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Institute of Medical Biology

In vitro reconstitution of proMatrix Metalloproteinase- 9/Chondroitin Sulfate Proteoglycan Complexes

Identification of motifs in proMMP-9 and the serglycin core protein involved in the complex formation

Rangita Dawadi

Master thesis in Biomedicine

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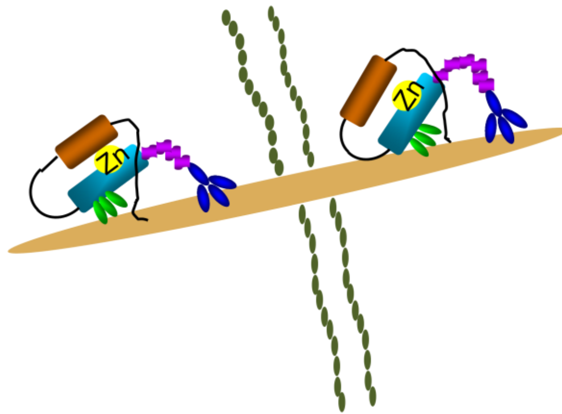

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UNIVERSITY OF TROMSO

DEPARTMENT OF MEDICAL BIOLOGY

FACULTY OF HEALTH SCIENCES

RANGITA DAWADI

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CONTENTS

Acknowledgements 8

Abbreviations 9

Summary 10

1. Introduction 13

1.1. The extracellular matrix 13

1.2. Proteoglycans 13

1.3. Proteolytic enzymes 17

1.4. Matrix metalloproteinase (MMPs) 18

1.4.1. Structures and classification of MMPs 18

1.4.2. Regulation of MMPs 21

1.4.3. MMPs in health and diseases 23

1.5. Gelatinases 24

1.5.1. Structure and regulation of MMP-9 24

1.5.2. Inhibitors of MMP-9 28

1.5.3. MMP-9 in health and diseases 28

1.5.4. MMP-9 and its complexes 29

1.5.4.1. ProMMP-9/CSPG complexes 30

1.5.4.2. Properties of proMMP-9/CSPG complexes 31

1.5.4.3. Factors affecting the formation of proMMP-9/CSPG complex 32

1.5.4.4. Physiological roles of proMMP-9/CSPG complexes 32

1.6. Aim 34

2.Materials and methods 35

2.1. Materials 35

2.2. Instruments and equipments 36

2.3. Buffers and solutions 37

2.3.1. General buffers and solutions 37

2.3.2. Buffers for gel filtration (Sephacryl S-400) 39

2.3.3. Gels, buffers and solutions used in Q-Sepharose ion exchange chromatography 39

2.3.4. Buffers and solutions used in the detection of CS-chains (Safranin O assay) 40

2.3.5. Buffers and solutions used in SDS gel zymography 41

2.3.6. Buffers and solutions used in western blotting 44

2.3.7. Buffers and solution for silver staining 45

2.4. Methods 47

2.4.1. Equilibration of the Sephacryl S-400 column for the determination of the separation properties of the column. 47

2.4.2. Determination of the separation properties of the Sephacryl S-400 column 47

2.4.3. Purification of proteoglycans using Sephacryl S-400 column 48

2.4.4. Safranin O method 49

2.4.4.1. Optimization of the quantitative determination of CS-chains by the use of the standard CS from shark cartilage using the Safranin O method 50

2.4.5. Q-Sepharose column chromatography 52

2.4.6. Desalting of the samples with help of Sephadex G-50 Fine gel 54

2.4.7. *In vitro* reconstitution 54

2.4.8. SDS-PAGE 55

2.4.8.1. Gelatin zymography 55

2.4.8.2. Western blotting analysis of the purified CSPGs 57

2.4.8.3. SDS- PAGE for silver staining and coomassie staining 58

2.4.8.3.A. Silver staining 59

2.4.8.3.B. Coomassie staining for mass spectrometry (M.S) analysis. 60

2.4.9. Peptide arrays 60

2.4.10. Mutation peptide arrays 62

2.4.11. *In vitro* reconstitution competition experiments 62

3.Results 64

3.1. Optimization of quantitative determination of CS-chains 64

3.2. Gel filtration with Sephacryl S-400 column 67

3.2.1. Determination of the separation properties of the Sephacryl S-400 column 67

3.2.2. Purification of CSPG from THP-1 cells using Sephacryl S-400 size exclusion chromatography 69

3.2.3. Purification of CSPG from MonoMac cells using Sephacryl S-400 size exclusion chromatography 73

3.3.4. Purification of CSPG from U-937 cells using Sephacryl S-400 size exclusion chromatography 77

3.4. *In vitro* reconstitution of proMMP-9/CSPG complexes 77

3.5. Separation of the proteins in purified CSPG fractions from THP-1 and MonoMac cells by SDS-PAGE 79

3.6. Peptide arrays 81

3.6.1. Peptide array based on sequences of serglycin probed with proMMP-9 82

3.6.2. Mutated peptide array based on the sequences of serglycin probed with proMMP-9 84

3.6.3. Peptide array based on the sequence of serglycin probed with TIMP-1 86

3.6.4. Peptide array based on sequences of FnII module of MMP-9 probed with His-tagged serglycin 87

3.6.5. Mutated peptide array based on the sequences of FnII module of MMP-9 probed with His-tagged serglycin 90

3.6.6. Peptide array based on sequences of HPX domain of MMP-9 probed with His-tagged serglycin 91

3.6.7. Mutated peptide array based on the sequences of HPX domain of MMP-9 probed with His-tagged serglycin 94

3.6.8. Peptide array based on the sequence of HPX domain of MMP-9 probed with TIMP-1 95

3.7. *In vitro* reconstitution competition experiments with soluble peptides 98

3.7.1. Peptides D1 and D2 99

3.7.2. Peptide D3 101

3.7.3. Peptide D4 102

3.7.4. Peptide D5 and D6 103

3.7.5. Peptide D7 and D8 104

3.7.6. Peptide D9 105

4.Discussion 107

4.1. Optimization of quantitative determination of CS-chains 107

4.2. Presence of serglycin and other CSPGs in THP-1 cells. 107

4.3. Presence of unknown CSPGs in MonoMac cells 110

4.4. Peptide array and competition experiments 111

4.4.1. Serglycin binds with MMP-9 and TIMP-1 111

4.4.2. FnII module binds with serglycin 114

4.4.3. HPX domain binds with serglycin and TIMP-1 115

4.5. Serglycin a substrate for MMP-9 117

5.Conclusion 118

6.Future Prospects 119

7.Appendix 121

7.1. Column Chromatography 121

7.1.1. Size exclusion chromatography / Gel filtration column chromatography 121

7.1.1.1. Sephacryl S-400 high resolution column chromatography 121

7.1.2. Ion-Exchange column chromatography 123

7.2. Slot blot apparatus used in the Safranin O method 124

7.3. Western Blotting 125

7.4. SDS-PAGE electrophoresis instruments 126

7.5. Purification of partly purified CSPGs from THP-1 and MonoMac cells.....127

7.6. Mass spectrometry.....128

7.7. Peptide array membranes.....130

7.8. Mutation scans for the MMP-9 Fn-II module, HPX domain and Serglycin.....138

7.9. List of peptides for *in vitro* reconstitution competition experiments.....142

8.References.....144

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Abbreviations

ECM - Extracellular Matrix

CS - Chondroitin sulfate

CSPG - Chondroitin sulfate proteoglycans

GAG - Glucosaminoglycan

MMP - Matrix Metalloproteinase

NaAc - Sodium Acetate

HAc - Acetic Acid

CPC - Cetylpyridinium Chloride

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

MES-SDS - 2-(*N*-morpholino)ethanesulfonic acid

TEMED - Tetramethylethylenediamine

DMSO - Dimethyl sulfoxide

DTT - Dithiothreitol

HRP - Horseradish Peroxidase

ECL - Enhanced chemiluminescence

PVDF - Polyvinyl difluoride

IAc - Iodoacetate

NEM - *N*-Ethylmaleimide

EDTA - Ethylenediaminetetraacetic acid

Summary

Previously it has been shown that different monocytic leukemic cell lines such as THP-1, MonoMac and U-937 can produce proMMP-9 and various types of proteoglycans. However, they do not produce an identical set of proteoglycans. THP-1 and U-937 cells produce serglycin, whereas THP-1 and MonoMac has been found to produce versican. At the mRNA level, these three cell lines also produced different variants of syndecan and glypican [Malla N et al (2013) FEBS J 280, 2870-2887]. When THP-1 cells were stimulated with PMA, they also produced a complex between proMMP-9 and one or several CSPGs, while unstimulated cells produced very small amount of this complex [Winberg J.O.et al (2000) J Mol Biol 304, 669-680; Malla N et al (2011) PLoS ONE 6(6), e20616]. Neither PMA stimulated nor unstimulated U-937 and MonoMac cells produced proMMP-9/CSPG complexes. However when proMMP-9 was mixed with isolated CSPGs from these three cell lines or pure serglycin and versican, proMMP-9/CSPG complexes were formed. These strong complexes were of two types, one was SDS-stable and reduction sensitive which was similar to the complexes isolated from PMA stimulated THP-1 cells and the other complex was SDS-soluble [Malla N et al (2013) FEBS J 280, 2870-2887]. The aim of the first part of this thesis was to determine if it is possible to separate the CSPGs as well as putative CSPG associated proteins, and to determine the nature of the proteins and determine which of these proteins can form proMMP-9/CSPG complexes. Different proteoglycans and proteins in the CSPGs isolated from THP-1 and MonoMac cells were partly separated by the use of size exclusion chromatography. Fractions with isolated CSPGs could to various extents form proMMP-9/CSPG complexes *in vitro*. In the separated CSPGs from THP-1 cells it was shown that the amount of SDS-stable complex produced by *in vitro* reconstitution followed the amount of serglycin, i.e. fractions with larger amounts of serglycin resulted in larger amounts of complex formed. In contrast to this, the amount of SDS-soluble complex was mainly produced by the fractions with the most serglycin and the fraction with the least amount of serglycin, but more of another larger CSPG or CSPG associated protein. As for the purified CSPGs from the THP-1 cells, it was the fractions with the smallest proteoglycans from the size exclusion chromatography that in the *in vitro* reconstitution experiments with proMMP-9 produced the largest amounts of both

SDS-stable and SDS-soluble complexes. So far it has not been possible to identify the nature of these CSPGs.

Both the FnII module and HPX domain of MMP-9 has been found to be involved in formation of proMMP-9/CSPG complexes [Winberg JO et al (2003) Eur J Biochem 270, 3996-4007; Malla N et al (2008) J Biol Chem 283, 13652-13665] and *in vitro* reconstitution of the complex between proMMP-9 and serglycin resulted in both SDS-stable and SDS-soluble complexes [Malla N et al (2013) FEBS J 280, 2870-2887]. The second part of this thesis has identified regions and motifs in the serglycin core protein, FnII module and HPX domain of MMP-9 that are involved in the complex formation between these two proteins. Identification was performed by peptide arrays, mutated peptide arrays and soluble peptides from the serglycin core protein, FnII module and the HPX domain of MMP-9. The soluble peptides were used in *in vitro* reconstitution experiments to determine if they acted as inhibitors of the complex formation. In Serglycin, the peptide arrays indicated that both the N- and C-terminal regions with respect to the location of the CS-chains were found to be involved in binding with proMMP-9. *In vitro* reconstitution with two different soluble peptides suggested that an SDS-stable complex could be formed at both the N- and C-terminal sides of the CS-chains. Peptide arrays indicated that all three repeats of FnII module seemed to be involved in binding with serglycin, but second repeat seemed to be more involved in the binding than the other two repeats. *In vitro* reconstitution in the presence of soluble peptides indicated that the FnII module was involved in the formation of both SDS-stable and SDS-soluble complexes. Also, all the four blades of HPX domain seemed to be involved in the formation of the proMMP-9/serglycin complexes, but most interactions occurred with blade 4. *In vitro* reconstitution of the proMMP-9/CSPG complexes in the presence of soluble peptides indicated that a region at the end of blade 4 in the HPX domain was involved in forming the SDS-stable complex. Furthermore, TIMP-1 has previously been shown to prevent the formation of the SDS-soluble proMMP-9/CSPG complex. This was assumed due to the binding of the C-terminal region of TIMP-1 to the HPX domain of proMMP-9, although it is not known where in the HPX region TIMP-1 binds [Malla N et al (2013) FEBS J 280, 2870-2887]. In the present thesis it is shown that TIMP-1 can bind the serglycin core protein as well as

to the HPX domain in proMMP-9. Peptide array showed that there were regions both N- and C-terminal to the CS-chains to which both TIMP-1 and proMMP-9 can bind and hence compete for binding. Furthermore, it seemed that TIMP-1 could compete with Serglycin for the binding to blade 1 in the HPX domain of MMP-9. This suggests that this part can be involved in the formation of the SDS-soluble proMMP-9/serglycin complex. Thus in this part of the thesis, regions, motifs and amino acids involved in the formation of the proMMP-9/serglycin complexes has been identified.

1. Introduction

1.1. The extracellular matrix

Our bodies are made up of organs which are made up of tissues and these tissues are made up of cells. The extracellular matrix (ECM) is such an element which is produced by various epithelial and stromal cells such as fibroblast, osteoblasts and basal epithelial cells. It provides a base for support and as a binding platform for all the cells to constitute together to form tissues and subsequently organs. The constitution of a cellular micro-environment; extracellular matrices are secreted molecules composed of a dynamic array of glycoproteins, collagens, elastins, proteoglycans and hyaluronans. Collagens are one of the most important molecules that make up the ECM. It is a large family of triple helical proteins which are present throughout our bodies. The main functions of collagens include, tissue scaffolding, cell adhesion, cell migration, angiogenesis, tissue morphogenesis as well as tissue repair. Collagen is the major tensile element that is predominantly occurs in the ECM as an elongated fibril especially in the tendons, cartilage, bone and skin [1]. The components of the ECM not only provide bulk but they also help ECM provide the shape and strength to many tissues such as basement membrane, bone and cartilage. ECM is a powerful mechanical and structural support for tissues. There are various proteins which have the capability of determining the behaviors, polarity, migration, differentiation, proliferation as well as survival of various cells and this is done via the communication with the intracellular cytoskeleton and transmission of growth factor signals [2].

The degradation of ECM is essential for the free cellular movement and tissue remodelling in various physiological and pathological conditions. These processes are carried out vastly by various proteases such as matrix metalloproteases along with heparanases [2].

1.2. Proteoglycans

There are certain proteins called proteoglycans which are present in ECM. These proteins are found intracellulary but are mainly designed for extracellular space and are substituted with linear polysaccharides glycosaminoglycans (GAGs) [3].These proteins

are highly negatively charged molecules [4]. These are present in various forms ranging from tissue specific to cell-type specific [5]. Proteoglycans (Fig. 1.) are not only diverse molecules they also represent different combinations of various types of core proteins with several classes of GAG chains. GAG chains are the glycosamine containing repeating disaccharides which are negatively charged and are linear. There are two major types of GAG chains that are present in most animals: heparin sulfate (HS) and chondroitin sulfate (CS). Others include dermatan sulfate and keratin sulfate. The GAG chains have the capability of interacting with various plasma proteins such as growth factors, cytokines, chemokines, proteases, protease inhibitors, coagulant and anticoagulant proteins, complement proteins, lipoproteins, and lipolytic enzymes [6].

Proteoglycans have been found to be in abundance, for example, there is an estimation that there could be as many as 1 million syndecan-I molecules at the surface of epithelial cells. There is a high chance of involvement of proteoglycans in various molecular interactions at the cell-surface, which could be cell-matrix, cell-cell, or ligand-receptor interactions. This involvement is due to their abundanacy at the site during these interactions and also due to their capability of binding proteins [3]. Various monocytic leukemia cell lines have been found to produce proteoglycans, such as: THP-1 is one of the cell lines that produces serglycin, versican, perlecan, glypican-1, CD44, thrombomodulin and syndecans-1, -2 and -3 [7-10]. U-937 cells have been found to produce serglcyin [4] and a little amount of versican. MonoMac is another cell line which is found to produce mostly versican [9].

Serglycin is one of the proteoglycans, which is mostly found in hematopoietic and endothelial cells. Although it is an intracellular proteoglycan, if can be secreted and then incorporated into the extracellular matrix. The molecular size of the core protein of serglycin is 17.6 kDa and it contains a 16-amino acid (serine/glycine) repeat region where the glycosaminoglycan chains especially HS-chains (in case of connective tissue mast cells) or CS-chains (mucosal mast cells as well as in macrophages) are attached. These proteoglycans are expressed by cells such as neutrophils, lymphocytes, monocytes, macrophages, platelets, megakaryocytes and mast cells. Serglycin have been found to have major roles in the immune cells intracellularly. It has been involved

dominantly in storing compounds for secretion, formation and homeostasis of various types of storage granules, apoptosis, blood coagulation and also to retent major inflammatory mediators inside storage granules and secretory vesicles. CD44 has been found to bind to chondroitin sulfate chains of serglycin [4, 11].

Serglycin is found to be expressed in various human leukemia cells including U-937 cells which are found to express serglycin highly. It is also expressed prominently in immature promelocyte as well as myelocyte cells in bone marrow. It has been found to be a major biomarker for various human myeloid leukemia. Not only that it has also been linked with epithelial-mesenchymal transition in tissue samples of metastatic nasopharyngeal cancer cells [11-13].

