 in the aggrecanase ELISA to estimate the quantity of enzyme. This information was needed to determine whether sufficient enzyme was present in fraction 8 for detection by mass spectrometry. The titration revealed that 6 μl of 7.2 nM N-TIMP-3 fully inhibited the activity of 0.25 μl of fraction 8 (Fig. 6.2). Assuming N-TIMP-3 inhibited the synovial aggrecanase in 1:1 stiochiometric ratio, and a molecular weight of 40 kDa for the enzyme, 1 μl of fraction 8 was calculated to contain 6.9 ng of the enzyme, and the whole 100 μl fraction 690 ng.

The elution profile (A_{280}) showed an intense absorbance peak in fraction 8 (Fig. 6.1A). Aliquots (5 μl) of the fractions were electrophoresed and the gel stained with silver (Fig. 6.1C). Fraction 8 (Fig. 6.1C, lane 3) showed multiple protein bands ranging from molecular weight 60 kDa to 25 kDa. The most prominent protein band (band 1) was of molecular weight ~ 45 kDa but a band of similar molecular weight and intensity was also present in the subsequent fractions 9 to 11 (Fig. 6.1C lanes 4-6). A doublet (band 2) with approximate molecular weight 40 kDa was seen below band 1 (Fig. 6.1C lane 3) but a similar doublet was also observed in fractions 10 and 11 (Fig. 6.1C lane 5 & 6). Therefore the protein bands 1 and 2 were unlikely to be synovial aggrecanase. However 3 unique protein bands with molecular weight ~ 37 kDa (band 3), ~ 35 kDa (band 4), and 25 kDa (band 5) were seen in fraction 8. One of these proteins might be a synovial aggrecanase (Fig. 6.1C lane 3).
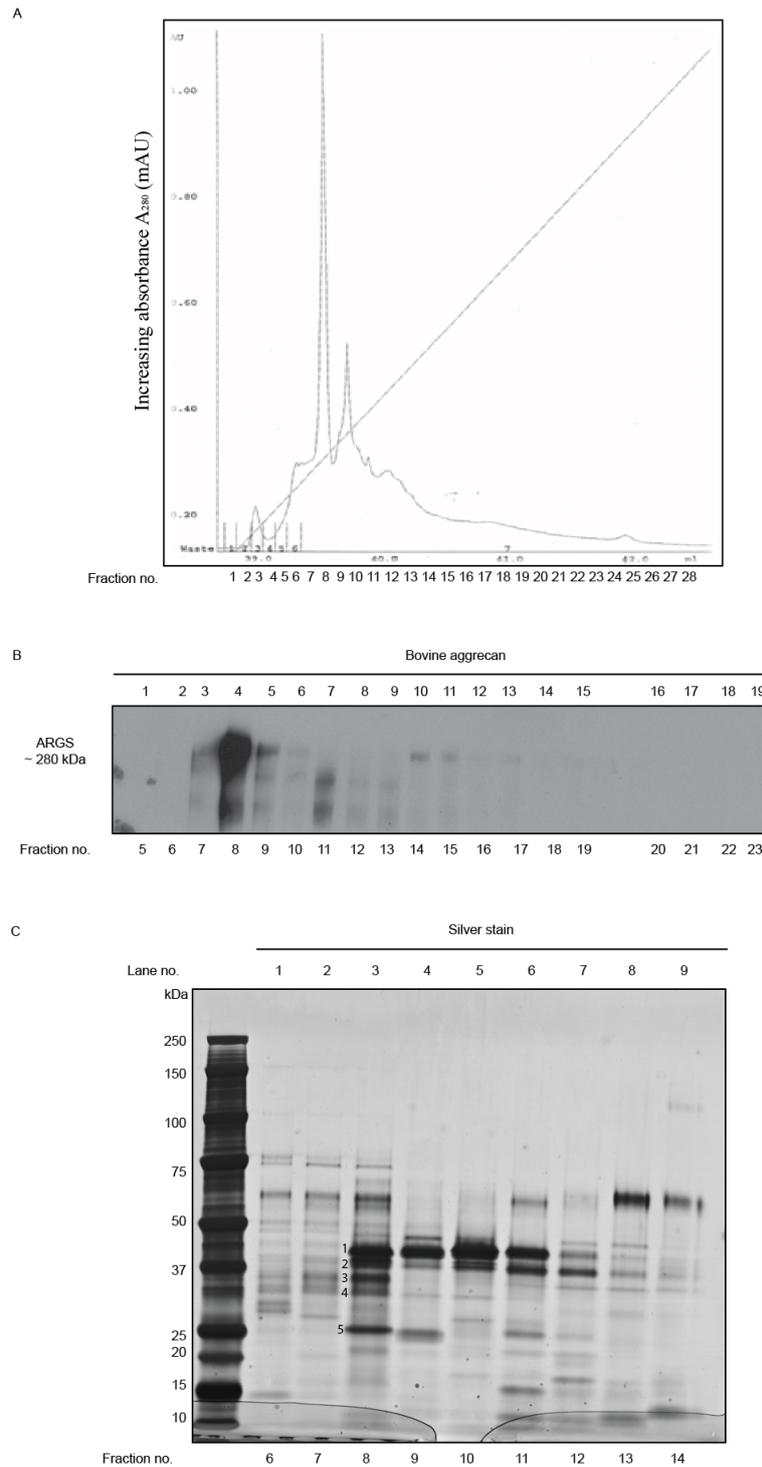
Fig. 6.1. Mono Q (SMART) chromatography of active material from size-exclusion column (Sephacyl S-200) A 60 ml aliquot of SYCM was used to partially purify synovial aggrecanase on a 1 ml HiTrap S column. The fractions showing aggrecan-degrading activity were pooled and concentrated to 5 ml using 3.5 kDa spin concentrator and applied to Sephacryl S-200 column (10 × 300 mm) that was equilibrated with a buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM CaCl$_2$). The enzymatically active fractions for low molecular species (~ 40 kDa) were pooled. A 25 ml aliquot was applied to mono Q column (100 µl) equilibrated with Tris buffer without NaCl (20 mM Tris-HCl pH 8.0, 10 mM CaCl$_2$) and the unabsorbed material was collected as flow through (A) A$_{280}$ trace of the material eluted from the mono Q column (B) Aliquots (5 µl) of the fractions were incubated with bovine aggrecan (50 µg) for 24 hours at 37 °C. The medium was probed for ARGS neo-epitope bearing fragment as described in Materials and Methods. (C) Aliquots (5 µl) were electrophoresed on a gradient gel (4-12 %) and protein stained with silver as described in Materials and Methods.