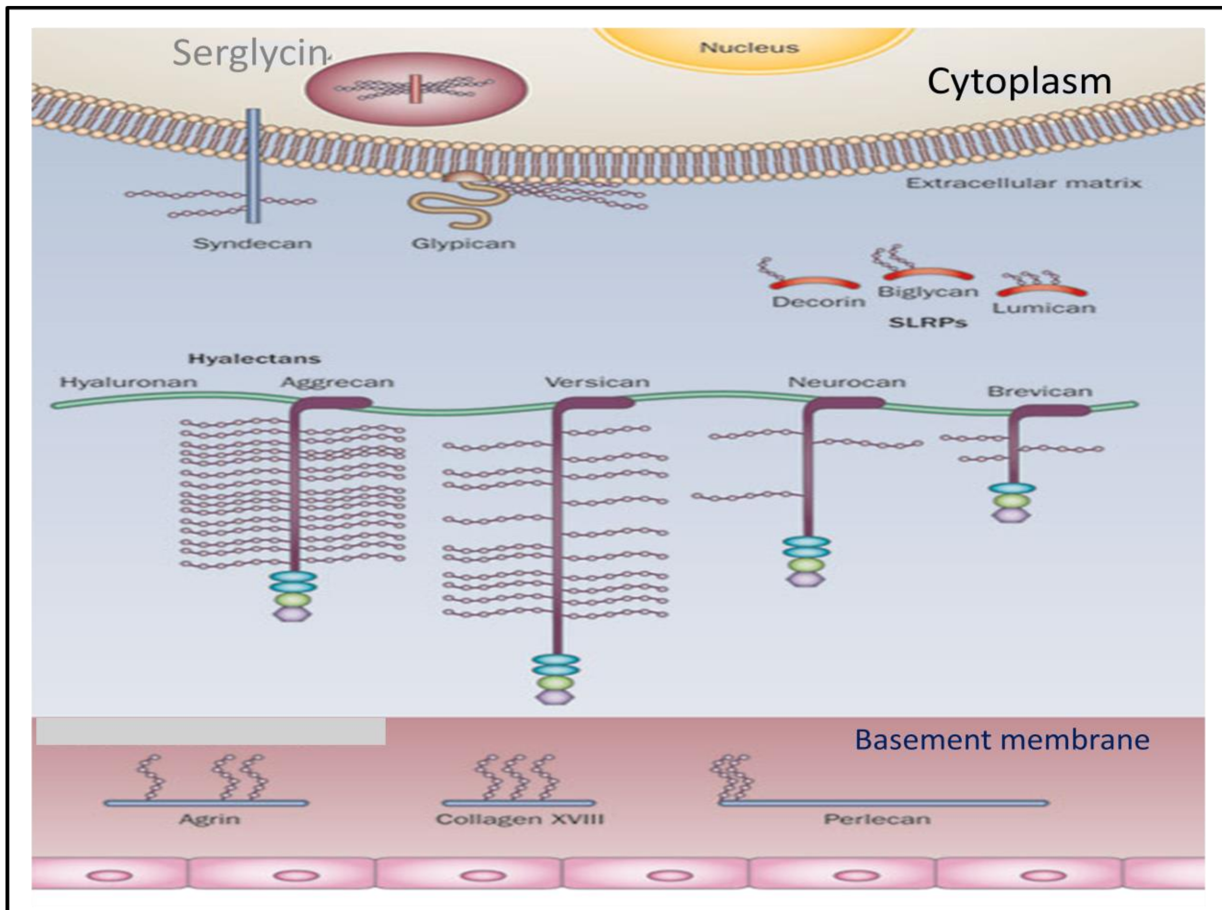

Fig.1. Different proteoglycan groups on the basis of the sites where they associate. Serglycin, unlike other proteoglycans is a intracellular proteoglycan (modified from [13]).

Versican is another proteoglycan which is found extracellularly and has four isoforms: V0, V1, V2 and V3 with core proteins ranging from 74-370 kDa. Versicans are found to be expressed along neural crest pathways and it influences neural cell migration. It is a very large proteoglycan that is found in various soft tissues. The glycosaminoglycan chains attached to this proteoglycan are CS-chains. Versicans are highly interactive, possess hygroscopic properties and it acts as a structural molecule which is why during several important developmental as well as abnormal events, they provide structural functions by creating loose and hydrated matrices. It also provides cell adhesion and survival to the cells by directly or indirectly interacting with them [14, 15].

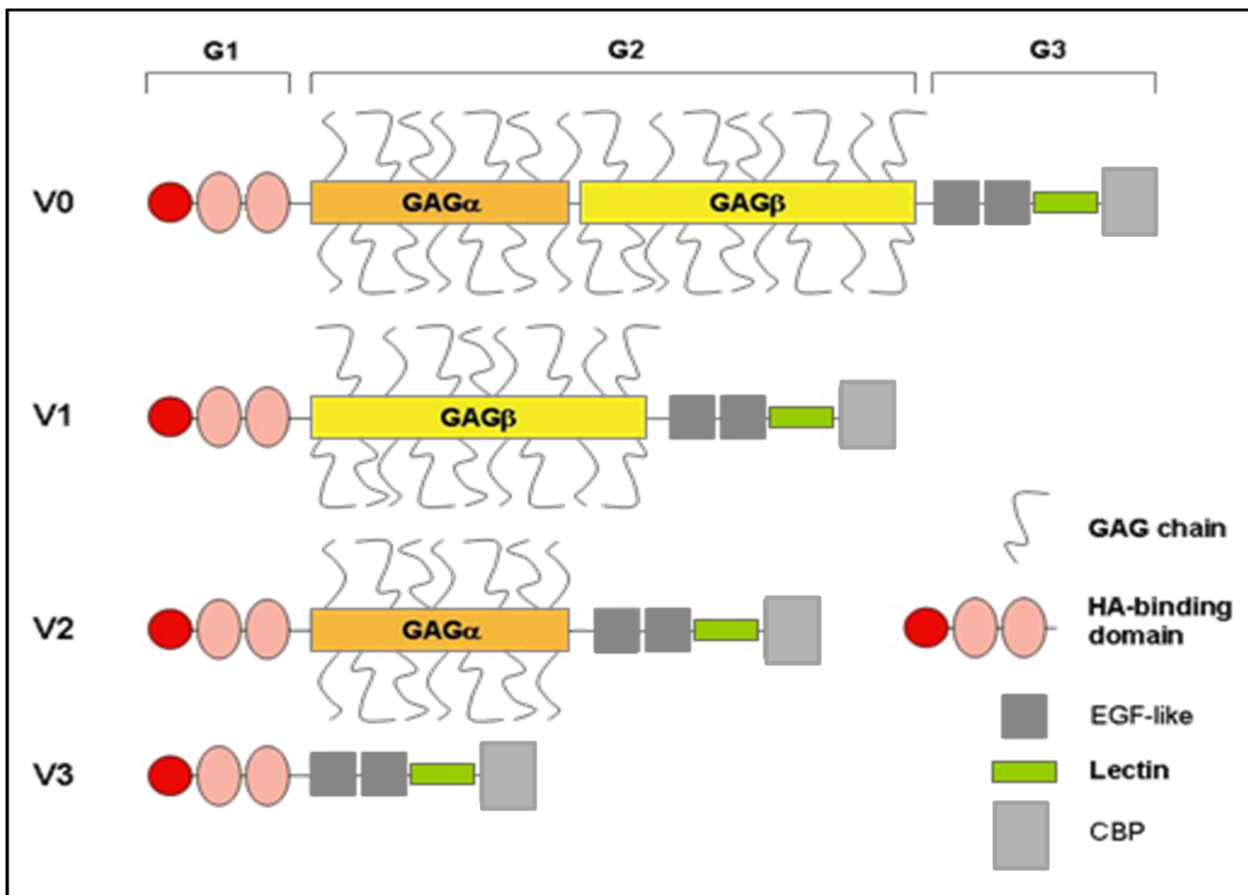

Fig. 2. Different isoforms of versican and their structures. (Modified from Atlas of Genetics and Cytogenetics in Oncology and Haematology).

V0 is the largest isoform of versican (Fig. 2.) and its core protein is divided into three different domains: N-terminal domain (G1), the central domain (G2) where the GAGs attach and the globular C-terminal domain (G3). As, we can see in Fig. 2, G3 consists of

two Epidermal growth factor (EGF)-like repeats, one lectin like subdomain and one compliment binding protein (CBP)-like subdomain. The G1 domain of the versican shows the anti-adhesive activity, carboxy-terminal domain activates focal adhesion kinase, promotes cells adhesion and prevents apoptosis of the β1 integrin of glioma cells. The G3 domain of versican has adhesive properties [14, 16]. Versicans have the capability of expanding the pericelullar ECM which is required for the proliferation and migration of cells [14, 17]. It is abundantly present in the blood vessels and its expression is regulated in several cardiovascular diseases. It has been involved in atherosclerotic and restenotic lesions, retention of inflammatory cells, thrombosis and aneurysms [18].

1.3. Proteolytic enzymes

Proteolytic enzymes (also known as: Peptidase or Proteinase) are the enzymes that cleave proteins by the hydrolysis of peptide bonds [19].

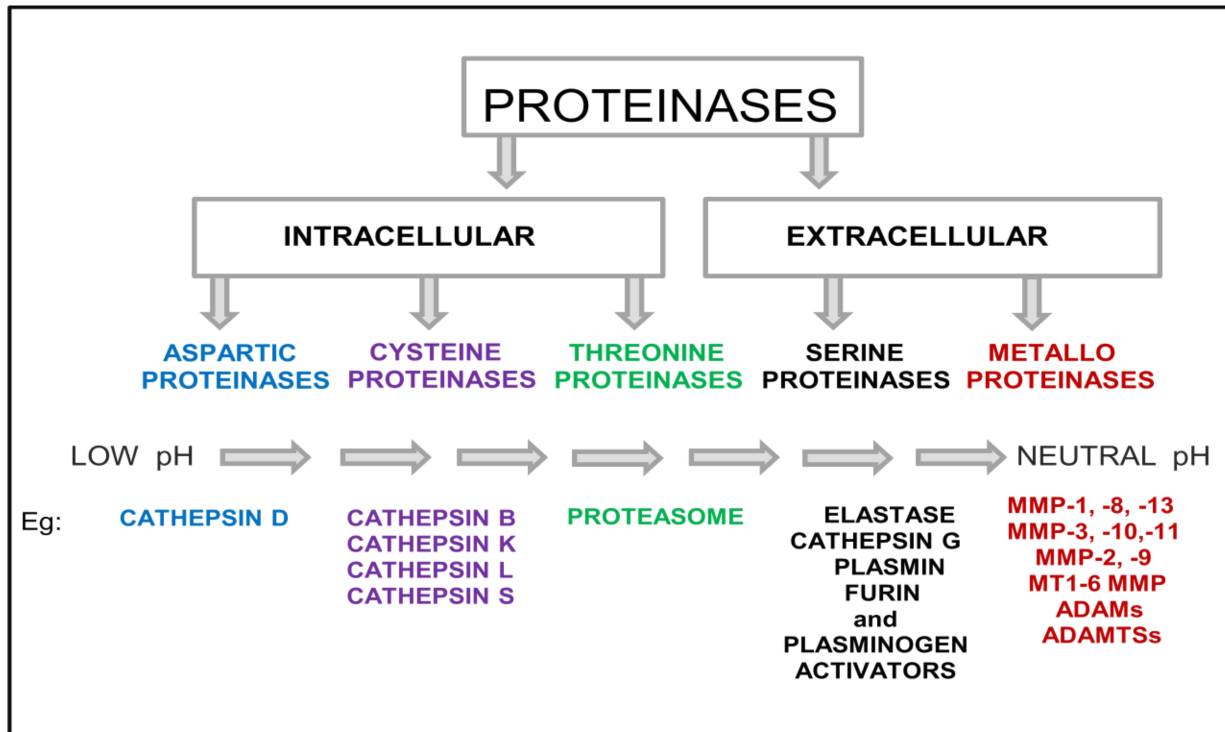

Fig. 3. Five classes of proteinase out of which three act predominantly inside cells (Aspartate, cycteine and threonine) and two act predominantly outside of cell (metallo and serine). Examples of some enzymes in each group are given. Modified from [19]

The classes of proteases (Fig. 3.) are divided according to the chemical group/amino acid that is involved in the hydrolysis of peptide bonds. They can be either endopeptidases or exopepdidases depending upon the position in the target protein chain where the hydrolysis of protein takes place [19].

1.4. Matrix metalloproteinase (MMPs)

For various processes such as embryonic development, morphogenesis, reproduction, tissue resorption as well as remodelling to occur, the breakdown of extracellular matrix at the right time is extremely important. MMPs or matrixins are a family of zinc-dependent endopeptidases which are involved in above mentioned processes. Matrixins comprises of large family of proteases sharing similar structures and funcitons. Synthesized as preproenzymes, all matrixins are secreted in latent forms called proMMPs. Growth factors, hormones, cytokines and cellular transformations regulate the expression of most of the matrixins [20, 21].

1.4.1. Structures and classification of MMPs

Almost all members of the MMP family are made up of three basic and distinctive domains as seen in figure 4. These domains are: an amino-terminal propeptide, a catalytic domain and a hemopexin-like domain at the carboxy-terminal [22]. The propetide is made up of approximately 80-90 amino acids containing a conserved motif with a cysteine residue called a ''cysteine switch''. This propeptide is quite important for maintaining the latency in the MMPs, as the cysteine coordinates with the catalytic zinc ion present in the catalytic domain. This coordination helps to prevent a water molecule necessary for catalysis from binding to the zinc ion thus keeping the enzyme inactive [23].The catalytic domain contains two zinc ions; a catalytic zinc ion and a structural zinc ion and one calcium. The structural zinc and calcium are important for the stability and expression of enzymatic activity. MT-MMPs, MMP-11,-21 and -28 contain a proprotein processing motif RX(K/R)R (Fig. 4.) which is present at the C-terminal of the propeptide and is found to be cleaved and hence activated intracellulary by furin [20]. The sequence of hemopexin-like domain (HPX domain) is similar to the plasma protein hemopexin, which is from where the name hemopexin like domain originates from. This domain is highly conserved and is functionally important in substrate binding as well as

interacting with tissue inhibitors of MMPs [21]. This C-terminal HPX domain is composed of four bladed β-propeller structures. These structures are especially important for collagenases in order to cleave the triple helical interstitial collagens [20].

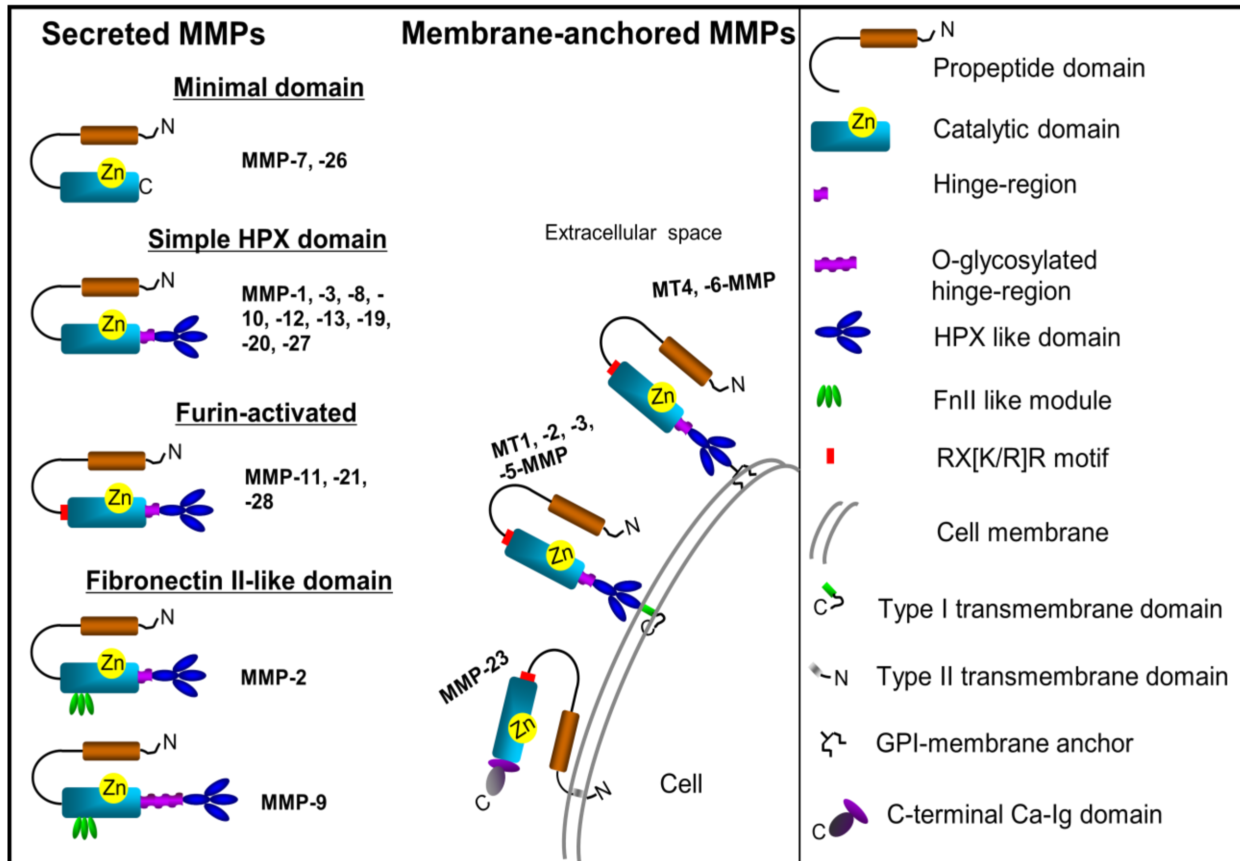

Fig. 4. Different MMPs with their domain structures. Almost all the MMPs contain four main components to their structures: propeptide, catalytic domain, hinge region and a HPX domain. MMP-7 and -26 do not have a hinge region or a hemopexin region while MMP-9 contains a heavily O-glycosylated hinge region. Only MMP-2 and MMP-9 contain a fibronectin module containing three fibronectin like repeats in the catalytic domain. At the C-terminal end of the prodomain of the membrane-anchored MMPs and the three furin activated MMPs contain a RX(K/R)R motif (modified from [22]).