124
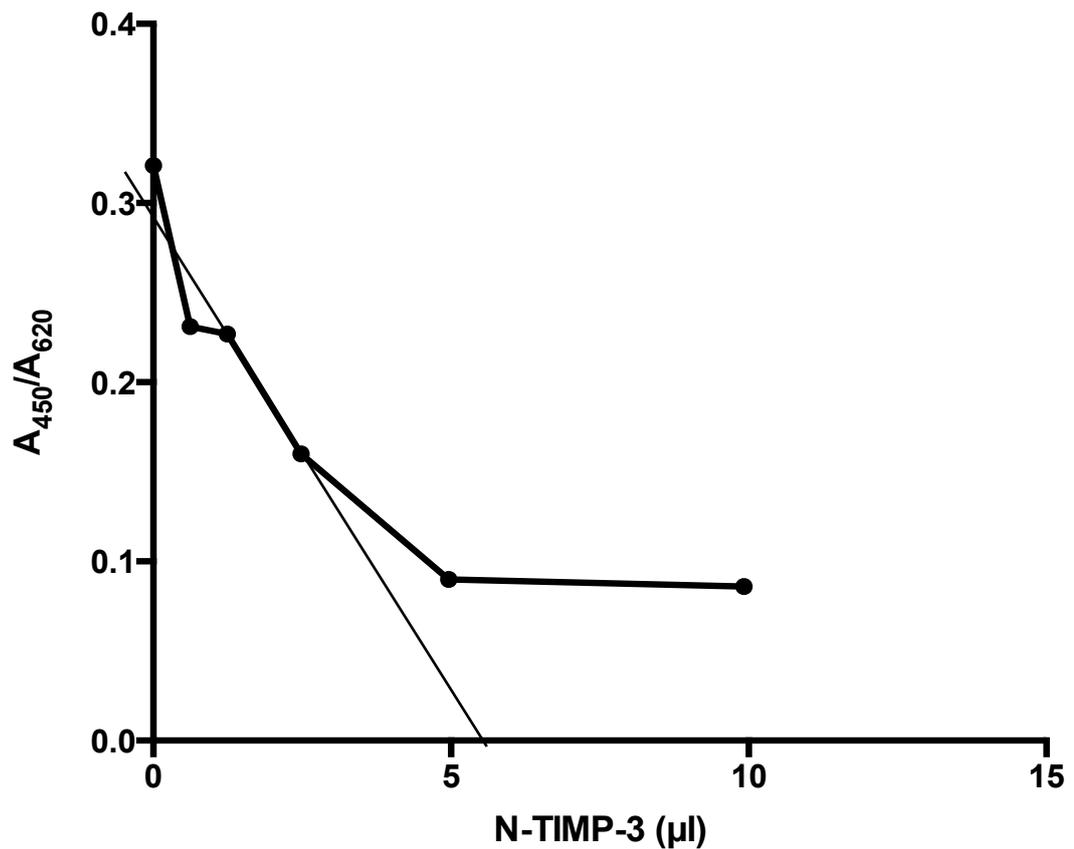
Fig 6.2. Estimating the amount of synovial aggrecanase in fraction 8 from SMART mono Q chromatography by aggrecanase ELISA. A 0.25 μl aliquot of fraction 8 was titrated with increasing volumes of 7.2 nM N-TIMP-3 (0 to 10 μl) in a 50 μl reaction volume. The incubations were left for 30 min at 37 °C. The materials were then applied to aggrecan IGD for 15 min at 37 °C. The release of ARGS bearing peptide in the medium was measured as described in Materials and methods.

6.3 Affinity purification of synovial aggrecanase

Initial pull downs of MMP1ΔC and ADAMTS-5 were carried out with a 50 μl aliquot of Ni^{2+} agarose beads (Chapter 5). A trial pull down with fraction 10 from the mono Q chromatography (Fig. 6.1) was carried out using the same volume of Ni^{2+} agarose beads as described in Chapter 5. A number of proteins were present in the eluted material suggesting that Ni^{2+} agarose beads may bind to some proteins in the fraction (data not shown). Ni^{2+} agarose beads have a very high binding capacity for His tagged protein (600 μg for 1 ml of agarose beads) so it was decided to bind a smaller aliquot of Ni^{2+} agarose beads (15 μl) with 100 μl of N-TIMP-3 ($\sim$ 2 μg) to reduce the presence of non-specifically bound proteins. The remaining 40 μl of fraction 8 was then applied to these beads, 60 μl having been used for enzyme assays and electrophoresis. A parallel pull down was carried out by applying 40 μl of fraction 9 (Fig. 6.1C, lane 4) to a 15 μl aliquot of Ni^{2+} agarose beads. It was hoped a comparison of proteins eluted from fraction 8 and fraction 9 pull downs would help distinguish those binding specifically to N-TIMP-3 from those binding to the Ni^{2+} agarose alone. MMP1ΔC (5 μg) was also incubated with another batch of N-TIMP-3 Ni^{2+} agarose beads (15 μl) as a positive control.