MMPs can be divided into six groups on the basis of substrate specificity and homology as shown in Table 1.

Table 1. Classification of MMPs on the basis of different classes [24].

Class	MMP name	Common name/ Descriptive name
Collagenases	MMP-1	Collagenase-1 / Interstitial collagenase
	MMP-8	Collagenase-2 / Neutrophil collagenase
	MMP-13	Collagenase-3
	MMP-18	Collagenase-4
Gelatinases	MMP-2	Gelatinase-A
	MMP-9	Gelatinase-B
Stromelysins	MMP-3	Stromelysin-1
	MMP-10	Stromelysins-2
	MMP-11	Stromelysins-3
Matrilysins	MMP-7	Matrilysin
	MMP-26	Matrilysins-2
MT-MMP	MMP-14	MT1-MMP
(membrane type)	MMP-15	MT2-MMP
	MMP-16	MT3-MMP
	MMP-17	MT4-MMP
	MMP-24	MT5-MMP
	MMP-25	MT6-MMP
Other enzymes	MMP-12	Macrophage metalloelastase
	MMP-19	RASI 1
	MMP-20	Enamelysin
	MMP-21	MMP identified on chromosome 1
	MMP-22	MMP identified on chromosome 1
	MMP-23	From human ovary cDNA
	MMP-27	Homology to Stromelysin-2
	MMP-28	Epilysin
	MMP-29	Unnamed

Collagenases are the group of MMPs that consists of MMP-1, -8,-13 and -18 and they specifically degrade collagens. The principle substrates of these MMPs are fibrillar collagens type 1, 2 and 3. Gelatinases consists of two MMPs: MMP-2 and -9, whose principle substrates are gelatin and non-fibrillar collagens type 4 and 5. Stromelysin consists of MMP-3, -10 and -11 and the principle substrates are proteoglycans, laminins, fibronectin and non-fibrillar collagens. Matrilysins consists of MMP-7 and -26. The group MT-MMPs consists of MMP-14, -15, -16, -17, -24 and -25. There are other enzymes which do not fall to any category and are listed as follows: MMP-12, -19, -20, -21,-22, -23, -27, -28 and -29 [25]. In addition to degrade extracellular matrix proteins, MMPs can degrade a large amount of non matrix proteins such as: growth factors, chemokines, cell adhesion proteins and other proteinases [22].

1.4.2. Regulation of MMPs

Normally the expression of most MMPs is low in tissue and only when the remodeling of the ECM is required the expression is induced [26]. MMP gene expression is regulated primarily at the transcriptional level. After transcription, mRNA stability is controlled by cytokines, nitric oxide and micro RNA [27]. Since MMPs regulate various biological processes, these enzymes are controlled in several critical steps, such as: synthesis and secretion, activation of the pro-enzymes, inhibition of active enzymes, localization and clearance of MMPs. Various cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α and IL-17 are responsible for stimulation of numerous cells types for production of MMPs [19].

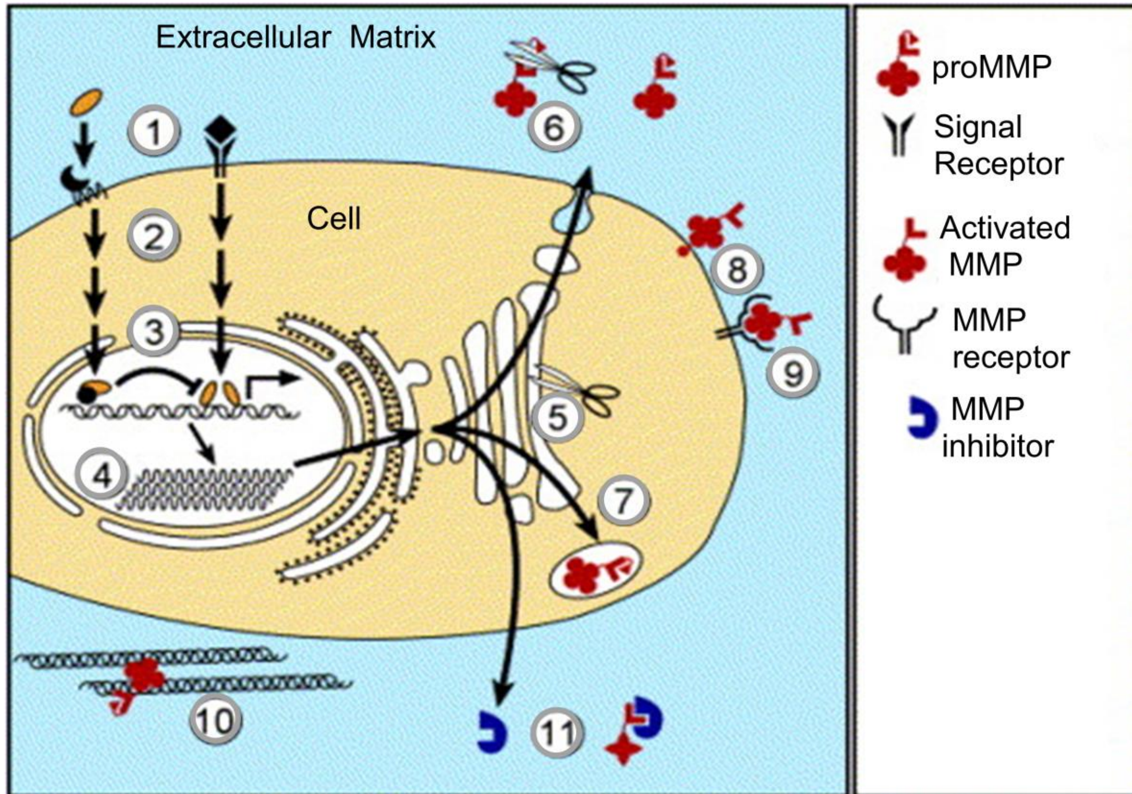

Fig. 5. Regulation of matrix metalloproteinase activity (modified from [19]).

In figure 5 we can see in (1) cytokines and growth factors can both upregulate and downregulate the MMP expression and also can act synergistically where in (2) shows different signalling pathways which combine to activate or suppress transcription (3). RNA can be unstable and rapidly processed as seen in (4).Then some of the proMMPs are activated intracellularly by furin (5) while others by proteases in the ECM (6). Then some MMPs are stored in the granules of some cell types (7) before the secretion. Then the secreted MMPs can be expressed on the cell surface as seen in (8) or can be bound to cell surface receptor proteins (9) or can be sequestered by extracellular matrix proteins as in (10). Tissue inhibitors of metalloproteinases (TIMPs) can inhibit all the active MMPs (11) [19].

Apart from these, MMPs can be controlled by other mechanisms too, such as secretion to specific regions of the plasma membrane, proteolytic processing and inactivation of MMPs, endocytosis as well as lysosomal breakdown [19]

1.4.3. MMPs in health and diseases

MMPs have been found to play a critical role in connective tissue turnover, bone extracellular matrix turnover, embryonic development, epiphyseal cartilage dysplasias, cancer metastasis, heart failure and in cerebral ischemia [28]. MMPs that appear to prominently regulate cellular migration, ECM protein transformation, ECM degradation and apoptosis in the growth plate are found to be MMP-9, MMP-13 and MMP-14 among other MMPs [29]. For the normal vascular and heart development an active and continuous changes in cell-cell adhesion, cell migration, cell proliferation, apoptosis and remodelling are required. For all these changes to occur, involvement of the MMP gene expression and activation of proMMPs are required. However, abnormal catalytic activities of MMPs have been found to be linked with various diseases and conditions such as atheroscleotic plaque formation and instability, vascular smooth muscle cell migration as well as restenosis, development of aortic aneurysm and progressive heart failures [28, 30].

MMPs have been found to degrade cartilage, tendons and bone ECM proteins in synovial joint arthritis. Not only that in the early T-cell mediated phase of rheumatoid arthritis, MMPs have been found to play a very critical role [31]. Also, in other prominent features of pathophysiology of arthiritis, MMPs play important roles such as; in the cytokine-induced inflammatory response which is largely involved in promotion of progression of ECM protein degradation and in dysfunctional apoptosis. MMP-1 and MMP-8 are responsible for mediating the degradation of Type I collagen (main interstitial collagen of tendon and bone) and Type II collagen (main collagen isotype of articular cartilage) [28, 32].

MMPs also play pivotal role in cancer and its progression. Gelatinases are prominently involved in the degradation of proteins in the ECM of basement membrane leading to the facilitation of the migration of the tumor cells into the blood vessels. The involvement of MMPs is also found in the diseases of central nervous system and in ischemic brain injury. MMPs are found to mediate disruption of the blood brain barrier, regulation of ECM protein destruction and remodelling. They also mediate tissue inflammation in response to the oxidative stress [28, 33].

1.5. Gelatinases

Proteolytic enzymes that have the capability of degrading or hydrolyzing gelatin are gelatinases. There are two types of gelatinases: A and B (MMP-2 and MMP-9 respectively). Structurally these two MMPs are quite different from the other MMPs as they contain FnII module which is made up of three fibronectin-II like inserts/repeats as we can see in figure 4. Although both gelatinases are structurally similar, they have a huge difference in their molecular size. ProMMP-2 has a molecular size of 72 kDa while proMMP-9 has 92 kDa. The reason behind this difference is due to a larger hinge region in MMP-9 that is heavily O-glycosylated in contrast to the small unglycosylated hinge region in MMP-2. MMP-2 are expressed by cells such as fibroblasts, endothelial cells and cancer cells and MMP-9 are expressed by cells such as monocytes, macrophages, neutrophils and epethelial cells [34].

1.5.1. Structure and regulation of MMP-9

Gelatinase B or MMP-9 has a structure (Fig. 6.) of a typical matrix metalloproteinase. It is composed of N-terminal pro-domain which is responsible for keeping active site present in the catalytic domain inactive. This N-terminal pro-domain is attached to the zinc containing catalytic domain which contains three FnII like repeats. The presence of these repeats occurs only in gelatinases and they facilitate the localization of gelatinases to connective tissue matrices and appear to be important for the degradation of macromolecules such as gelatin, elastin, and collagens (IV, V and XI), but do not influence the degradation of small chromogenic substrates [35-39]. The catalytic domain is attached to the HPX domain at the C-terminal with the help of a heavily O-glycosylated hinge region. HPX domains are involved in substrate specificity and they interact with various inhibitors and cell surface receptors [34, 40].

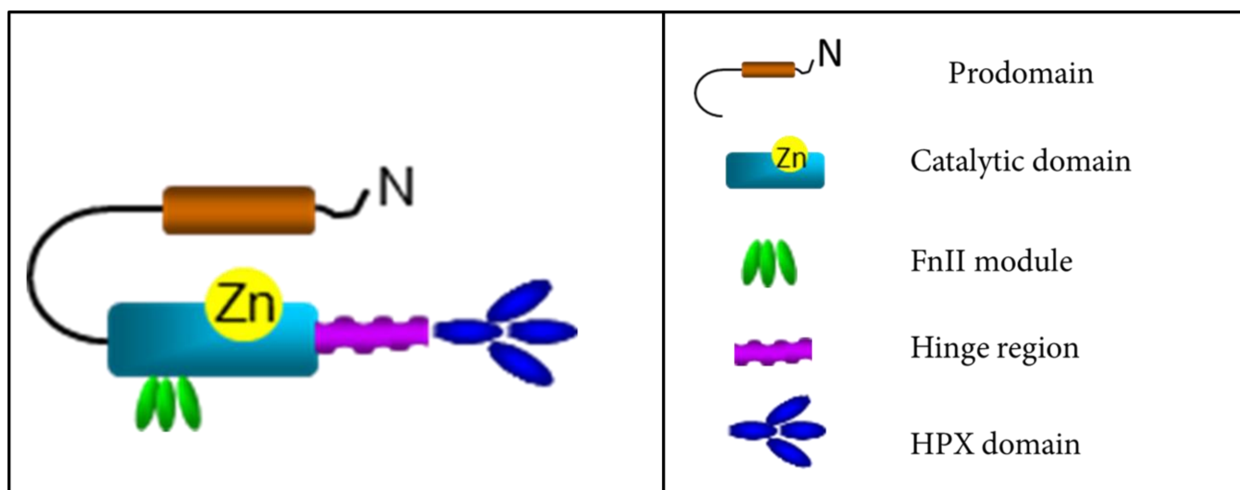

Fig. 6. Structure of a ProMMP-9 built up with various domains, modules and motifs.

The catalytic domain consists of a metal binding site and the active site. The active site in MMP-9 consists of a Zn^{2+} ion (Fig. 6.) and Glu402 which is catalytically essential [41]. The pro-peptide domain in MMP-9 is very important for latency. This domain contains approximately 80 amino acids which contain ''the cysteine switch'' that interacts with the catalytic zinc ion. This interaction keeps the whole enzyme inactive [42]. When the pro-peptide is cleaved off, the enzyme is activated. The most efficient activator of MMP-9 is found to be MMP-3. Apart from that other activators of MMP-9 are MMP-2, trypsin, kallikrein, plasmin, neutrophil elastase, hypochlorous acid and organomercurial compounds such as $HgCl_2$ (mercury chloride) and APMA (amino phenyl mercury acetate) [40, 43].

A cluster of three identical fibronectin repeats is present between the active site and the metal binding site in the catalytic domain. These repeats each contain two intramolecular disulfide bonds [44]. The catalytic domain is attached to HPX domain by the help of O-glycosylated hinge region which is very unique in MMP-9. It is 64 amino acids long and mostly contains proline, serine and threonine residues [45]. This hinge region provides intradomain and interdomain flexibility to MMP-9 [46]. This region is rich in O-glycans and its removal can cause limitations in MMP-9 mediated cell migrations [47].

The HPX domain consists of a four-bladed β-propeller where the blades are connected by disulfide bridges [48]. This domain is involved in complex formation (as described later in 1.5.4.) and for interaction with substrates such as gelatin, collagen type I and IV, elastin and fibrinogen. HPX domain is also involved in inducing auto-activation of the enzyme [40, 49].

The release of 92 kDa MMP-9 is regulated by a membrane-anchored hRECK protein which is present on the plasma membrane. The down regulation of RECK gene can result in increased secretion of MMP-9 that leads to morphological transformation [50]. RECK has also been demonstrated to be an inhibitor to MMP-9 [51].

The amino acid sequence of preproMMP-9 is seen in figure 7. The preproMMP-9 contains 707 amino acid residues in which there are 19 amino acid residues in signal peptide, 166 amino acid residues in FnII module and 187 amino acid residues in HPX domain. The X-ray structures of full length MMP-9 are not available which is due to the flexibility of the heavily O-glycosylated hinge region in MMP-9. However, the X-ray structures for the prodomain linked to the catalytic domain and for the HPX domain are available [44, 48].

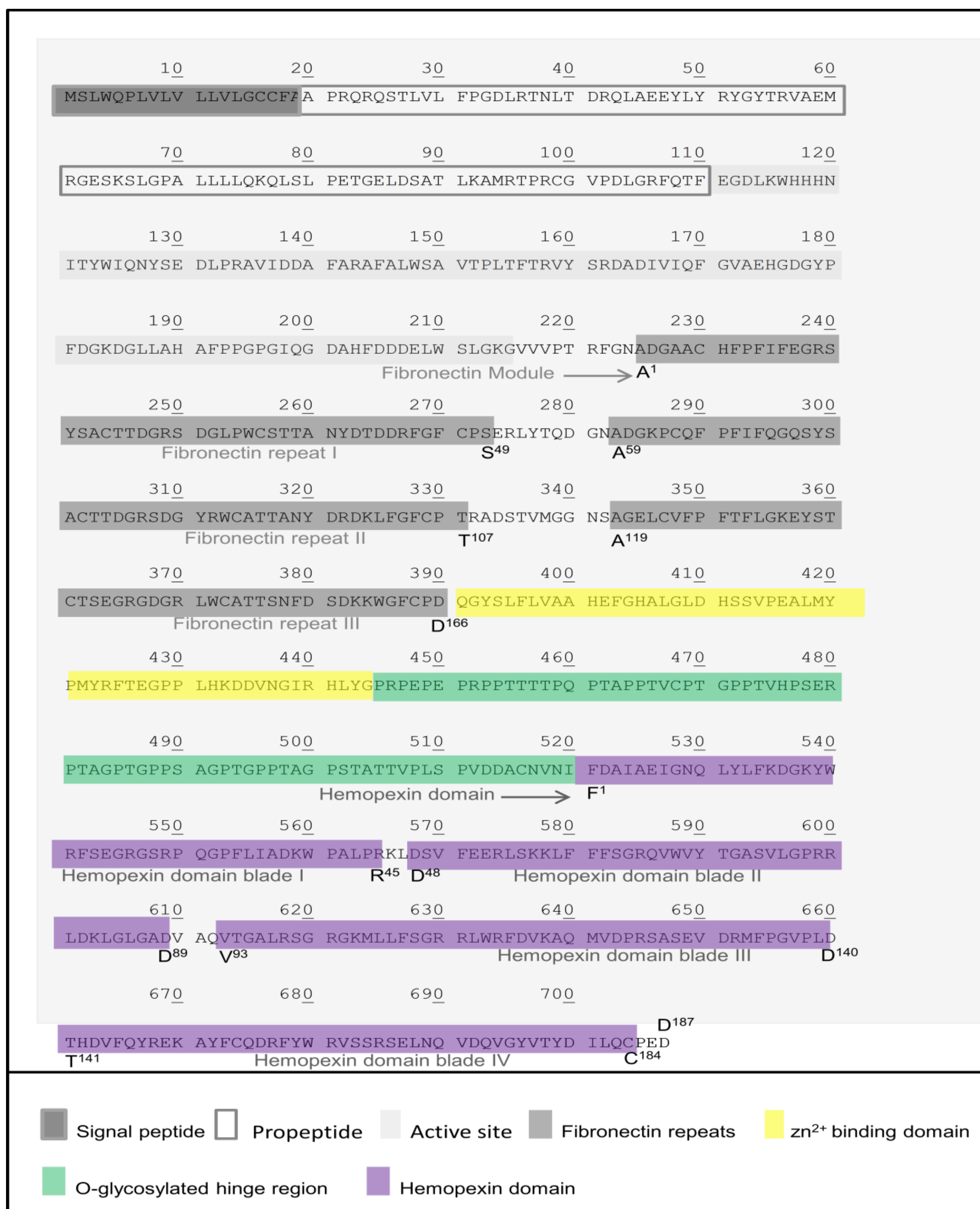

Fig. 7. Amino acid sequence of MMP-9. The numbering of the sequences of fibronectin module and hemopexin domain is indicated along with the three fibronectin repeats and the four hemopexin blades. In the figure we can see the signal peptide, propeptide, active site, fibronectin module and zinc binding domain present in catalytic site, O-glycosylated hinge region and hemopexin domain in different color

stretches. The amino acids in FnII module and HPX domain are numbered 1 and 166 (in case of FnII module) and 187 (in case of HPX domain) which corresponds to the numbering in the peptide arrays used in this thesis. In the FnII module, A^{225} is represented by A^1 and the last amino acid D^{390} is represented by D^{166}. Similarly in HPX domain, F^{521} is represented by F^1 and the last amino acid of the sequence D^{707} is represented by D^{187}. The indication of the domains are based on reference [40]. The sequence of human MMP-9 was taken from the MEROPS database [52].