The beads from all three incubations were gently pelleted and the supernatants collected. The supernatant of fraction 8 was assessed for activity using aggrecanase ELISA. More than 95 % of the enzymatic activity was removed of fraction 8 by the N-TIMP-3 agarose beads (Fig. 6.3). The elution of agarose beads was carried out in a buffer containing 0.5 M imidazole. The eluted materials of fraction 8, fraction 9 and the MMP1ΔC pull down were electrophoresed in lanes 1, 2 and 3 respectively of a gradient gel and proteins were visualized by silver stain (Fig. 6.4A, lanes 1, 2 and 3). Fig. 6.4A lane 1 shows that MMP1ΔC pull down was successful as the eluted material contained both MMP1ΔC (20 kDa) and N-TIMP-3 (15 kDa) proteins.

Aggrecanolytic activity of fraction 8
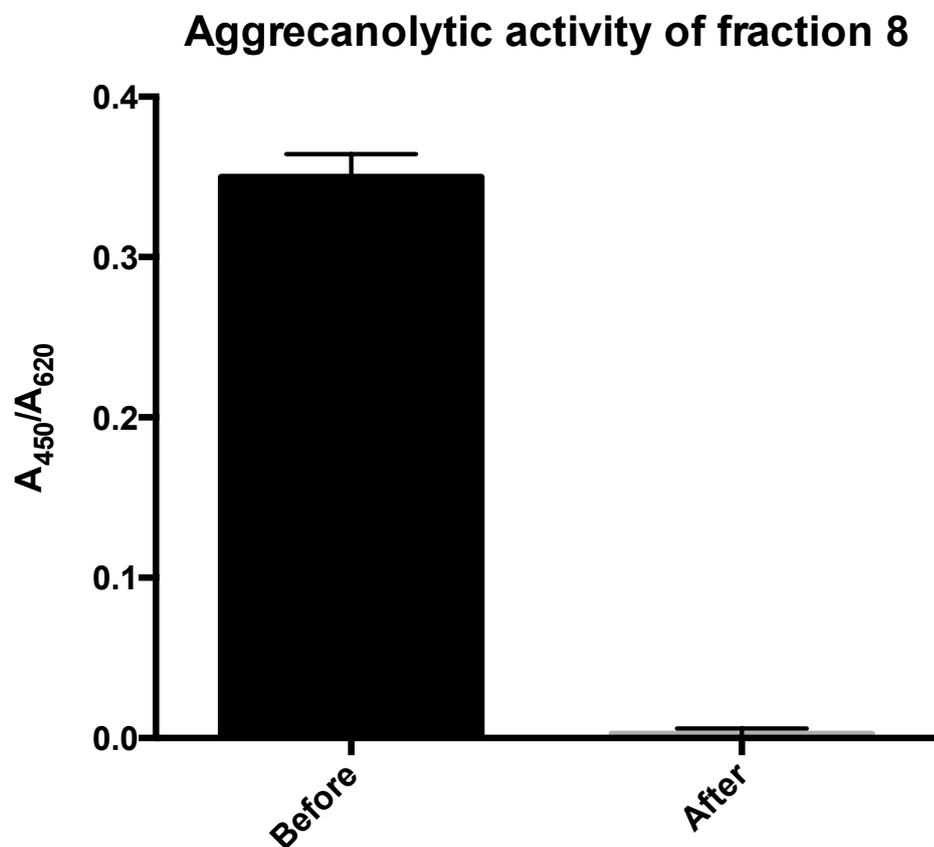
Fig 6.3. Aggrecan-degrading activity of fraction 8 measured on aggrecanase ELISA before and after incubation with N-TIMP-3 coupled agarose beads. The fraction 8 from mono Q chromatography was incubated with aggrecan-IGD at 1:200 dilution for 15 min at 37 °C. The ARGS bearing peptide was quantified using aggrecanase ELISA as described in Materials and Methods. n= 1, error bar represents the standard deviation of the triplicates for one experiment.

The eluted material of fraction 8 pull down contained 4 protein bands (Fig. 6.4A lane 2, bands 1-4). The majority of the proteins in fraction 8 were not bound by N-TIMP-3 coupled Ni^{2+} agarose beads and were collected as supernatant (Fig. 6.4A, lane 5). Lane 3 shows proteins from fraction 9, which bound to Ni^{2+} agarose beads in the absence of TIMP-3. However the majority of fraction 9 proteins did not bind and were found in the supernatant (Fig. 6.4A, lane 6). The protein with molecular weight 15 kDa in lane 2 (band 4) represented monomeric N-TIMP-3 (15 kDa). This band was absent in the eluted material from the pull down carried out with fraction 9 (Fig. 6.4A, lane 3). Another protein band (band 3) with a molecular weight of 30 kDa was also seen in lane 2 which may represent the dimeric form of N-TIMP-3 (Fig. 6.4A lane 2). A strong band (band 2) that appeared to be unique to lane 2 had a molecular weight of ~ 35 kDa (Fig. 6.4A lane 2). This band was absent from lane 1 indicating that it originated from fraction 8 (Fig. 6.4B) and was not a multimer of N-TIMP-3 protein. However a fainter band of similar molecular weight was also observed in lane 3 suggesting that the band 2 protein may represent a non-specific interaction of the protein with Ni^{2+} agarose beads. Protein bands in the region of 50-60 kDa (Band 1) were observed in all three lanes 1, 2 and 3. It was felt these may represent contamination by keratins (50-60 kDa) that could be present either in the sample buffer used to solubilize the eluted material for gel electrophoresis or in the buffer used to resuspend the Ni^{2+} agarose beads. However band 1 in lane 2 was stronger than lane 1 or lane 3. Perhaps there were more keratins in fraction 8 or it may represent an interaction of 50-60 kDa protein with N-TIMP-3. The silver stained gel under the light box didn't suggest the appearance of any discrete bands in the region of 50-60 kDa. Therefore it was decided not to select this region for mass spectrometric analysis. The prominent appearance of band 2 (predicted molecular weight of the synovial aggrecanase) made it an interesting candidate for identification by mass spectrometry. A protein band with similar molecular weight (marked with B) was also located in the silver stained proteins of fraction 8 and selected for identification by mass spectrometry (Fig. 6.4B lane 3, Fig. 6.4B is a

duplication of Fig.6.1C). Altogether 4 protein bands were selected for analysis by mass spectrometry as shown in Fig. 6.4.
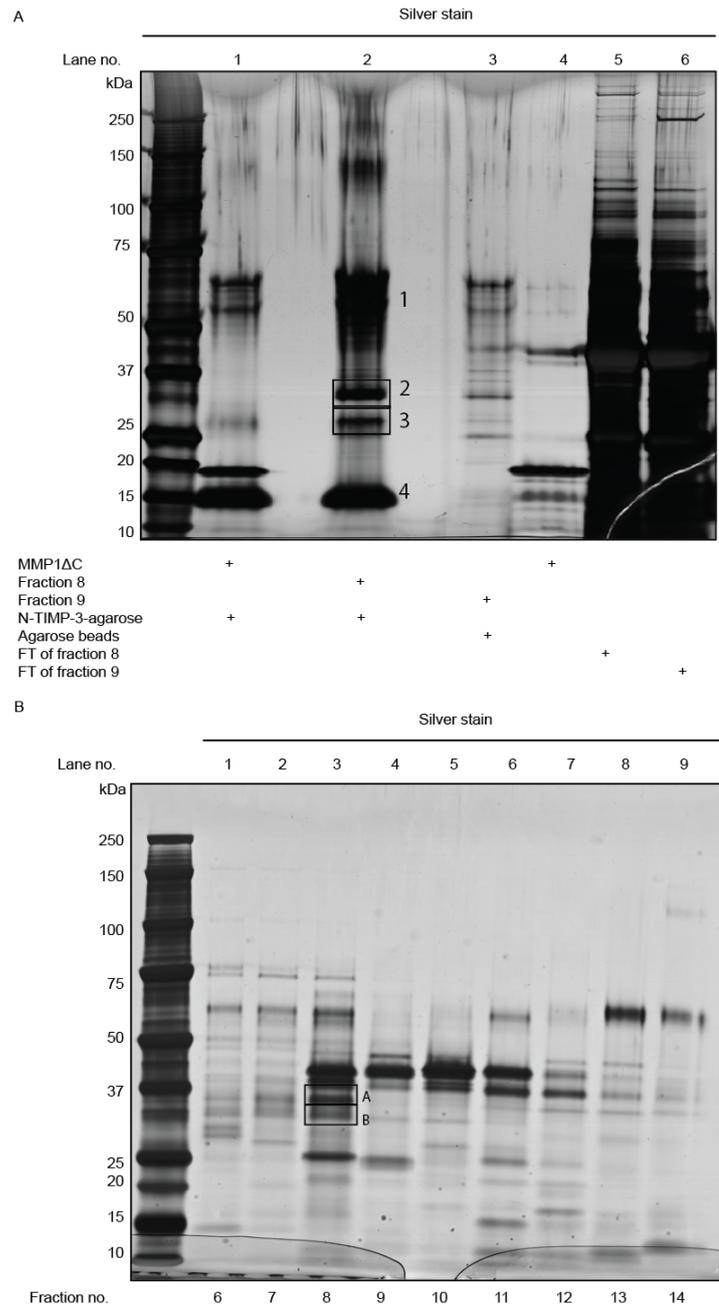
Fig. 6.4 Use of TIMP-3 to affinity purify synovial aggrecanase. (A) A 15 μl aliquot of Ni^{2+} agarose beads (~ 10 μg binding capacity) was incubated with 100 μl of N-TIMP-3 protein (2 μg active N-TIMP-3 protein) for 30 min at 25 °C. Aliquots of N-TIMP-3 Ni^{2+} agarose beads (15 μl) were incubated separately with fraction 8 (40 μl) and MMP1ΔC (5 μg) in separate ependorfs while Ni^{2+} agarose beads were incubated with fraction 9 for 2 hours at 25 °C. The beads were gently pelleted and supernatants collected. The beads were then washed three times with buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM $CaCl_2$). The beads were eluted with buffer containing 0.5 M imidazole. The eluates of MMP1ΔC, fraction 8 and fraction 9 pull downs were loaded in lanes 1, 2 & 3 respectively and electrophoresed on a 4-12% polyacrylamide gradient gel. The supernatants from fraction 8 and 9 pull downs were loaded in lane 5 and 6 respectively. The gel was stained with silver (B) Aliquots (5 μl) of fractions obtained after mono Q anion exchange chromatography (Fig. 6.2A) were electrophoresed and stained as for (A). Bands with boxes indicates the bands selected for mass spectrometry analysis.