1.5.2. Inhibitors of MMP-9

For the control of the level of MMP-9, various inhibitors come to use such as α_{2}-macroglobulin which is a major inhibitor of MMP-9 in circulation. These globulins trap the active forms of MMP-9 and remove them from circulation with the help of scavenger receptors. There are tissue inhibitors of matrix metalloproteinases (TIMPs) which are secreted in a non covalent complex with MMP-9. There are four types of TIMPs: 1 to 4 out of which TIMP-1 is the most unique inhibitor for MMP-9. It can form a complex with the inactive proMMP-9 by binding in the C-terminal and it can also form a complex with the active form of MMP-9 by binding its N-terminal to the catalytic domain of the MMP-9 [53]. Other inhibitors that are found to inhibit MMP-9 are RECK, MMP-2 and MT1-MMP [51].

1.5.3. MMP-9 in health and diseases

MMP-9 has been linked with many physiological and pathological conditions. MMP-9 along with other enzymes such as MMP-13 and MMP14, appears to be prominently regulating cellular migration, ECM protein transformation, ECM degradation and apoptosis in the growth plate [29]. MMP-9 along with MMP-2 plays an important role in cancer and is prominently responsible for the ECM degradation in the basement membrane which leads to migration of tumor cells to blood vessels. These enzymes have been found to facilitate the tumor growth and metastasis. MMP-9 specifically appears to actively release tissue-bound fibroblast growth factor as well as vascular endothelial growth factor resulting in tumor growth [21, 28]. Several researches have also shown the correlation between the level of MMP-9 in plasma and various cancers such as: breast cancer, gastric cancer, lung cancer, head and neck squamous cell carcinoma, hepatocellular carcinoma, colorectal cancer and ovarian cancer [54-56].

MMP-9 is highly expressed in astrocytic glioma and its expression can be linked to the aggressiveness of this disease. Also, it has been found that MMP-9 can be a mediator for oncogenic functions in the progression of this disease suggesting that MMP-9 could be a potential diagnostic as well as therapeutic target for patients with astrocytic glioma [57].

In the synovial fluid of patients with different inflammatory diseases, the raised level of MMP-9 has been found and its increase in level is associated with the severity of such inflammatory diseases such as rheumatoid arthritis. The level of MMP-9 is elevated in rheumatoid arthritis because it is produced by the local macrophages that are highly produced in inflamed synovial fluid [58].

MMP-9 is also important for wound healing and can be proved by its active involvement with the migration and repair of human bronchial epithelial cells (HBEC). The higher the migration rate of HBEC the higher the expression of MMP-9 inside these cells so, the MMP-9 mediated cell migration of HEBC is very important in wound healing in respiratory epithelium [59].

1.5.4. MMP-9 and its complexes

MMP-9 is secreted from cells as monomers or in various dimeric forms such as homodimer (two MMP-9 molecules are linked together) or heterodimers. Certain MMP-9 dimer cannot be dissociated by SDS and are reduction sensitive, which means that these proteins are linked by either disulfide bonds or could be due to a strong reversible interaction where intramolecular disulfide bonds are needed [34].

The proMMP-9 homodimer is formed inside the cell [60] with the help of non-covalent, hydrophobic interactions between the two C-terminal hemopexin domains of the enzymes [40, 48]. And for forming the dimer, the involvement of heavily glycosylated hinge region is very important [40]. TIMP-1 can form complex with proMMP-9 with the help of C-terminal domains of both molecules and an internal disulphide bond in the HPX region of proMMP-9. However, to be able to form such complex glycosylation of proMMP-9 needs to take place [34, 40, 53, 60].

MMP-9 has also been found to form complex with MMP-1 and the formation of such heterodimer involves the HPX region of both enzymes. This heterodimer has the ability of degrading gelatin and fibrillar type I collagen [53]. MMP-9 produced in human neutrophils can form complex with gelatinase B-associated lipocalin (NGAL) and the HPX region of MMP-9 is presumed to be involved in forming this dimer. This heteromer can also bind with TIMP-1 [61, 62]. MMP-9 has been found to form complex with haptoglobulin, fetuin-A as well as membrane proteins such as megalin, cell surface lipoprotein-receptor-related protein1 (CD91), DNA repair protein Ku and various isomers of CD44 [34]. The heterodimer complex formed between MMP-9 and haptoglobin is suggested to be a part of acute inflammatory response as these complexes are found to be formed only in the sera of cows with acute inflammation or even in healthy cows [63].

1.5.4.1. ProMMP-9/CSPG complexes

MMP-9 has been found to form complexes with chondroitin sulfate proteoglycans (CSPG) [64]. PMA stimulated monocytic cell line THP-1 was found to produce proMMP-9 not only as a monomer and homodimer but also as a reduction sensitive heterodimer in which it is strongly linked to the core protein of one or several proteoglycans [64, 65]. Two other cell lines U-937 and MonoMac however, were not found to produce any significant amount of proMMP-9/CSPG complexes and it was the same even when these cells were treated with PMA [9]. These proMMP-9/CSPG complexes were first believed to be formed with the help of disulphide links between MMP-9 and the core protein of one or several proteoglycan CSPGs [64] but later it was found that the complex could be reconstituted *in vitro* in the presence of compounds that prevent disulfide exchange and thiol oxidation and hence the two proteins are not linked together by disulfide bridges [9]. The CSPGs from all three cell lines THP-1, MonoMac and U-937 could form a complex with proMMP-9 *in vitro* and gave rise to two types of complexes, SDS-stable and SDS-soluble complexes [9].

SDS-stable complexes are those which remain stable even when it is passed through SDS as the name suggests and SDS-soluble complexes are those whose components are separated or dissolved when being introduced to SDS, hence SDS-soluble. A mixture of Triton X-100 and NaCl prevented the formation of both SDS-stable and SDS-

soluble complexes suggesting that the formation of such complexes involves compound forces. The SDS-stable complexes ranging from 300 kDa to 1000 kDa as well the SDS-soluble complexes involve binding between different regions of HPX domain in MMP-9 and the core protein of the CSPG [9]. Along with the HPX domain in the C-terminal, FnII module in the catalytic domain of MMP-9 is also found to involve in binding with the CSPGs in formation of both SDS-stable and SDS-soluble complexes [66].

1.5.4.2. Properties of proMMP-9/CSPG complexes

The Binding of proMMP-9 with the core protein of CSPG may alter the activity of the enzyme [67]. The proMMP-9 heteromers produced from treated or untreated THP-1 cells are seemed to have altered biochemical properties when compared with the monomeric forms of proMMP-9. It has been suggested that the MMP-9 in complex with CSPG and the MMP-9 monomer could have different substrate specificity as new exosites maybe exposed in the complex [9].

The presence of calcium has been found to activate the proMMP-9 in the complex and also thereafter cleave a part of the core protein of proteoglycan as well as the C-terminal hemopexin domain of the enzyme resulting in release of the activated enzyme forms from the complex [34]. These studies also indicated that the HPX domain of proMMP-9 was involved in complex formation.

The binding properties of proMMP-9/CSPG complex are different with collagen and gelatin [66]. MMP-9 and proMMP-9/CSPG complex seems to bind to different regions in gelatin [67]. Both collagen and gelatin bind to the FnII module of proMMP-9 but it has been found that FnII module is not involved in binding of collagen I and gelatin to the proMMP-9/CSPG complex [68] as formation of this complex results in hiding of the FnII module for binding. There seems to be a same or an overlapping binding site in the proMMP-9/CSPG heteromer for binding collagen I and gelatin. It appears that the proMMP-9 and proMMP-9/CSPG complex seems to bind to different and non-overlapping isotopes in gelatin [66]. The proteglycans that most commonly seem to complex with proMMP-9 are serglycin and versican which are produced by THP-1 cells. MonoMac is another cell line which does not produces serglycin but produces versican that can form proMMP-9/versican complexes *in vitro* [9]. It seems that formation of

proMMP-9/CSPG complex has been found to dissociate TIMP-1 which are bound to the proMMP-9 and this dissociation is facilitated by presence of Triton X-100, NEM and IAc [9].

1.5.4.3. Factors affecting the formation of proMMP-9/CSPG complex

PMA is the only agent found to stimulate the production of proMMP-9/CSPG complexes in THP-1 cells. Various agents such as TNF-α, M-CSF, etc were not found to be sufficient in increasing the synthesis of the proMMP-9/CSPG complex. The PMA stimulation of THP-1 cells leads to upregulation of synthesis of proMMP-9 monomer and homodimer, and proMMP-9/CSPG heteromer, but it was also found to increase cell death. However it is appears that the PMA induced upregulation of synthesis of proMMP-9 monomer, homodimer and proMMP-9/CSPG heteromer was not due to release of intracellular stored molecules from those dying cells. The signaling pathways responsible for the synthesis of CSPG were found to be different than those which were responsible for synthesis of the proMMP-9/CSPG heteromer and proMMP-9 [65].

Formation of SDS-soluble proMMP-9/CSPG complexes *in vitro* are prevented by TIMP-1 and this was assumed because the epitopes present in HPX region of MMP-9 that are involved in binding with TIMP-1 are the same or overlapping epitopes that are involved in formation of SDS-soluble complex. However, SDS-stable complexes are not affected by TIMP-1 [9]. Presence of gelatin has been found to inhibit the formation of both SDS-stable and SDS-soluble complexes which also suggests that FnII module is involved in complex formation [9].

The *in vitro* formation of proMMP-9/CSPG complexes have been found to be affected by the pH. More complexes were formed at pH 7.7 than in pH 5.5.This also suggested that complexes can be formed inside the cells if there is a co-localization of specific CSPG and proMMP-9 in the same secretory vesicles [9].

1.5.4.4. Physiological roles of proMMP-9/CSPG complexes

Proteoglycans interact with various structural molecules in the ECM as well as with cell surface receptors such as: collagen, laminin, fibronectin, fibrin, elastin, fibrillin, hyaluronic acid, CD44, epidermal growth factor receptor, intergins and selectins. The

interaction of proteoglycans with these molecules takes place through their core protein or the GAG chains. [69].

Different molecules such as proteinases (MMP-2 and MMP-7), proteinase inhibitors, growth factors, cytokines and chemokines bind to the GAG chains of the proteoglycans and this type of binding appears to alter the activity of these bound molecules which leads to their involvement in signaling events. These bindings can sometimes prevent degradation of the bound molecules too [70-72]. Active MMP-9 can form complex with certain GAG chains such as heparin sulfate, for example; highly metastatic colon carcinoma cells contain glypican like proteoglycans at their cell surface. The GAG chains present in these proteoglycans are heparin sulfate which forms complex with active MMP-9 and results in the cell migration and invasion of these proteoglycans. However, if there is presence of proMMP-9, it competes with these active MMP-9 for binding with the HS chains and thus results in inhibition of cell migration and invasion of the carcinoma cells [55].

Serglycin is one of the CSPGs which is found to be involved in forming complex with MMP-9 produced from THP-1. Functionally role of serglycin in forming complex with MMP-9 is unknown but it has been suggested that since both MMP-9 and serglycin bind to cD44 receptor, serglycin in proMMP-9/serglycin complex allows MMP-9 to link to cD44 receptor so that MMP-9 can attach to the cell surface of a normal or malignant cells. Thus serglycin is thought to act as a carrier molecule for MMP-9 [64].

Also, proMMP-9/CSPG complexes seem to be involved in cellular migration and invasion in tumors for example; the invasiveness of THP-1 cells increases after it is stimulated with PMA leading to increased production of MMP-9 along with other molecules [73].

1.6. Aim

Previous researches have shown that different monocytic leukemia cell lines such as THP-1, MonoMac and U-937 can produce proteoglycans. Different cell lines have been found to produce different proteoglcyans for example: THP-1 and U-937 produces serglycin and THP-1 and MonoMac produces versican. These proteoglycans have been found to form complexes with proMMP-9 *in vitro* [9]. Our aim was to separate different proteoglycans and proteoglycans associated proteins present in partly purified CSPGs from THP-1, MonoMac and U-937 cells and then perform *in vitro* reconstitution experiments to determine which proteoglycans/proteoglycan associated proteins that is most likely involved in the formation of the SDS-stable and SDS-soluble proMMP-9/CSPG complexes.

In the formation of the proMMP-9/CSPG complexes, it has been shown that the FnII module and HPX domain of proMMP-9 are involved in binding. Serglycin is one of the CSPGs that has been found to form both SDS-stable and SDS-soluble complexes with proMMP-9 *in vitro* [9]. Another aim in this thesis was to identify the motifs and amino acids in the serglycin core protein and regions, motifs and amino acids in the FnII and HPX domains in MMP-9 that are involved in the complex formation, as nothing is known about this.

2. Materials and methods

2.1. Materials

Materials	Producer (City, Country)
TRIS, urea, DMSO, citric acid, sodium acetate and Coomassie Brilliant Blue G-25 stain, Triton X-100	Merck (Darmstadt, Germany)
Acrylamide	BDH (Poole, UK)
Safranin O (no.S-2255), cetylpyridinuim chloride, HEPES, silver nitrate, alkaline phosphatase-conjugated antibodies, gelatine bloom 300, bovine serum albumin (BSA), blue dextran, apoferitin, alcohol dehydrogenase (ADH), acetic acid, Metahnol, goat anti-mouse antibody against versican (A2554)	Sigma-aldrich Chemical Co (St Louis, MO, USA)
Proteinase free chondroitin ABC lyase (cABC) and antibody against versican (2-B-1)	Seikagaku Kogyo Co (Tokyo, Japan)
Q-Sepharose, Sephadex G-50 (fine), Sephacryl S-400	GE-Healthcare Bio-Sciences (Uppsala, Sweden)
Magic marker molecular weight standards, NuPAGE MES-SDS running buffer 20X, pre-casted polyacrylamide gels (NuPAGE Novex 4-12% Bris-Tris gels)	Invitrogen (Carlsbad, CA, USA)
Western Blotting Luminol reagent	Santa Cruz Biotechnology (CA, U.S.A)
HRP-conjugated goat anti-rabbit secondary antibody	Southern Biotech (Birmingham, AL, U.S.A)
Recombinant TIMP-1	Calbiochem (San Diego, CA, U.S.A)
Rabbit polyclonal antibody against TIMP-1	Abcam (Cambridge, UK)
Rabbit antibody against MMP-9	Eurogentec (Liege, Belgium)
Rabbit antibodies against C-terminal, mid-region and N-terminal of serglcyin	Antibodies-online Inc. (Atlanta, U.S.A)
Ponceau stain	Wallert and Provost Lab (Minnesota State University Moorhead, Minnesota, U.S.A)
Silver staining kit	Pierce (Rockford, IL, U.S.A)
Peptide arrays, mutation peptide arrays and	Peptide Synthesis: Core Facility, The Biotechnology Centre of Oslo, University of

the soluble peptides	Oslo (Oslo, Norway)
Coomassie imperial protein stain, Spectra multicolor low range protein ladder and high range protein ladders	Thermo Scientific (Rockford, IL, U.S.A).
Recombinant serglycin and versican	Gift from Dr. A. Theocharis (University of Patras, Greece).