6.4 Mass spectrometric analysis of protein bands

Three major proteins were identified in band 2 of the N-TIMP-3 pull down of the active fraction (Fig.6.4A, lane 2): annexin A1 & A2, carboxypeptidase B2 and TIMP-3. Annexins A1 and A2 were by far the most abundant proteins in band 2 as indicated by a very high Mascot scores (annexin A1: 1935 and annexin A2: 1495) and more than 15 distinct sequence matches, seq(sig) (Fig. 6.5B). The Mascot score indicates how well the experimental data (peptide sequences) match the database sequence. The Mascot score for a protein is the summed score for the individual peptides (peptide masses for all peptides) matching a given protein. A very high Mascot score for annexins A1 and A2 indicate strong confidence in the matching of the peptides arising from tryptic cleavages of these proteins against the database sequence of annexin A1 and A2. Another measure known as emPAI, which offers relative quantitation of the protein in a mixture based on protein coverage by peptide matches, was also very high for annexins A1 (7.38) and A2 (4.51) (Fig. 6.5B). Only one metallopeptidase was observed in band 2 namely carboxypeptidase B2 (CBP2). However, the sequence coverage for carboxypeptidase B2 was very low, only two distinct sequence matches (Fig. 6.5B), along with low Mascot score and emPAI number. TIMP-3 was another protein identified in band 1 with 2 distinct peptide sequence matches albeit with low Mascot score (63) and sequence matches (2) (Fig. 6.5B). TIMP-3 was also the only significant protein identified in band 3 (Fig. 6.5C). No peptides matching ADAMTS enzyme were found in either band 2 or band 3.
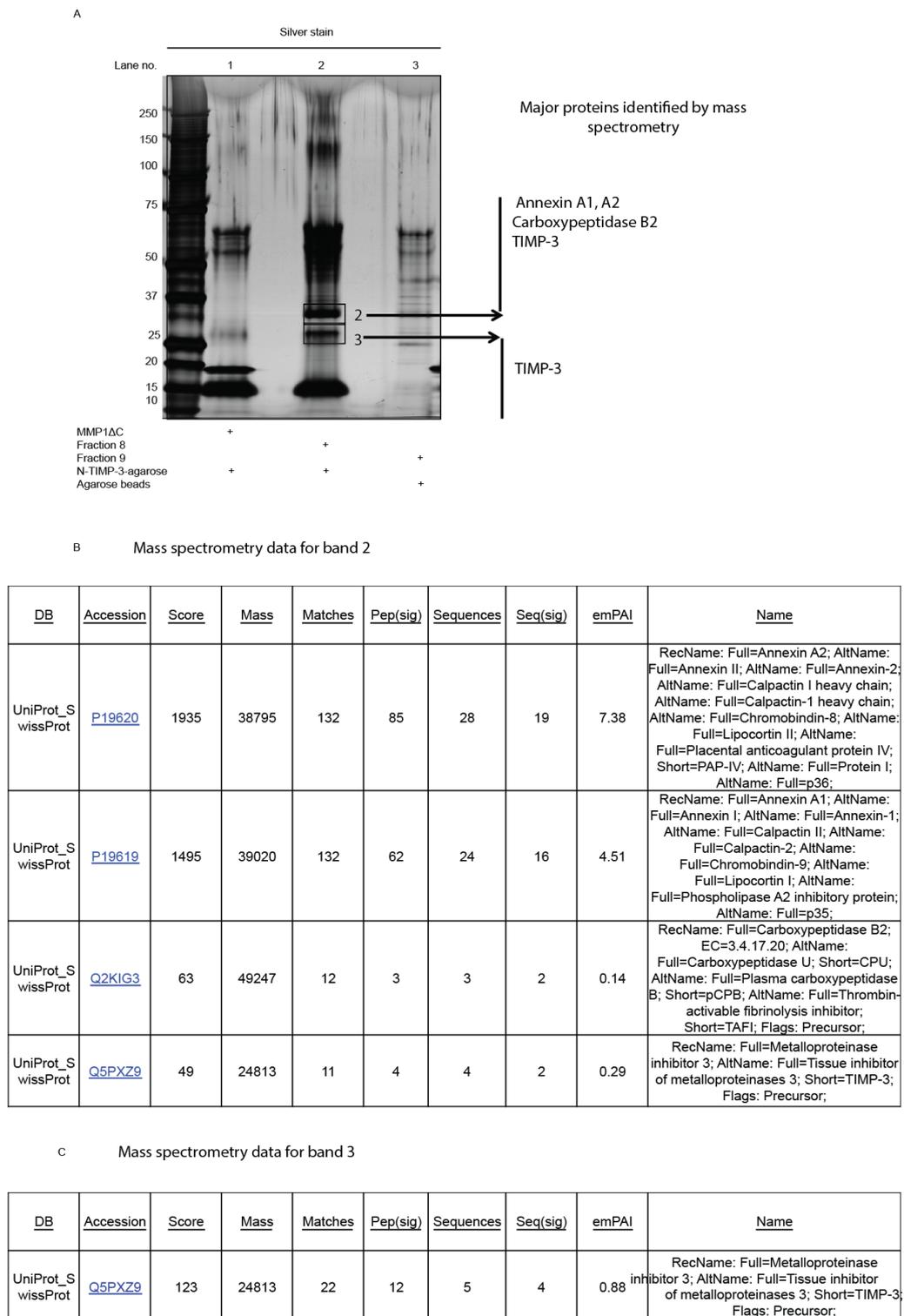
Fig. 6.5 Mass spectrometric analysis of selected protein bands. (A) The bands 2 and 3 were excised using scalpel. The processing of gel discs and mass spectrometric identification of proteins was carried in the proteomics laboratory of Prof. Benedikt Kessler, University of Oxford. The list of major proteins identified through database searching from the peptide sequences obtained through MS analysis of band 1 (A) and band 2 (C).