2.2. Instruments and equipments

Equipments used:	Producer (City, Country)
Spectrophotometer (Ultraspec III)	Pharmacia (Uppsala, Sweden)
Western blot electrophoresis instrument (PowerEase 500) for SDS-PAGE	Invitrogen Life Technologies (California, USA)
Image quant LAS4000 image reader	GE Health Care Bio-Sciences AB (Uppsala, Sweden)
Fujifilm LAS3000 image reader	FujiFilm (Tokyo,Japan)
Slot blot (Minifold II)	Schleicher & Schuell Inc (Keene, NH, USA)
Versa max tunable microplate reader	Molecular devices (Sunnyvale, California, U.SA)
Vortex mixer (Ser no: 110627098)	VWR international (Germany)
Speed vac concentrator (model: RH40-11)	Savant Instruments Inc (Farmingdale, N.Y, U.S.A)
Fraction collector (2211 superrac), hydrostatic pump	LKB Pharmacia (Bromma, Stockholm, Sweden)

2.3. Buffers and solutions

2.3.1. General buffers and solutions

1 M NaAc (pH 6.0), 1 L

NaAc (M.W: 82.03 g/ml) - 82.03 g

Milli-Q water added to give an approx. volume of 0.6 litres

pH adjusted to 6.0 using HAc (approx. 3 M)

Milli-Q water added to give a total volume of 1 litre

pH was controlled and if necessary adjusted to the correct pH

50 mM NaAc (pH 6.0), 1 L

1 M NaAc - 50 ml

Milli-Q water - 950 ml

1 M Tris (pH 6.8), 100 ml

Tris (base; M.W: 121.14 g/mol) - 12.1 g

Milli-Q water added to give an approx. volume of 80-90 ml

pH adjusted to 6.8 using HCl (approx. 1.0 M)

Milli-Q water added to give a total volume of 100 ml

pH was controlled and if necessary adjusted to the correct pH

2% NaAzide 20 ml

NaAzide - 0.4 g

Milli-Q water - 20 ml

1 M HEPES (pH 7.5) 1 L

HEPES (M.W: 238.301 g/mol) - 238.30 g

Milli-Q water added to give an approx. volume of 0.4 litres

pH adjusted to 7.5 using NaOH (approx. 10 M)

Milli-Q water added to give a total volume of 1 litre

pH was controlled and if necessary adjusted to the correct pH

0.1 M HEPES 500 ml

1 M HEPES - 50 ml

Milli-Q water - 450 ml

10% Ammonium persulphate 100 ml

Ammonium persulphate - 10 g

Milli-Q water - 100 ml

5X sample buffer 40 ml (0.25 M Tris/Hcl, 10% SDS, 50% glycerol, 0.5% Bromophenol blue) 40 ml.

1.0 M Tris pH 6.8 - 10 ml

87% Glycerol - 23 ml

SDS - 4 g

Bromphenol blue - 0.2 g

Milli-Q water - 7 ml

1X sample buffer 25 ml

5X sample buffer - 5 ml

Milli-Q water - 20 ml

Sample buffer with 0.5 M DTT 100 µl

5X sample buffer - 80 µl

2.5 M DTT - 20 µl

2.3.2. Buffers for gel filtration (Sephacryl S-400)

Equilibration buffer 1 (50 mM Tris, 150 mM NaCl, 100 mM $CaCl_2$, pH 7.5), 2 L

Tris (base; M.W: 121.14 g/mol) - 12.114 g

NaCl (M.W: 58.44 g/mol) - 17.532 g

$CaCl_2$ (M.W: 110.99 g/mol) - 22.198 g

Milli-Q water added to give an approx. volume of 1.4 litres

pH adjusted to 7.5 using HCl (approx. 1.0 M)

Milli-Q water added to give a total volume of 2 litres

pH was controlled and if necessary adjusted to the correct pH

Equilibration buffer 2 (4 M Guanidine hydrochloride, 50 mM NaAc, pH 6.0), 2 L

Guanidine hydrochloride (M.W: 95.53 g/mol) - 764.24 g

NaAc (M.W: 82.03 g/mol) - 8.203 g

Milli-Q water added to give an approximate volume of 1.4 L

pH adjusted to 6.0 using HAc (approx. 3 M).

Milli-Q water added to give a total volume of 2 litres

pH was controlled and if necessary adjusted to the correct pH

2.3.3. Gels, buffers and solutions used in Q-Sepharose ion exchange chromatography

Wash buffer (6 M urea, 50 mM NaAc, 0.35 M NaCl, pH 6.0), 1L

Urea (M.W: 60.06 g/mol) - 360.36 g

NaCl (M.W: 58.44 g/mol) - 20.45 g

NaAc (M.W: 82.03 g/mol) - 4.101 g

Milli-Q water added to give an approximate volume of 900 ml

pH adjusted to 6.0 using HAc (approx. 3 M).

Milli-Q water added to a the total volume of 1.0 L

pH was controlled and if necessary adjusted to the correct pH.

Elution buffer (6 M Urea, 50 mM NaAc, 1.5 M NaCl, pH.6.0), 1L

Urea (M.W: 60.06 g/mol) - 360.36 g

NaAc (M.W: 82.03 g/mol) - 4.101 g

NaCl (M.W: 58.44 g/mol) - 87.66 g

Milli-Q water added to give an approximate volume of 900 ml

pH adjusted to 6.0 using HAc (approx. 3 M).

Milli-Q water added to give a total volume of 1 litre.

pH was controlled and if necessary adjusted to the correct pH.

2.3.4. Buffers and solutions used in the detection of CS-chains (Safranin O assay)

Safranin O solution

0.02% Safranin O - 0.2 g

50 mM NaAc (pH 4.75) -100 ml

Extraction solution

CPC - 4.5 g

Milli-Q water - 45 ml

2.3.5. Buffers and solutions used in SDS gel zymography

Stacking gel buffer (0.5 M Tris, pH 6.8, 0.4% SDS), 10ml

Tris (base; M.W: 121.14 g/mol) - 0.605 g

SDS - 0.04 g

Milli-Q water added to give an approximate volume of 6 ml

pH was adjusted to 6.8 using 5 M HCL.

Milli-Q water added to give a total volume of 10ml.

pH was controlled and if necessary adjusted to the correct pH.

2% sodium azide (100 µl) added.

Separating gel buffer (1.5 M Tris, pH 8.8, 0.4% SDS), 100 ml

Tris (base; M.W: 121.14 g/mol) - 18.2 g

SDS - 0.4 g

Milli-Q water added to give an approximate volume of 60 ml

pH was adjusted to 8.8 using 5 M HCL

Milli-Q water added to give a total volume of 100ml.

pH was controlled and if necessary adjusted to the correct pH.

2% sodium azide (100 µl) added.

Separating gel for zymography (4507 µl)

Separating gel buffer - 1120 µl

2% gelatin - 225 µl

Milli-Q Water - 2266 µl

Acrylamide 40% - 874 µl

TEMED - 7 µl

10% Ammonium persulfate - 15 µl

Stacking gel for zymography (1501 µl)

Stacking gel buffer - 186 µl

2% gelatin - 145 µl

Milli-Q water - 1.0 ml

Acrylamide 40% - 155 µl

TEMED - 7 µl

10% ammonium persulfate - 8 µl

Electrophoresis Buffer, pH 8.3 (10X) 1L

Tris (base; M.W: 121.14 g/mol) - 30 g

Glycine (M.W: 75.07 g/mol) - 144 g

SDS (final conc. 1.0%) - 10 g

Milli-Q water added to give an approximate volume of 0.6 litres.

pH adjusted to 8.3 with conc. HCL

Milli-Q water added to give a total volume of 1 L.

pH was controlled and if necessary adjusted to the correct pH.

Gelatin (2%)

Gelatin bloom 300 - 0.2 g

Milli-Q water -10 ml

Staining solution stock (200 ml)

Coomassie Brilliant Blue - 0.4 g

Methanol – 120 ml

Milli-Q water – 80 ml

Staining solution

Staining solution stock - 20 ml

20% HAc - 20 ml

Staining stock solution is first filtered and then mixed with 20% HAc.

Washing buffer (400 ml)

Triton X-100 (warm) – 10 ml

Milli-Q water - 390 ml

Destaining solution (400 ml)

Methanol -120 ml

100% Acetic acid – 40 ml

Milli-Q water – 240 ml

Developing buffer 10x (0.5 M Tris, 2.0 M NaCl, 0.05 M $CaCl_2$, 0.2% Brij-35, pH 7.8),1L

Tris (base; M.W: 121.14 g/mol) -12.1 g

Tris HCl (M.W: 157.60 g/mol) - 63.0 g

NaCl (M.W: 58.44 g/mol) - 117 g

$CaCl_2$-2H_2O (147.01 g/mol) - 7.4 g

30% Brij-35 – 6.7 g

Milli-Q water added to give the total volume of 1litre.

2.3.6. Buffers and solutions used in western blotting

1M Tris pH 8.0, 1 L

Tris (base; M.W 121.14 g/mol) – 121.1 g

Milli-Q water added to give an approximate volume of 0.6 litres

pH adjusted to 8.0 using HCl (approx. 5 M)

Milli-Q water added to give the total volume of 1 L

pH was controlled and if necessary adjusted to the correct pH

1x TBST (Tris Buffered Saline solution with Tween) 1 L

5 M NaCl - 30 ml

1 M Tris pH 8.0 - 20 ml

100% Tween 20 - 1 ml

Milli-Q water added to give the total volume of 1L

Blocking buffer

Non- fat milk powder - 2 g

1x TBST - 40 ml

1xNuPAGE Running Buffer (MES-SDS) 20X

MES-SDS running buffer (20X) - 30 ml

Milli-Q water - 570 ml

Blotting buffer (0.05 M Tris, 0.4 M Glycine, 20% Methanol, pH 8.6), 1 L

Tris (base; M.W: 121.14 g/mol) - 5.8 g

Glycine (M.W: 75.07 g/mol) - 29 g

Milli-Q water - 800 ml

Methanol – 200 ml

2.3.7. Buffers and solution for silver staining

Fix 1 (50% methanol,10% HAc), 0.1 L

100% (Approx.) Methanol - 50 ml

100% (Approx.) HAc -10 ml

Milli-Q water - 40 ml

Fix 2 (7% HAc), 0.1L

100% (Approx.) HAc -7 ml

Milli-Q water - 93 ml

Silver nitrate (0.1%)

Silver nitrate - 50 mg

Milli-Q water - 50 ml

Development solution (3% $NaCO_3$, 0.037% Formalin) 0.1 L

$NaCO_3$ – 3 g

37% Formalin – 100 µl

Milli-Q water – 100 ml

2.4. Methods

2.4.1. Equilibration of the Sephacryl S-400 column for the determination of the separation properties of the column.

The general principle for column chromatography and the equipments used is described in appendix 7.1. Sephacryl HR (high resolution) was the preferred gel that was used during the gel filtration. This gel was obtained in suspension and approx. 200 ml of the gel was mixed with equilibrating buffer 1 and was applied to a column (length: 100 cm, diameter: 1.6 cm) resulting in 80 cm long Sephacryl S-400 gel (appendix 7.1.1.1 shows a figure of Sephacryl S-400 column used in the experiments). Immediately after the gel was packed in the column, it was equilibrated with equilibration buffer 1 using around 10 column volumes with the flow rate of 30 ml/hour. All experiments were done at 4ºC.

2.4.2. Determination of the separation properties of the Sephacryl S-400 column

The separation properties of the column was determined by determining the void volume (V_0) of the column with blue dextran (molecular mass: 2000 kDa), total volume (V_t) with bromophenol blue (molecular mass: 669.99 Da), and the elution volume (V_e) of three globular protein standards: bovine serum albumin (BSA: molecular mass of 29 kDa), alcohol dehydrogenase (ADH: molecular mass of 150 kDa), apoferitin (molecular mass: 443 kDa).

V_0 was determined by applying 1ml blue dextran (10 mg/ml) and V_t was determined by applying 1 ml bromophenol blue (0.5 mg/ml). For determination of V_e of the different globular proteins we did separate experiments by applying 1 ml of each protein to the column in separate experiments with these standards: BSA (60 mg/ml), ADH (4 - 30 mg/ml) and apoferitin (10 mg/ml). For each standard, 2-3 separate experiments were conducted. All the standards were made with equilibration buffer 1. A flow rate of 30 ml/hour was used and 1.5 ml fractions were collected. The absorbance of BSA, ADH and Apoferitin were determined at 280 nm. The absorbance of blue dextran and bromophenol blue was determined at 610 nm. Absorbance measurements were performed with a Spectrophotometer (Ultraspec III) from Pharmacia.

2.4.3. Purification of proteoglycans using Sephacryl S-400 column

Equilibration of the column was done with approx. 1L of equilibration buffer 2. The same buffer was used for preparing the samples. The CSPGs samples used in my experiments had previously been isolated and partly purified (Q-Sepharose ion exchange chromatography) from THP-1, U-937 and MonoMac cell conditioned media by our lab engineer Eli Berg as described in [9, 64]. These partly purified CSPGs were then further purified by me on the Sephacryl S-400 column.

Two separate experiments with THP-1 samples were performed. In both experiments, 1 ml CSPG (3 mg/ml) in equilibration buffer 2 was loaded to the column. Also, two separate experiments with U-937 samples, 0.5 ml CSPG (3 mg/ml) prepared in equilibration buffer 2 were loaded to the column. And three different experiments with MonoMac samples were done. In all experiments with media from MonoMac cells, 0.5 ml CSPG (2 mg/0.5 ml) in equilibration buffer 2 was loaded in the column. In each experiment, the concentration of samples used was based on the amount of GAG chains.

Different flow rates were used in different experiments and it ranged from 15 to 30 ml per hour. Safranin O method was applied to the fractions collected from the experiments to determine where the CSPGs were eluted. To do this, at first every second fraction were applied to the Safranin O method. Then if necessary, every fraction of the determined peak of CSPG was then applied to the Safranin O method. The fractions which contained CSPGs were pooled and diluted 20-25 times with 50 mM NaAc (pH 6.0) and applied to Q-Sepharose ion exchange chromatography. The dilution of these pooled samples was done to ensure the concentration of Guanidine hydrochloride (equilibration buffer 2) was at 0.35 M or lower, in order to assure that the CSPGs would bind to the column.10 fractions were collected from the eluted samples from the Q-Sepharose column. The Safranin O method was used to determine which fractions contained the CSPGs and so only the CSPG containing fractions were pooled, desalted on Sephadex G-50 fine columns and thereafter were concentrated using a vacuum centrifuge (Speedvac).

2.4.4. Safranin O method

The quantification of glycosaminoglycan chains bound to the proteoglycan is done reliably as precipitates by the help of cationic dye Safranin O [74]. These precipitates are formed after mixing proteoglycans and glycosaminoglycans with Safranin O and collected on a nitrocellulose membrane with the help of a slot blot apparatus attached to a vacuum-aided filtration (appendix 7.2, Fig. A3). The colored dots are cut out and solubilized in extraction solution. The intensity of the colored precipitates are measured spectrophotometrically at 536 nm using either a spectrophotometer (Ultraspec III) or a microplate reader (Versa Max tunable microplate reader coupled to computer which used the Soft Max Pro software).

A brief description of the Safranin O method is as follows: First the nitrocellulose membrane was cut according to the number of samples and was kept in 40% ethanol for 2 seconds and then was kept in water. The membrane was then kept between the two plates of the slot blot apparatus over a filter paper. The apparatus was fixed and connected to the vacuum pump. 300 µl of Safranin O solution was pipetted into the wells and the samples (15-30 µl) were added in the wells. The vacuum pump was turned on and the wells were washed with 250 µl water. After all the solution was sucked out of the wells, the vacuum pump was stopped and the membrane was taken out. These dots (precipitates) were then cut out and transferred to 1.5 ml eppendorf tubes containing 200 µl of extraction solution. These tubes were vortexed and then incubated at 37^{0} C in a shaker. The absorbance of the solubilized color was determined at 536 nm by using spectrophotometer or with the help of a micro plate reader by using Softmax Pro program 5.4.1.

The amount of GAG chains in the samples from the experiments were estimated from a standard curve of 0-2.5 µg of chondroitin sulfate (shark cartilage) per well. Then the elution profiles of the fractions from different samples from different experiments were created with the help of Sigma Plot 12 (Systac Software Inc).

2.4.4.1. Optimization of the quantitative determination of CS-chains by the use of the standard CS from shark cartilage using the Safranin O method

The quantification of sugar chains in the samples was done with the help of CS-chains from shark cartilage with known concentration. In order to do that we first needed to optimize our standards for better quantification of our purified samples from gel filtrations.

To do this first we prepared a stock solution of 19.5 mg/ml CS-chains in milli-Q water. From this stock solution, a new stock solution containing 1 mg/ml CS-chains in milli-Q water was made. This solution was used to prepare standards ranging from 0 to 5 µg/ 30µl as shown in table 2.

Table 2. Scheme for the preparation of shark cartilage CS standards

CS (µg/ 30 µl)	1 mg/ml shark cartilage CS (µl)	Milli-Q water (µl)
0	0	100
0.5	1.66	98.34
1	3.33	96.67
1.5	5	95
2	6.6	93.4
2.5	8.33	91.67
3	10	90
3.5	11.66	88.34
4	13.33	86.67
4.5	15	85
5	16.66	83.34

These standards were prepared in the total volume of 100 µl. The Safranin O test was done (procedure as 2.4.4) and three parallels of the standards were used. However, the procedure was repeated again with more accuracy in mind. From the stock

concentration of 1mg CS/ml, standards: 0 - 2.5 µg CS/ 30 µl were prepared in milli-Q water to give a total volume of 100 µl. The scheme for the preparation of these standards is shown in table 3.

Table 3. Scheme for the preparation of the shark cartilage CS standards.

CS (µg/ 30 µl)	1 mg/ml Shark cartilage CS (µl)	Milli-Q water (µl)
0	0	100
0.25	0.83	99.17
0.5	1.66	98.34
0.75	2.5	97.5
1	3.33	96.67
1.25	4.16	95.84
1.5	5	95
1.75	5.82	94.18
2	6.6	93.4
2.25	7.5	92.5
2.5	8.32	91.68

The Safranin O method (as in 2.4.2) was repeated with these standards. The procedure was repeated again but this time we prepared a fresh stock solution of 24 mg/ml CS-chains in water. From this stock solution a new stock solution of 10 mg/ml was made from which 1 mg/ml stock solution was made. From this stock solution of 1 mg/ml we prepared an end stock solution of 0.1 mg/ml and this end stock solution was used to prepare the standards: 0, 0.75, 1.5, 2.0 and 2.5 µg/ 30 µl. The total volume of the standards was changed from usual 100 µl to 200 µl. The scheme for the preparation of the standards is shown in table 4.

Table 4. Scheme for the preparation of shark cartilage CS standards.

CS (µg/ 30 µl)	0.1 mg/ml Shark cartilage CS (µl)	Milli-Q water (µl)
0	0	200
0.75	50	150
1.5	100	100
2.0	133.3	66.77
2.5	166.7	33.3

The Safranin O method (as in 2.4.2) was repeated with these standards.

2.4.5. Q-Sepharose column chromatography

Q-Sepharose column chromatography is a type of ion-exchange column chromatography (see appendix 7.1.2 for information about ion-exchange column chromatography). The Q-Sepharose gel matrix is positively charged and is in the state of equilibration with negatively charged particles. But when we add a negatively charged protein solution to the gel matrix, these proteins in the solution displaces the negatively charged particles and binds to the matrix. For the elution of these bound proteins, higher concentration of negatively charged solute such as NaCl needs to be added so that the negatively charged particles competes with the proteins for the binding site at the matrix and then displaces them. Displaced bound proteins are eluted from the column.

The different pools of CSPG and proMMP-9/CSPG complexes from the different experiments were passed through Q-Sepharose columns. Before applied to the columns the samples were diluted 10-25 times with 50 mM NaAc (pH 6.0) to reduce the high salt concentration (guanidine hydrochloride) of Equilibration buffer 2 (from Sephacryl S-400 column chromatography) to be less than 0.35 M in order to avoid that

a high salt concentration prevent the binding of the CSPG to the column. However, samples from *in vitro* reconstitution experiments are not diluted due to the low salt concentrations in the samples and these columns are not attached to a pump due to the small column size and sample volume.

For the large sample volumes obtained from the Sephacryl S-400 gel chromatography a 4 ml plastic column was used where small glass wool filter rolls were made and put in the bottom of the column to prevent the gel material to leak out of the column. Then the gel bed volume of 1ml was applied to the column. The column was attached to a pump and to the flask containing our diluted sample with a thin rubber pipes (as seen in appendix Fig. A2.). The column was then equilibrated with 50 mM NaAc (pH 6.0) for 10 minutes. The column was taken to 4ºC where the column was washed with wash buffer for 10 minutes. The sample pool was then applied to the column. After that the column was washed for 10 minutes with wash buffer. Then the sample was eluted with elution buffer using an elution volume of 1.5 ml per fraction (total of 10 fractions were collected) and the Safranin O test of these fractions were performed to assure the presence of CSPGs. The fractions with largest amount of CS were selected and pooled to be desalted in Sephadex G-50 fine gel.

Similarly, in case of small sample volumes (in *in vitro* reconstitution experiments), the experiment was carried out at room temperature. 4 ml plastic column were prepared with small glass wool filter rolls and the gel bed volume of 0.1 ml was applied to the columns. The columns were then fixed on a plastic stand and were washed with 200 - 500 µl wash buffer for 3-4 times, after which the 50 µl of samples was applied to the columns. The pass-through fraction was discarded and then again the rest of the sample was applied to the column. The column was washed with the 200 - 500 µl wash buffer for 4-6 times. 50 µl of elution buffer was applied to the column and then the pass through fraction was discarded. This was done to displace the column volume. The CSPGs were thereafter eluted by 180 µl elution buffer. The eluted fractions were desalted in Sephadex G-50 fine gel.

2.4.6. Desalting of the samples with help of Sephadex G-50 Fine gel

Sephadex fine G-50 is a gel formed by beads and this gel is made up of dextran cross linked with epichlorohydrin. This gel has the tendency of swelling in aqueous solutions, especially water. The bead size in this column ranges from 20 to 80 µm. The fractionation range of the globular proteins ranges from 1500 to 30000 and the fractionation range of dextrans ranges from 500 to 10000 [74].

First a glass wool filter was rolled and was put inside 10 ml plastic columns. Then Sephadex G-50 fine gel was gently stirred to get a homogenous mixture. 600 µl of the gel was applied to the column. After the gel was set, the column was washed with Milli-Q water for 2-3 times. Then 1.0 ml samples were applied to separate columns. Samples were eluted with 1.5 ml Milli-Q water. The eluents were concentrated using vacuum centrifuge (Speedvac) down to 100-1000 µl (in case of large volumes of samples).

2.4.7. *In vitro* reconstitution

The *in vitro* reconstitution is a procedure where, the different components of the proMMP-9/CSPG complex are mixed together in order to form the complex in a test tube. In some of the experiments, in addition to the proMMP-9 and the CSPG, various peptides (short sequences of Serglycin, the FnII module and the HPX domain of MMP-9) were also added to the mixture to determine if they inhibit the complex formation. Such reconstitutions are called *in vitro* reconstitution competition experiments.

In *in vitro* reconstitutions, 5-26 µg proteoglycan (CS-chains) were mixed with 12.5 µM MMP-9, 10 µl 1 M HEPES (pH 7.5) and Milli-Q water (to make the total volume of 100 µl). These mixtures were then incubated for 2-24 hours at 37°C. Then the samples were passed through Q-Sepharose columns (as in 2.4.5). The eluents were then desalted using Sephadex G-50 fine gel columns (with 600 µl gel bed) as described in 2.4.6. The desalted samples were concentrated in vacuum centrifuge until they were dry. These dried samples were dissolved in sample buffer and used in gelatin zymography.

2.4.8. SDS-PAGE

Gel electrophoresis is a technique in which the separation of different charged molecules take place on the basis of their physical properties such as charge or molecular mass. These molecules are separated in the gel matrix by the application of an electric current. So, for the identification of individual proteins in samples, proteins are separated using polyacrylamide gel electrophoresis (PAGE). If the gel contains sodium dodecyl sulfate (SDS), it is called as SDS-PAGE. Before a sample is applied to SDS-PAGE the protein samples are either heated in the presence of a reducing agent, or not heated or treated with reducing agents. For zymography the samples are not heated or treated with reducing agents as these techniques are used to observe the proteolytic activities of enzymes. In SDS-PAGE where the gels either is going to be used in western blotting or detect protein bands, samples can be treated in the various ways as stated above. Reducing agents such as DTT and mercaptoethanol breaks disulfide bonds in these proteins. By comparing reduced and non-reduced samples, information is obtained of a molecular size of a protein subunit and if the protein occurs in either monomeric or oligomeric forms where the protein chains are linked by disulphide bridges. Various sets of proteins with known molecular weights are loaded alongside protein samples so that these proteins can act as references to the molecular masses of our sample proteins. The protein samples give bands in different sites of the gel, according to their molecular masses. The proteins with higher molecular masses travel more slowly through the gel matrix than proteins with lower molecular masses [75].

2.4.8.1. Gelatin zymography

Gelatin zymography is an electrophoretic technique which is based on SDS-PAGE. In gelatin zymography, gelatin a substrate for MMP-9 and other proteinases is copolymerized with the polyacrylamide gel matrix. For the detection of proteolytic activity of enzymes especially of gelatinases, this technique proves to be a valuable and very sensitive tool. Enzyme degradation of the gelatin substrate appears as clear bands against a deep blue background. The merit of using this technique is that it allows an estimation of the molecular weight of the enzymes. Not only that, it helps in the

identification and monitoring of specific and non-specific enzymatic activities in various biological and clinical samples. However, the major limitation of this technique is that it cannot provide an exact information on the net proteolytic activity of gelatinases as it does not take account of endogenous inhibitors. The special characteristic about gelatin zymography is that, it allows us to see the activity of normally inactive forms of MMPs. This is because in the presence of SDS, the conformation of these enzymes change and when SDS is removed during washing process, the enzyme refolds. However, during the refolding the prodomain binds to the active site in such a way that the enzyme has the capability to cleave off the prodomain, leading to its autoactivation. This property of MMPs is very unique. SDS also disrupts the non-covalent interactions between the inhibitors (TIMP) and the enzymes, which helps furthermore in detection of gelatinolytic activity as the MMPs and TIMPs has been separated during the electrophoresis [76]. Various molecular weight standards are used to identify the molecular mass of the unknown bands one wishes to analyze.

During this experiment it is very important to wear gloves, lab coat and work under hood as polyacrylamide is neurotoxic. First the zymography plates between which the gel was prepared were cleaned with 96% ethanol and were fixed horizontally in a zymography apparatus. The gelatin gel was prepared which composed of stacking and separating gel with 4% and 7.5% of polyacrylamide respectively. First, separating gel was prepared and loaded between the plates. To prevent air to interfere with the polymerization of the gel, water was added on the top of the unpolymerized acrylamide. After the separating gel was set, the water was removed and stacking gel was prepared on top of the separating gel. A 15 tooth comb was pressed into the stacking gel before it had polymerized. This comb was pressed so that 15 wells could be formed in the stacking gel. After the stacking gel was set, the plates were taken out and fixed in the electrophoresis chamber. 70 ml of Electrophoresis buffer 1x was poured on the upper part of the electrophoresis chamber which supported the gel and 70 ml was poured in the lower part of the electrophoresis chamber. The combs were taken out and the wells were cleaned by the buffer. The gel was ready to be loaded with samples.

The samples dried in the vacuum centrifuge were mixed with 8 µl of 1X sample buffer. Then the samples along with the 3 - 5 µl standards (mixed with 2-4 µl 5X sample buffer) were loaded into wells of the gelatin zymography gel that was prepared as described above. The gel was run at 20 mA per gel for 2 hours. Cold water (approx. 4ºC) was being circulated through electrophoresis chamber while the gel was running. After the electrophoresis the gel was taken out and was washed twice in100 ml wash buffer for thirty minutes each time. The wash buffer was removed and 100 ml of incubating buffer was added and the gel was incubated at 37ºC overnight. The next day the incubating buffer was removed and the gel was stained with staining solution. The gel was kept in a shaker. After an hour the gel was destained with destaining buffer. This was done till the bands in the gel were clear and observable after which the gel was photographed.

2.4.8.2. Western blotting analysis of the purified CSPGs

For the principle of western blotting see appendix 7.3.

The purified CSPGs samples were prepared as in 2.4.8.3 and same treatment was done for standards (purified serglycin and versican from Dr.A.Theocharis (University of Patras, Greece). 4-12% SDS-polyacrylaminde gel (commercially available gel) was taken out of the gel bag. The gel was then rinsed with milli-Q water, the white tape in the bottom of the cartridge was taken out and the comb was removed which was rinsed with 1XNuPAGE MES-SDS running buffer (20X). The gel was arranged in the mini-cell rack orienting inwards (In case of only one gel, buffer dam can be used as gel number two).1X NuPAGE running buffer was prepared and the inner chamber of electrophoresis tray was filled with 1X NuPAGE running buffer. Leakage was checked and then the rest of the buffer was added to the outer chamber. The samples, along with magic marker molecular weight standards, were loaded to the gel. The gel was run for 35 minutes at 200 V.

After 35 minutes, the PVDF-membrane was cut in the same size as the gel and then the membrane was kept in methanol for 3 seconds followed by 10 seconds in water. After that, the membrane was kept in blotting buffer for more than 5 minutes. When the gel was ready, the casket was cut open by breaking the sides. A gel-knife was used to break the gel casket. The gel was taken out and was placed on top of a pre-wetted filter

paper. The lowest part of the gel was removed by cutting it off. Then pre-wetted PVDF-membrane was placed on top of the gel and then a pre-wetted filter paper was placed on top of the membrane. The air bubbles were removed by slowly rolling a glass rod on the membrane. The pre-wetted pads and filter paper and the membrane were arranged according to the blotting module in the cathode core for blotting. The inner chamber of the cathode core was filled with the blotting buffer and the outer with water for cooling. After the lid was put on, blotting was done according to the desired program. After the blotting was done, the membrane was taken out from the pads and kept inside a tube and was washed in 5 ml 1x TBST for 5 minutes in a rotating wheel. The blocking of the membrane was done by adding 5 ml blocking buffer for an hour. The membrane was incubated with primary antibodies (5 µl mouse antibody against versican diluted 1:1000 and 1µl rabbit antibody against serglycin diluted 1:10000) diluted in 5 ml blocking buffer. The next day, after removing the primary antibody solution the membrane was washed with 1x TBST for three times each 5 minutes. The membranes were incubated with HRP conjugated secondary antibodies (1µl goat anti-mouse antibody diluted 1:5000 and 2.5 µl goat anti-rabbit antibody diluted 1:2000) in 5 ml for one hour in room temperature. The membrane was thereafter washed with 1x TBST for 3 times each five minutes. At last the membrane was developed in 1:1 ratio of luminal reagent A and luminal reagent B (Western Blotting Luminol reagents) for antibody detection and images were obtained using the luminescent image analyser (Image quant LAS 4000 or Fujifilm LAS 3000).

2.4.8.3. SDS- PAGE for silver staining and coomassie staining

Bands in SDS-PSGE can be detected by using various techniques, two of which has been used in this project are: silver staining and coomassie staining.

The samples (CSPG samples from purified THP-1 and MonoMac cells) were prepared in 1.5 ml eppendorf tubes where 2.5 - 88.5 µg samples were added. The samples were dried completely or dried until the volume was 12 µl. 1.5-10 µl 0.1 M HEPES buffer and 0-12 µl milli-Q water was added. Then 1µl cABC was added and incubated at 37ºC for 2-24 hours. A control sample was made with a mixture of 1µl cABC, 1.5-10 µl 0.1M HEPES and 0-12.5 µl milli-Q water. Then all the samples including the control sample were treated with 3-5 µl sample buffer containing 0.5 M DTT and were incubated in

boiling water for approx. 4 minutes. The tubes with these samples were then centrifuged for 30 seconds at 1000 rpm and were loaded in 4-12% NuPAGE gel (readymade gel) along with 5 µl of each spectra multicolor high range and low range protein ladders. First the wells of the gel were cleaned with MES-SDS running buffer. 600 µl MES-SDS running buffer was added to the electrophoresis tray. Then the machine was set for 35 minutes at 200V. See appendix 7.4 (Fig. A4.) for the setup of SDS-PAGE electrophoresis. After the program was finished, the gel was treated according to the choice of staining process.

2.4.8.3.A. Silver staining

Silver staining is technique in which proteins in polyacrylamide gels are visualized with the help of metallic silver. The principle behind silver staining is that the proteins in the samples bind to the silver ions when the gels are incubated with silver solution. These silver ions are reduced to metallic silver when they come in contact with macromolecules in polyacrylamide gels. Once they are reduced to metallic silver, they are insoluble and visible and hence the proteins are visualized. The main steps in this procedure are: fixation for getting rid of interfering compounds, sensitization, silver impregnation with silver nitrate solution and finally the development to help build up the silver metal image [77, 78].

The gel was taken out and kept in a tray with Fix 1 for 20-30 minutes. It was removed and Fix 2 was added for another 20-30 minutes. The gel was washed for 20 min to 2 hours. After that 0.1% silver nitrate was prepared freshly and added to the gel for 20 minutes. The gel was washed for 2 times in a short span after which it was developed with 3% sodium carbonate. Around 10ml of 7% acetic acid was added in the development solution for approx. one minute and was removed. 100 ml of fresh 7% acetic solution was prepared and added. The bands were observed and the picture was taken.

2.4.8.3.B. Coomassie staining for mass spectrometry (M.S) analysis.

Coomassie staining is a process that allows the detection of protein bands in PAGE, SDS-PAGE, isoelectric focusing as well as in 2D gels. This staining process detects less than 3 ng protein per band and is compatible with mass spectrometry analysis of bands as well as for protein sequencing [78].

The gels after SDS-PAGE (as described in 2.5.9) were handled with care as the bands that were produced in the gels were sent to M.S for analysis. The gels were washed in Milli-Q water for 3 times each 5 minutes. Then 25 ml imperial protein stain was added to the gel and was incubated for one hour. Then the gel was destained with Milli-Q water overnight. Next day the bands were observed and the picture was taken. The bands which were selected for MS analysis (refer to appendix 7.6 for information about mass spectrometry) were cut off using a clean sterile blade. It is very important to wear an apron, gloves, mask and a cotton cap to cover the head. This was done to prevent contamination of the gels with keratin from the skin and hair.

2.4.9. Peptide arrays

Peptide array is an important tool that helps to discover various protein-protein interactions. These arrays are made up of hundreds to even thousands of immobilized peptides in various supports such as filter paper, glass or monolayer. Peptides are used because various protein binding and enzymatic activities are mostly directed towards peptides and so, these peptides provide a platform for the identification of several biologically active motifs and hence to the identification of protein-protein and protein-peptide interaction motifs. Not only that, peptide arrays have the wide range of applicability such as: to identify the action of enzymes, the adhesion of cells, the binding of metals and many more [79].