The electrophoresis gel of fraction 8 of mono Q chromatography (Fig. 6.4B) was also analysed by mass spectrometry. Two bands, A & B, were cut from the 37 kDa region (Fig.6.6, lane 3). Band B was thought to correspond to the band 2 of the N-TIMP-3 pull down (Fig. 6.4A, lane 2) and band A contained a prominent protein that apparently did not bind to N-TIMP-3. Annexins A1 and A2 were the two most abundant proteins in both bands A and B as indicated by a very high Mascot score, peptide sequence matches and emPAI scores (Fig. 6.6B). Methionine aminopeptidase 1 (MAP 1) was the only proteinase identified in both bands A and B with relatively high Mascot score (Fig. 6.6B). Another proteinase, carboxypeptidase B2 was also found in band B and was the only common proteinase between band 2 and band B (Fig. 6.5B vs. Fig. 6.6B). No ADAMTS enzyme was identified in any of the 4 protein bands analyzed by mass spectrometry.

A

Silver stain

B   Mass spectrometry data for band A

| DB	Accession	Score	Mass	Matches	Pep(sig)	Sequences	Seq(sig)	emPAI	Name
UniProt_SwissProt	P19620	1126	38795	78	59	22	16	4.13	RecName: Full=Annexin A2; AltName: Full=Annexin II; AltName: Full=Annexin-2; AltName: Full=Calpactin I heavy chain; AltName: Full=Calpactin-1 heavy chain; AltName: Full=Chromobindin-8; AltName: Full=Lipocortin II; AltName: Full=Placental anticoagulant protein IV; Short=PAP-IV; AltName: Full=Protein I; AltName: Full=p36;
UniProt_SwissProt	P19619	543	39020	27	21	10	9	1.25	RecName: Full=Annexin A1; AltName: Full=Annexin I; AltName: Full=Annexin-1; AltName: Full=Calpactin II; AltName: Full=Calpactin-2; AltName: Full=Chromobindin-9; AltName: Full=Lipocortin I; AltName: Full=Phospholipase A2 inhibitory protein; AltName: Full=p35;
UniProt_SwissProt	Q5RBF3	279	44114	19	13	5	5	0.54	RecName: Full=Methionine aminopeptidase 1; Short=MAP 1; Short=MetAP 1; EC=3.4.11.18; AltName: Full=Peptidase M 1;
UniProt_SwissProt	Q15113	74	48797	8	3	3	2	0.14	RecName: Full=Procollagen C-endopeptidase enhancer 1; AltName: Full=Procollagen COOH-terminal proteinase enhancer 1; Short=PCPE-1; Short=Procollagen C-proteinase enhancer 1; AltName: Full=Type 1 procollagen C-proteinase enhancer protein; AltName: Full=Type I procollagen COOH-terminal proteinase enhancer; Flags: Precursor;

Fig. 6.6 Mass spectrometric analysis of selected protein bands. PART1 (A) The bands A and B were excised using scalpel. The processing of gel discs and mass spectrometric identification of proteins was carried in the proteomics laboratory of Prof. Benedikt Kessler, University of Oxford. The list of major proteins identified through database searching from the peptide sequences obtained through MS analysis of band A **(B)** and band B **(C).**

C   Mass spectrometry data for band B

DB	Accession	Score	Mass	Matches	Pep(sig)	Sequences	Seq(sig)	emPAI	Name
UniProt_SwissProt	P19620	2887	38795	195	138	32	28	23.25	RecName: Full=Annexin A2; AltName: Full=Annexin II; AltName: Full=Annexin-2; AltName: Full=Calpactin I heavy chain; AltName: Full=Calpactin-1 heavy chain; AltName: Full=Chromobindin-8; AltName: Full=Lipocortin II; AltName: Full=Placental anticoagulant protein IV; Short=PAP-IV; AltName: Full=Protein I; AltName: Full=p36;
UniProt_SwissProt	P19619	1284	39020	68	42	19	12	1.88	RecName: Full=Annexin A1; AltName: Full=Annexin I; AltName: Full=Annexin-1; AltName: Full=Calpactin II; AltName: Full=Calpactin-2; AltName: Full=Chromobindin-9; AltName: Full=Lipocortin I; AltName: Full=Phospholipase A2 inhibitory protein; AltName: Full=p35;
UniProt_SwissProt	O08628	131	50837	3	3	1	1	0.06	RecName: Full=Procollagen C-endopeptidase enhancer 1; AltName: Full=Procollagen COOH-terminal proteinase enhancer 1; Short=PCPE-1; Short=Procollagen C-proteinase enhancer 1; AltName: Full=Type 1 procollagen C-proteinase enhancer protein; AltName: Full=Type I procollagen COOH-terminal proteinase enhancer; Flags: Precursor;
UniProt_SwissProt	Q2KIG3	42	49247	7	2	3	1	0.07	RecName: Full=Carboxypeptidase B2; EC=3.4.17.20; AltName: Full=Carboxypeptidase U; Short=CPU; AltName: Full=Plasma carboxypeptidase B; Short=pCPB; AltName: Full=Thrombin-activable fibrinolysis inhibitor; Short=TAFI;
UniProt_SwissProt	Q5RBF3	75	44114	5	4	2	2	0.24	RecName: Full=Methionine aminopeptidase 1; Short=MAP 1; Short=MetAP 1; EC=3.4.11.18; AltName: Full=Peptidase M 1;

Fig. 6.6 Mass spectrometric analysis of selected protein bands. PART 2 (C) The list of major proteins identified through database searching from the peptide sequences obtained through MS analysis of band 4.