Peptide arrays based on the amino acids sequences of serglycin (131 amino acids), the MMP-9 FnII module (166 amino acids) and the MMP-9 HPX domain (187 amino acids) were ordered from The Peptide Synthesis: Core Facility, The Biotechnology Centre of Oslo, University of Oslo (see appendix 7.7 for the amino acid sequences of the peptides of the peptide arrays and the figure of ponceau stained peptide array membranes). The first peptide in the peptide arrays was made up of first twenty amino acids and each

successive peptide had a two amino acid shift. The membranes containing the peptide arrays for serglycin, FnII module and hemopexin domain were first ponceau stained, which is basically staining the membrane with 0.1% ponceau stain in 5% HAc for about 5 minutes at room temperature. These membranes with the arrays were then dried, marked and cut out as individual arrays for serlgycin, Fn-II module and hemopexin domain. The membranes were then fitted inside a 50 ml plastic tube and the membrane was washed with water in a rotating wheel for 5 minutes. Then the membranes were washed again with 10 ml 1x TBST for 5 minutes after which the membranes were blocked with 10 ml blocking buffer (blocking buffer used in western blot) for one hour in a rotating wheel. This step was done to prevent nonspecific binding of our proteins to the membrane.

After this step, the serglycin array membrane was incubated in 10 ml blocking buffer containing 8.8 µl proMMP-9 (stock conc: 1.13 ng/ml), the arrays of Fn-II module and hemopexin domain were incubated in 10 ml blocking buffer with 10 µl His-tagged Serglycin (stock: 1 ng/µl) and the arrays of serglycin and hemopexin domain were incubated in 10 ml blocking buffer with 30 µl human TIMP-1(stock 15 ng/µl, 0.5 mM). These incubations were done for 1-4 hours at room temperature.

Then the membranes were washed 3 times for 5 minutes with 10 ml 1x TBST. Then different primary antibodies according to the type of arrays were diluted in 10 ml blocking buffer and were added to the tubes with the arrays. In the tube with the array of serglycin incubated with proMMP-9, 10 µl primary antibody against human MMP-9 (rabbit antibody against MMP-9, stock: 5.1 mg/ml, diluted 1:1000) was added in 10 ml blocking buffer. In tubes with arrays of Fn-II module and hemopexin domain incubated with His-tagged serglcyin , rabbit antibodies against the C-terminal, mid-region and the N-terminal of the Serglycin were added: 2 µl of C-Terminal (0.25 mg/ml, ABIN656226), 8 µl of N-terminal (1 mg/ml, ABIN971784) and 8 µl of Mid-region (1 mg/ml, ABIN501963) were added in 10 ml blocking buffer. In tubes with arrays of serglycin and hemopexin domain incubated with TIMP-1, rabbit polyclonal antibody against TIMP-1 (ab-11795, 1:1000 dilution) was added with 10 ml blocking buffer. The membranes were then incubated overnight in the rotating wheel at - 4ºC.

The next day, the membranes were washed with 10 ml 1x TBST for 3 times for 5 minutes. Then 2.5-10 µl HRP conjugated goat anti-rabbit secondary antibodies were diluted in 10 ml blocking buffer and the arrays were incubated in this mixture for one hour at room temperature. Then the membranes were washed with 10 ml 1x TBST for 3 times for 5 minutes. At last the membranes were developed in 1:1 ratio of luminal reagent A and luminal reagent B (Western Blotting Luminol reagents) for antibody detection and images were obtained by luminescent image analyzer (Image quant LAS 4000 or Fujifilm LAS 3000).

2.4.10. Mutation peptide arrays

After the analysis of the peptide arrays, we ordered a set of peptide arrays with mutated amino acids (see appendix 7.8) assumed to be involved in binding. These peptide arrays of mutated amino acids of serglycin, Fn-II module and hemopexin domains were ordered from the same place from where the peptide arrays were ordered and these arrays were treated in same way as described for the peptide arrays in section 2.4.9.

2.4.11. *In vitro* reconstitution competition experiments

In case of *in vitro* reconstitution competition experiments, different peptides based on sequences in serglycin, FnII and HPX (3 peptides based on sequence of serglycin, 3 peptides based on sequence of Fn-II repeats, 3 peptides based on sequence of HPX domain) were ordered from The Peptide Synthesis: Core Facility, The Biotechnology Centre of Oslo, University of Oslo. One peptide (D9) based on the sequence of serglycin was ordered from J. Eksteen (Institute of Medical Biology, Faculty of Health Sciences, UiT-The Arctic University of Norway, Tromso, Norway). For the detailed description of these peptides, see appendix 7.9. (Table A1.)

All peptides were dissolved in 100% DMSO giving a peptide concentration of 10 mM based on the purity of the peptides. From this solution two new stock solutions were made in 100% DMSO with the following peptide concentrations: 1.0 mM and 0.1 mM. The samples for the *in vitro* reconstitution competition experiments were prepared as shown in Table 5.

Table.5 Scheme for *in vitro* reconstitution competition experiments.

Tubes numbers:	**1**	**2**	**3**	**4**
Peptide (µM)	**0**	**10**	**100**	**1000**
10 mM Peptide (µl)				10
1.0 mM Peptide (µl)			10	
0.1mM Peptide (µl)		10		
100% DMSO (µl)	10			
26 mg/ml CSPG from untreated THP-1 cells (µl)	5	5	5	5
1 M HEPES buffer (µl)	10	10	10	10
Milli-Q water (µl)	73.5	73.5	73.5	73.5
12.5 µM MMP-9 (µl)	1.5	1.5	1.5	1.5

These samples were incubated for 2-4 hours and then passed through small Q-Sepharose column followed by desalting on a Sephadex G-50 column. The eluted samples were then dried using the vacuum centrifuge and then mixed with 8 µl 1X sample buffer. These samples were then applied to gelatin zymography along with standards.

3. Results

3.1. Optimization of quantitative determination of CS-chains

The first part of this thesis deals with the purification of CSPGs from the leukemic monocytic cell lines THP-1 and MonoMac. The amount of CSPGs in the purified material was estimated from the amount of CS-chains by using the Safranin O method. To do this we needed CS-chains with known concentration and therefore CS from shark cartilage was used. In the first standard curve the concentration of CS ranged from 0 - 5 µg per slot as described in methods (2.4.4 and 2.4.4.1). In this experiment the extraction of dye from the nitrocellulose membrane was performed at 37 °C without stirring. The standard curve from this experiment is shown in figure 8. This standard curve showed an unacceptable variation with a very low regression coefficient (r^2) of 0.809.

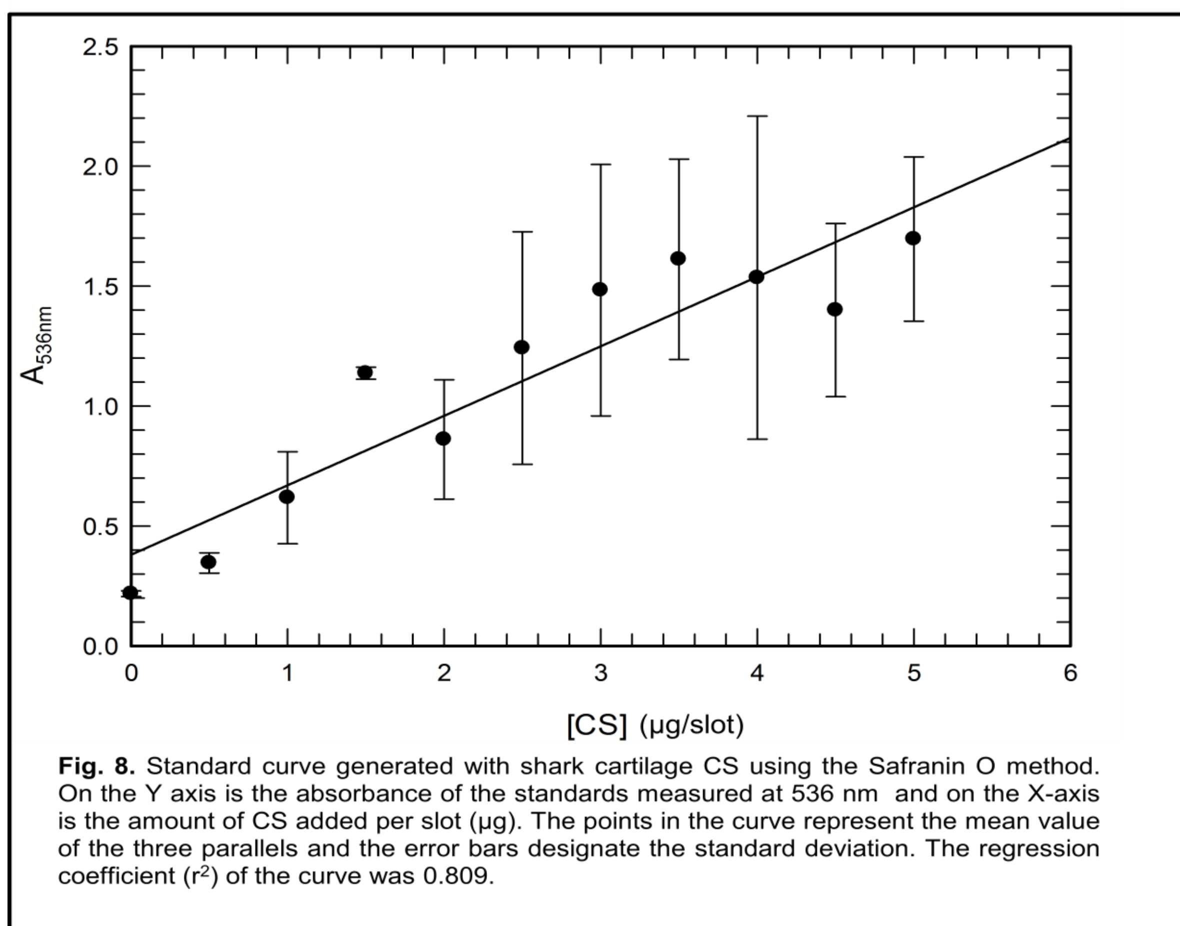

Fig. 8. Standard curve generated with shark cartilage CS using the Safranin O method. On the Y axis is the absorbance of the standards measured at 536 nm and on the X-axis is the amount of CS added per slot (µg). The points in the curve represent the mean value of the three parallels and the error bars designate the standard deviation. The regression coefficient (r^2) of the curve was 0.809.

Since the first standard curve was unreliable we decided to do another optimization test but for good accuracy we decided to take the standards with concentration ranging from 0 - 2.5 µg per slot as described in methods (2.4.4 and 2.4.4.1). The extraction of the dye from the nitrocellulose membrane was performed in the same way as for the standard shown in figure 8. As shown in figure 9, there was still unacceptable variation with even a lower regression coefficient (r^2) than before, 0.473.

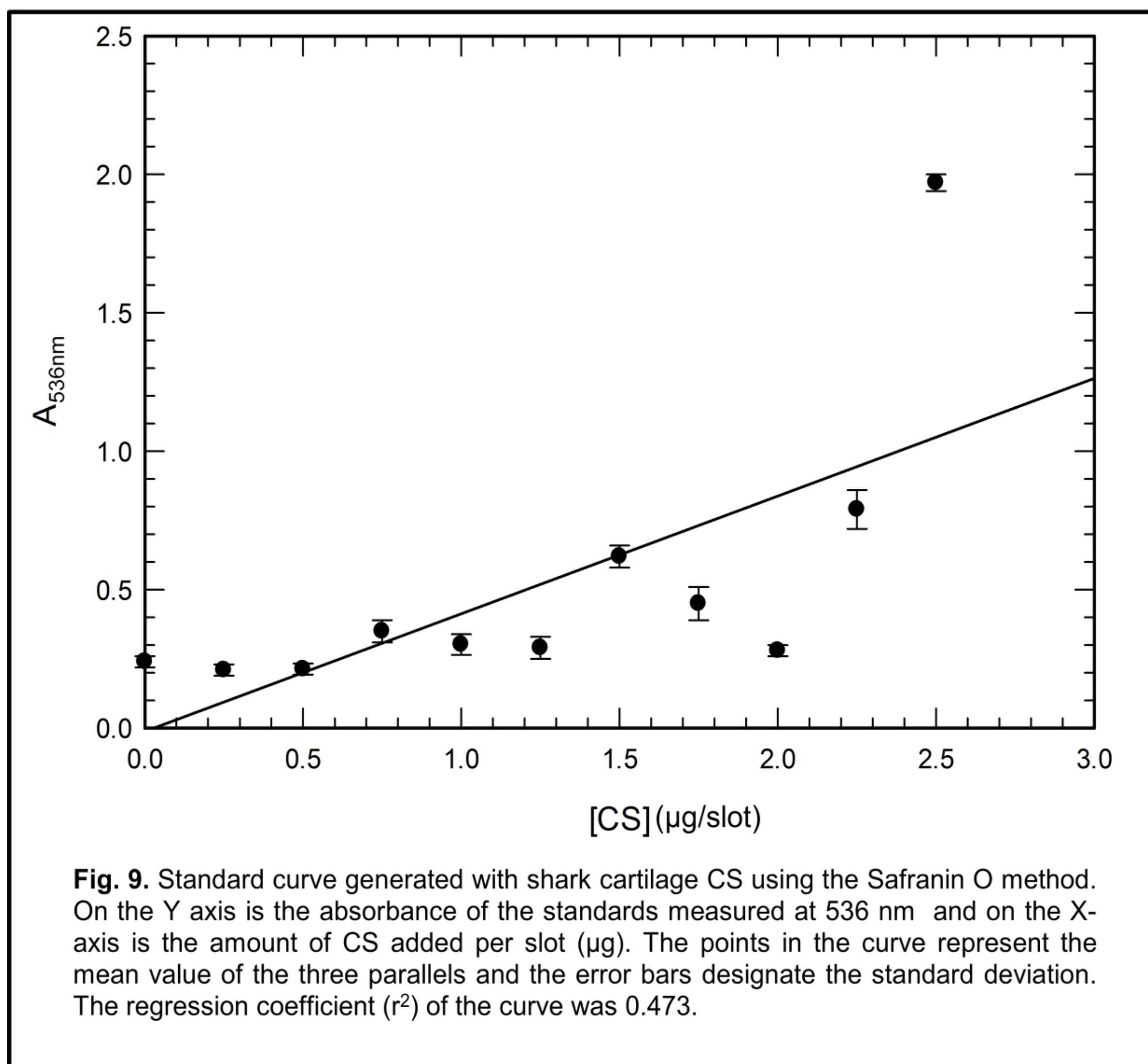

Fig. 9. Standard curve generated with shark cartilage CS using the Safranin O method. On the Y axis is the absorbance of the standards measured at 536 nm and on the X-axis is the amount of CS added per slot (µg). The points in the curve represent the mean value of the three parallels and the error bars designate the standard deviation. The regression coefficient (r^2) of the curve was 0.473.

We suspected that the reason behind the low linear regressions in both standard curves might have been due to the total volume (100 µl) of the standards and also the dilution of the CS-chain stock solution. So, we decided to prepare a new stock solution of 24

mg/ml and diluted 2 more times to give end stock solution of 0.1 mg/ml. This new stock solution was made to prepare standards with the total volume of 200 µl and we used standards with the concentration ranging from 0 - 2.5 µg per slot for generating a standard curve. Also, another step which we changed during this experiment was the extraction of the dye from the nitrocellulose membrane using CPC at 37ºC. Instead of letting it stand without stirring the whole time, we kept the mixtures in a shaker during the time of incubation (as described in method section 2.4.4.1). This may result in a more efficient extraction of the dye from the membrane. The standard curve we obtained from these standards gave the best regression coefficient (0.997) as seen in figure 10.

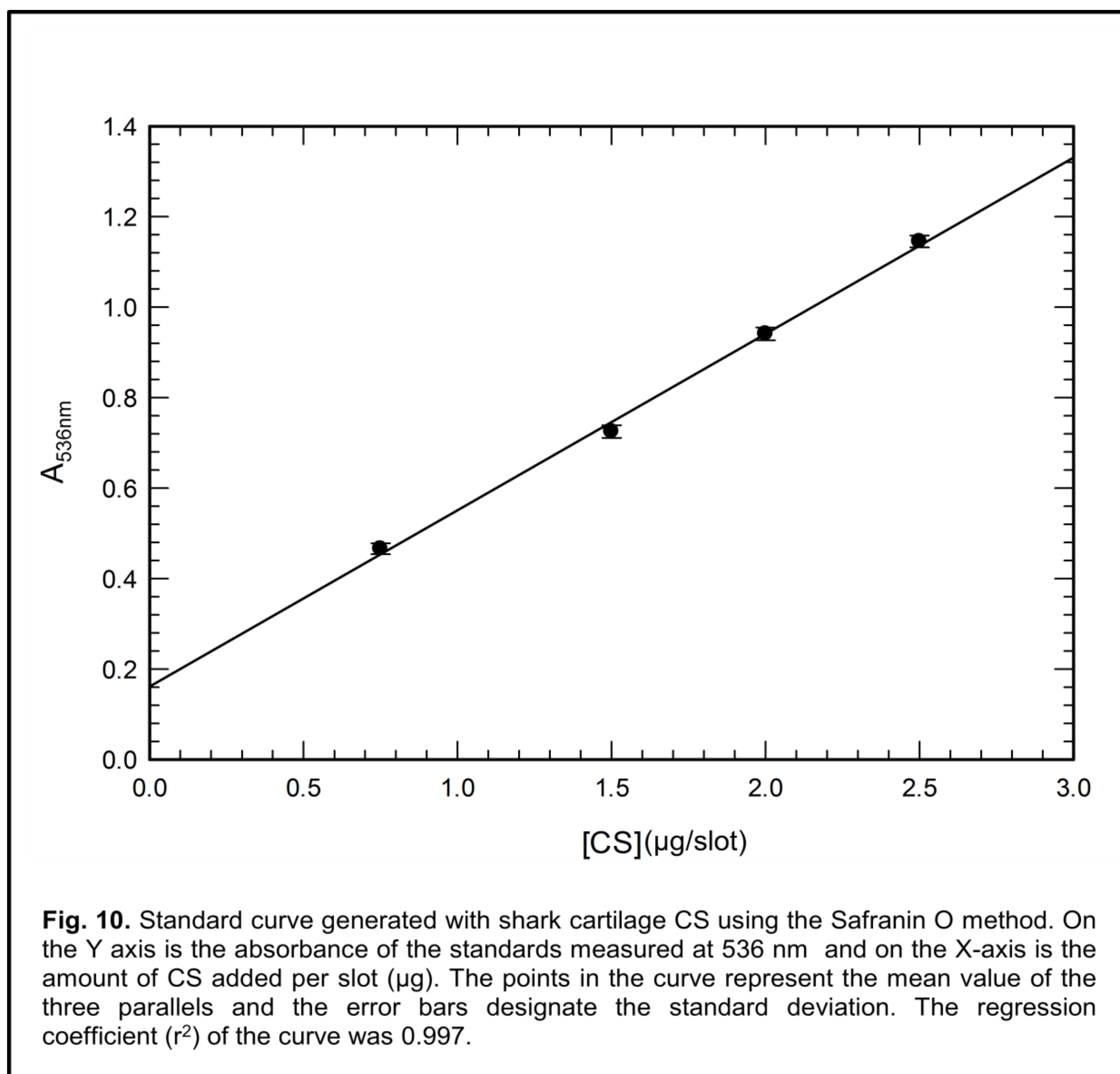

Fig. 10. Standard curve generated with shark cartilage CS using the Safranin O method. On the Y axis is the absorbance of the standards measured at 536 nm and on the X-axis is the amount of CS added per slot (µg). The points in the curve represent the mean value of the three parallels and the error bars designate the standard deviation. The regression coefficient (r^2) of the curve was 0.997.