6.5 Discussion

The mass spectrometric analysis showed that annexins A1 and A2 were the dominant proteins in bands 1 to 4 of the N-TIMP-3 pull down. The annexins are 35-40 kDa cellular proteins that associate with negatively charged phospholipids in a calcium-dependent manner, a property that resides in the conserved calcium binding domains of annexins (Gerke *et al.* 2002). There are more than 160 members of the annexin family with diverse functions including endocytosis, phagocytosis, membrane trafficking, Ca^{2+} ion dependent assembly of lipid rafts etc. No catalytic activity against aggrecan has been reported for them (Gerke *et al.* 2002). Given the affinity of annexins for Ca^{2+} ions, they might conceivably bind non-specifically to Ni^{2+} charged agarose beads and hence appear as major protein bands in band 1. They are unlikely to be synovial aggrecanase.

Methionine aminopeptidase 1 (MAP1) and carboxypeptidase B2 (CPB2) were two metalloenzymes identified in bands 1 to 4. Methionine aminopeptidase 1 (MAP1) releases N-terminal methionine residues from the nascent proteins in the translation stage of protein synthesis (Kendall *et al.* 1992). Although MAP1 is a metalloenzyme, there is no evidence for the enzyme involved in aggrecanolysis. Carboxypeptidase B2 (CPB2) is a 36 kDa plasma protein. Its precursor is a 60 kDa zymogen expressed by hepatocytes and platelets (Eaton *et al.* 1991). Upon thrombin or plasmin cleavage of the zymogen, a 36 kDa enzymatically active fragment, thrombin activatable fibrinolysis inhibitor (TaFIa) or CPB2, is released. This fragment plays a role in the fibrinolytic system. Effective fibrinolysis results from the formation of a ternary complex between tissue plasminogen activator (tPA), plasminogen and C-terminal lysine residues on fibrin. Plasminogen bound to fibrin is more effectively converted to plasmin, thereby localizing the lytic activity to the area of the clot. Plasmin degradation of fibrin generates additional C-terminal lysine residues thereby amplifying the system locally. TAFIa cleaves C-terminal lysines on fibrin resulting in down-regulation of

fibrinolysis by reducing the number of plasminogen and tPA binding sites on fibrin.   CPB2 is a plasma protein and therefore unlikely to be synovial aggrecanase, which is actively synthesized by cells in the synovial tissue culture. In order to eliminate MAP1 and CPB2 as candidates of synovial aggrecanase, it would be worth testing the recombinant proteins on bovine aggrecan nd assessing the aggrecan degradation using anti-ARGS and anti-AGEG antibodies.

The combination of the three chromatography steps gave a purification of 384-fold (Table 4). Each step lead to significant loss of the enzyme. Such losses of protein are part and parcel of chromatography. The proteins are lost on the surface, in the chromatographic media and on the agarose beads. The recovery of activity after cation exchange and gel filtration chromatography was approximately 56 %. The worst loss occurred at the mono Q step as only 25% of the activity from gel filtration step was recovered. The anion exchange (HiTrap S) and gel filtration (Sephacryl S200) chromatographic steps were carried out sequentially whereas mono Q step was carried after a time period of one month. It is possible that the enzyme lost some of its activity on storage at 4 $^{\circ}$C prior to its application to the mono Q column. A better procedure would be to carry out all three purification steps sequentially with minimal storage time between each step. All the chromatography steps were carried out in a buffer with pH 7.0-8.0 and a salt concentration of 150 mM NaCl to obtain the maximal activity of the enzyme. Experiments carried out to determine the stability window of the synovial aggrecanase suggest that aggrecan degrading activity decreased with pH values below 5.0 and maximal activity was observed in the range of pH 7.0 to 8.0 (data not shown). Similar analysis with increasing salt concentrations (0-1 M NaCl) suggests that the enzymatic activity was maximum in the range of 100 mM to 300 mM NaCl (data not shown). The stability of synovial aggrecanase appears to be similar to aggrecanases produced by IL-1 stimulated bovine cartilage cultures (Arner *et al.* 1999).

Purification step	Total Protein (mg)	Total activity (arbitary units)	Specific activity (units/mg)	Purification factor	Recovery of activity (%)
SYCM	240	2500	10.42	1	100
HiTrap S	30	2000	66.67	6.4	80
Sephacryl S200	7	1400	200.00	19.2	56
Mono Q	0.1	400	4000.00	384	16

Table 4. Purification of synovial aggrecanase from 60 ml of SYCM. Aggrecanase ELISA was used to measure the activity of the medium at each chromatography step. The A_{450}/A_{620} measurement from aggrecanase ELISA was taken as an arbitrary unit of activity. Recovery of activity for each chromatography step was calculated as a percentage of the original material. The protein content of mono Q fraction was estimated using A_{280} value from the absorbance trace.

Chapter 7

Final discussion and future work

7.1 Final discussion

A number of steps would be taken to improve the purification of synovial aggrecanase(s). A new batch of Ni^{2+} beads will be obtained to ensure no keratin is present. A mass spectrometry grade sample buffer from Invitrogen will be used to solubilize the proteins for gel electrophoresis, which could further reduce the problem of keratin contamination. Another resin (TALON), which uses Co^{2+} rather Ni^{2+} as divalent cation for binding His tagged proteins could also be tried. An alternative would be to use a His tag pull down kit (Dynabeads His tag pull down kit) available from Invitrogen. The kit contains optimized buffers for affinity isolation of native proteins expressed in small amounts.

As discussed earlier, annexins A1 and A2 were the most abundant proteins in the fraction 8 of mono Q cation exchange chromatography (Fig. 4.12) and appeared to bind Ni^{2+} agarose beads. An anti- annexin A1 and A2 antibody coupled to protein A or G beads could be used to deplete the active fractions of annexins A1 and A2 or a pre-clearing step with Ni^{2+} agarose beads could also be used to remove the annexin proteins. An alternative approach would be to further chromatograph the enzyme in fraction 8 on a high resolution gel filtration column using a SMART system (SMART Superdex 75). This step should give a good separation of proteins in the region of 60 to 20 kDa, thus separating the enzyme from annexin proteins.

Fraction 8 of mono Q chromatography was applied to N-TIMP-3 bound Ni^{2+} agarose while fraction 9 was applied to Ni^{2+} agarose beads only (Chapter 6 section 6.1) to distinguish proteins binding specifically to N-TIMP-3 from those binding to the Ni^{2+} beads. A better procedure would be to incubate half of the fraction 8 material with N-TIMP-3 Ni^{2+} agarose beads and the other half with Ni^{2+} beads. The N-TIMP-3 specific protein bands would be selected for mass spectrometry but unlike my earlier experiment where only 37 kDa region was selected for mass spectrometry, the whole lane should probably be cut for analysis by

mass spectrometry. In addition to improving the individual procedures, the starting material volume could be scaled up to give a higher yield of enzyme after sequential chromatography.

If improved chromatography does not help in the identification of the enzyme, antibodies against the catalytic domain of ADAMTS-4/ADAMTS-5 (discussed in Chapter 4 introduction) could be tested on purified mono Q fractions or material obtained after N-TIMP-3 pull down. Any antibody reactive bands could be subjected to mass spectrometry analysis to ensure they do not represent non-specific interaction of antibody with antigens in the medium. Recently the matrix biology lab at Kennedy Institute of Rheumatology has successfully developed ADAMTS-5 neutralizing antibodies. The antibodies would be tested against synovial aggrecanase for neutralizing its aggrecanolytic activity. A positive result could establish that synovial enzyme is ADAMTS5. The antibodies could then be used to immunoprecipitate the aggrecanolytic enzyme and subjected to mass spectrometry for positive identification of the aggrecan degrading species.

From my results, synovial aggrecanase appears in two forms: low and high molecular weight. They may represent one or two different enzymes. LRP1 did not appear to be involved in the formation of the higher molecular weight form. The expected size of ADAMTS proteins is about 100 kDa, significantly larger than the 37 kDa form protein I identified. This smaller enzyme could represent the truncated form of ADAMTS protein or a novel enzyme. Both forms were active in the SYCM that contained abundant plasma proteins (Fig. 4.3A). The plasma proteins accumulated in the culture medium presumably through the passive release of the contents of capillaries present in the synovial tissue. One group of plasma proteins is proteinase inhibitors of which the most abundant is alpha-2 macroglobulin (α_2-M), which inhibits all classes of enzymes including the metalloproteinase family of proteins. The ability of high and low molecular weight forms of synovial enzymes to evade inhibition by plasma proteinase inhibitors is interesting. In synovial joint, the synovial tissue is attached to joint capsule on one side while the other side opens up to synovial fluid, which is an ultrafiltrate of

141

blood plasma. Joint trauma may produce synovial enzymes, which can pass the synovial fluid uninhibited and destroy the ECM of cartilage. Further study is needed to investigate the inhibition of the enzymes by α_2-M, their lack of interaction with the LRP1 and the composition of the high molecular weight species.

The synovial explant cultures differ from those of cartilage explant cultures. Little or no soluble aggrecanase activity is observed in the medium of IL-1 stimulated cartilage explant cultures. We currently think this is because the enzymes are continually being internalised by LRP-1, or interact with TIMP-3, which is also internalised by LRP1 (Yamamoto *et al.* 2013). Why proteinase(s) accumulate in the synovial cultures is unknown. The original purifications of aggrecanase 1 and 2 (ADAMTS4 and ADAMTS5) were carried out from IL-1 stimulated bovine nasal cartilage cultured for a week. Since this tissue has vascular channels, the cellular origin of the enzymes was uncertain. Moreover, these enzymes have not been convincingly demonstrated in chondrocyte cultures.

To what extent the production of the aggrecanase by synovial tissue is a response to damage can be investigated when the enzyme is identified. Whether it is produced generally by damaged connective tissue also needs to be investigated. Lohmander and his colleagues found significantly higher levels of ARGS- and AGEG- neo-epitope bearing fragments in the synovial fluid of patients with acute and chronic knee injuries (Struglics *et al.* 2011). The synovial enzyme(s) could be involved in the generation of these fragments which could erode the ability of the cartilage to resist compressive forces and hence predispose to OA.

Once the enzyme is identified then its mRNA regulation can be studied. Is it induced by tissue explantation? Can it be regulated by cytokines or other agents? It will be important to determine the cell of origin (by immunolocalisation and by possibly by *in situ* hybridisation) and to find the relationship of this enzyme to those responsible for IL-1 mediated, or disease mediated, aggrecan degradation. The enzyme may be regulated at the

mRNA level (transcriptional regulation & stability) or the mRNA may be pre-existent and the protein production be regulated translationally. Its post-translational processing will also be investigated. These steps would all present possible therapeutic targets if the enzyme is involved in pathological tissue destruction.

Chapter 8

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