Therefore, this process for producing standard curves was followed each time the isolated CSPGs were needed to be quantified. Furthermore, the extraction procedure used for this standard curve was also used in all the extraction of purified CSPGs.

3.2. Gel filtration with Sephacryl S-400 column

We have carried out at least two separate gel filtration experiments for the partly purified CSPGs from all three cell lines: THP-1, MonoMac and U-937. But first we determined the separation properties of the Sephacryl S-400 column that was used in the purifications of the CSPG from these cell lines.

3.2.1. Determination of the separation properties of the Sephacryl S-400 column

To determine the separation properties of the Sephacryl S-400 column, void volume (V_0), total volume (V_t) and elution volume (V_e) was determined. Blue dextran was used to determine V_0 and bromophenol blue to determine V_t. The globular proteins apoferitin, yeast alcohol dehydrogenase (ADH) and bovine serum albumin (BSA) was used to determine V_e. Figure 11 shows the chromatograms for these standards and Table 6 summarize the results.

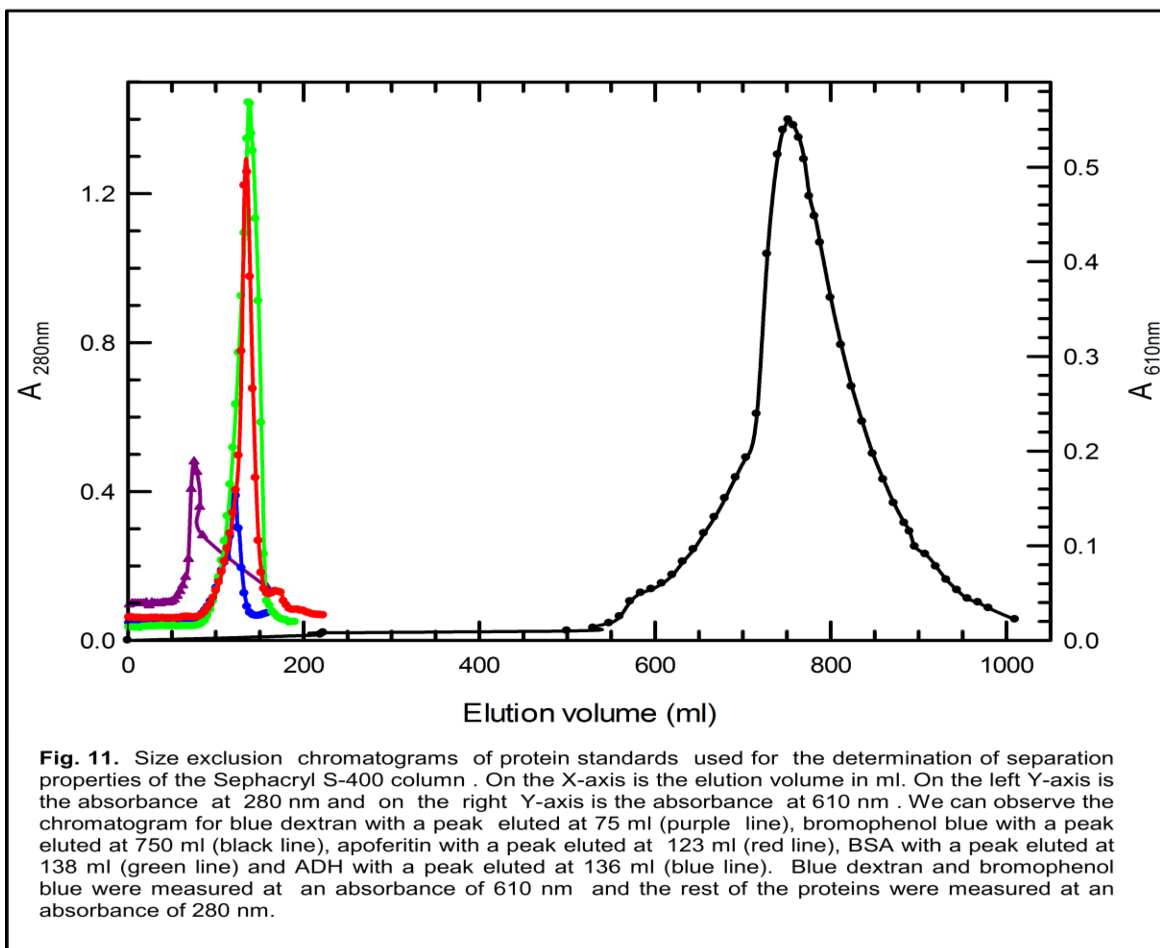

Fig. 11. Size exclusion chromatograms of protein standards used for the determination of separation properties of the Sephacryl S-400 column . On the X-axis is the elution volume in ml. On the left Y-axis is the absorbance at 280 nm and on the right Y-axis is the absorbance at 610 nm . We can observe the chromatogram for blue dextran with a peak eluted at 75 ml (purple line), bromophenol blue with a peak eluted at 750 ml (black line), apoferitin with a peak eluted at 123 ml (red line), BSA with a peak eluted at 138 ml (green line) and ADH with a peak eluted at 136 ml (blue line). Blue dextran and bromophenol blue were measured at an absorbance of 610 nm and the rest of the proteins were measured at an absorbance of 280 nm.

Table 6. A summary of the separation properties of the Sephacryl S-400 column. The elution volume (V_e) and the wavelength used for determination of the protein standards are shown. The molecular size of the protein standards are given in kDa. The elution volume was measured in ml.

Standards (molecular size in kDa)	Wavelength (nm)	Elution Volume V_e (ml)
ADH (150)	280	136
BSA (66)	280	138
Apoferitin (443)	280	123
Blue Dextran (2000)	610	75
Bromophenol Blue (0.67)	610	750

The elution volume (V_e) of the standards used for calibration of the Sepharyl S-400 column are summarized in Table 6. The elution volume (V_e) of ADH came to be at 136 ml, BSA at 138 ml and apoferitin at 123 ml. Blue dextran used for determination of the void volume (V_0) of the samples came out at 75 ml. The total volume (V_t) of the column was measured with the help of bromophenol blue which came out of the column at 750 ml.

Although ADH and BSA have huge difference in their molecular sizes, their V_e was approximately the same. This suggests that BSA might have existed as a dimer during the conditions used in the experiment. Normally, it is used as a standard and it occurs as a monomer.

3.2.2. Purification of CSPG from THP-1 cells using Sephacryl S-400 size exclusion chromatography

The first purification was done from partly purified CSPGs from the THP-1 cells. As seen in figure 12, CSPGs was eluted in one broad peak. A small fraction of the CSPG is eluted at around V_0, but the main peak occurs after V_0. The CSPGs were eluted in one broad peak which might indicate that there are CSPGs of different sizes in THP-1 media. Therefore, we divided the eluted materials into four pools as shown in figure 12. The first fractions containing CSPGs which were eluted around V_0 was joined together and named pool I. Similarly other fractions containing CSPGs were joined together to give three more pooled fractions.

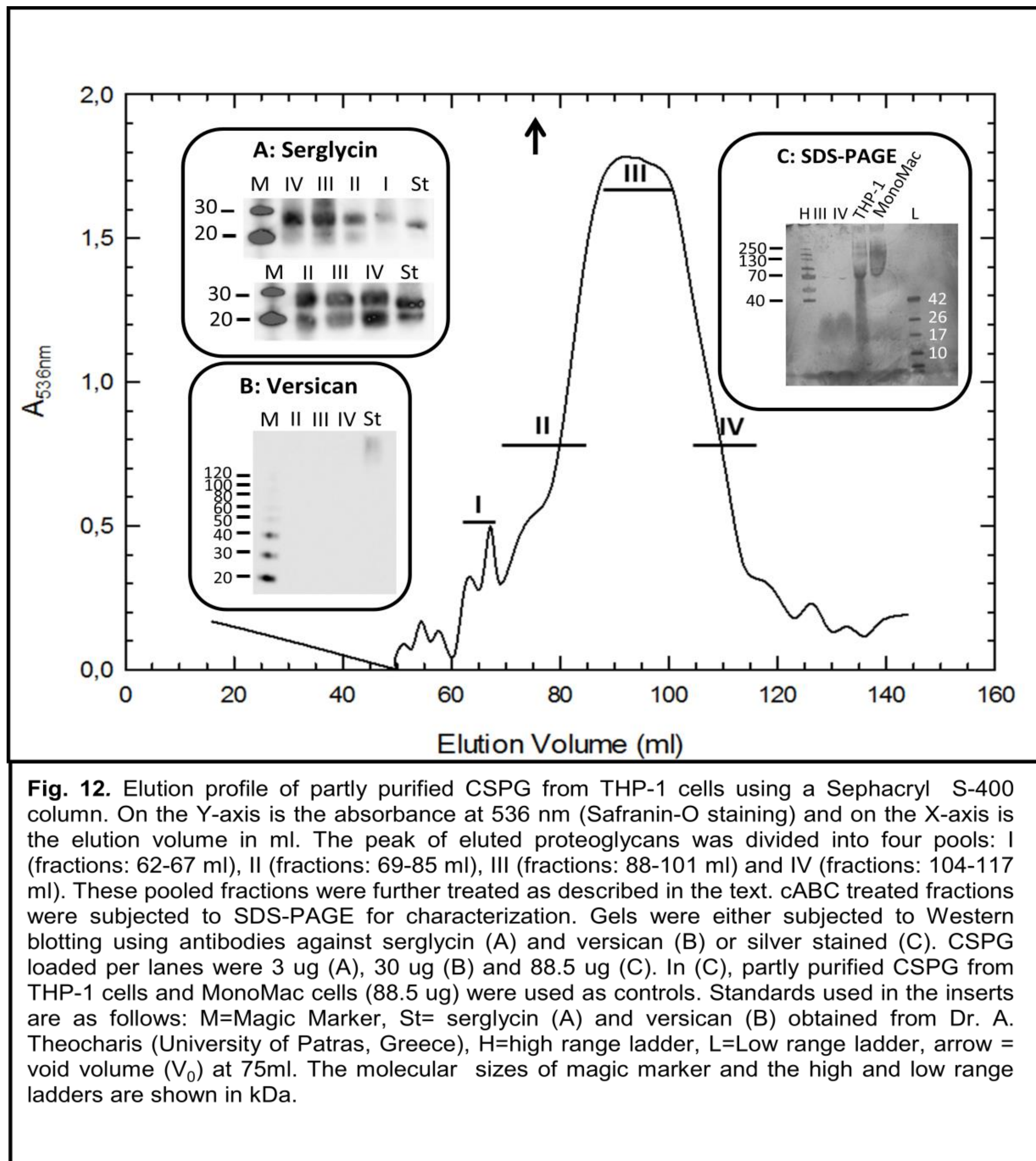

Fig. 12. Elution profile of partly purified CSPG from THP-1 cells using a Sephacryl S-400 column. On the Y-axis is the absorbance at 536 nm (Safranin-O staining) and on the X-axis is the elution volume in ml. The peak of eluted proteoglycans was divided into four pools: I (fractions: 62-67 ml), II (fractions: 69-85 ml), III (fractions: 88-101 ml) and IV (fractions: 104-117 ml). These pooled fractions were further treated as described in the text. cABC treated fractions were subjected to SDS-PAGE for characterization. Gels were either subjected to Western blotting using antibodies against serglycin (A) and versican (B) or silver stained (C). CSPG loaded per lanes were 3 ug (A), 30 ug (B) and 88.5 ug (C). In (C), partly purified CSPG from THP-1 cells and MonoMac cells (88.5 ug) were used as controls. Standards used in the inserts are as follows: M=Magic Marker, St= serglycin (A) and versican (B) obtained from Dr. A. Theocharis (University of Patras, Greece), H=high range ladder, L=Low range ladder, arrow = void volume (V_0) at 75ml. The molecular sizes of magic marker and the high and low range ladders are shown in kDa.

The silver staining (seen in insert C of Fig. 12.) was done with pools III and IV shows a band around 26 kDa. Then western blots (seen in insert A of Fig. 12) of all the pools with the purified CSPGs were performed. This showed the presence of serglycin in all the pools, but with the largest amount in pool IV and the lowest amount in pool I. The elution curve produced from the second experiment as seen in appendix 7.5 (Fig. A5.) gave almost identical results as the elution curve from the first experiment. Also a

western blot of the pooled fractions from the second experiment was conducted using antibodies against serglycin. Similarly as in the first experiment, largest amount of serglycin was detected in pool IV. The collection of the four pools and the total elution volume from both experiments are summarized in Table 7.

Table 7. Summary of pooled fractions from experiments 1 and 2. Four pools were collected from two different runs of gel filtration of the partly purified CSPGs from THP-1 cells. The fractions pooled are shown in figure 12 and appendix 7.5 (Fig. A5.).

Run no.	Pool	Total volume (ml)
1	1	8
	2	20
	3	18
	4	18
2	1	8
	2	12
	3	14
	4	15

The pooled fractions from both runs were diluted 20 - 25 times so that the concentration of guanidine hydrochloride (equilibration buffer 2) was maintained at 0.3 M or less than that (so that it did not affect the binding of the CSPGs to the Q-Sepharose columns). These diluted pools were then applied to Q-Sepharose ion-exchange chromatography and thereafter desalted through Sephadex G-50 (fine) columns. The pools were concentrated and the CS-chains in these pools were determined by the Safranin O method. The amount of CSPGs present in all four pools from both runs is summarized in Table 8.

Table 8. The amount and volume of the four pools of CSPG from two different Sephacryl S-400 gel filtrations of the partly purified CSPGs from THP-1 cells. The table shows the four final pools of the two runs, the final volume of the purified samples, the final CSPG concentration and the total amount of CSPG.

Run no.	Pool	Total volume (µl)	[CSPG] (µg/µl)	Total amount of CSPG (µg)
1	1	500	0.0317	16
	2	900	0.225	203
	3	300	5.9	1770
	4	400	1.3	520
2	1	300	0.0274	8
	2	600	0.2813	169
	3	800	0.5573	446
	4	700	0.2375	166

These four pools from each Sephacryl S-400 gel filtrations of the partly purified CSPGs from THP-1 cells were joined to give a final four pools and the average CSPG content was calculated with the help of table 8 and summarized in Table 9.

Table 9. The joined pools of purified CSPG from the two different Sephacryl S-400 gel filtrations of the partly purified CSPGs from THP-1 cells. Pool1 from both runs were joined, final pool1 and similar was done for other three pools. The table shows the four final pools and the final volume of the purified samples after joining the pools together. The average concentration of CSPG and the total amount of the CSPG after the joining of pools are given in the table.

Final pool	Total volume (µl)	[CSPG] (µg/µl)	Total amount of CSPG (µg)
1	800	0.030	24
2	1500	0.248	372
3	1100	2.014	2216
4	1100	0.6236	686

Both from Table 8 and Table 9 we can see that the largest amount of CSPG is present in pool 3, while the Western blots (Fig.12. insert A and appendix 7.5 Fig. A5.) showed that pool 4 had the largest amount of serglycin.

3.2.3. Purification of CSPG from MonoMac cells using Sephacryl S-400 size exclusion chromatography

Three separate experiments were done with equal amount of isolated CSPGs from MonoMac cells. Elution curves (Fig. 13. and appendix 7.5 Fig. A6.) show the first two purifications out of three total runs of the samples (partly purified CSPGs) from MonoMac cells. After the CSPGs were pooled and quantified, we saved the samples for the M.S analysis. We did not decide to conduct any test to observe presence of serglycin in the pooled samples as a previous research done in our research group had already confirmed the absence of serglycin in partly purified CSPG from MonoMac cells [9].

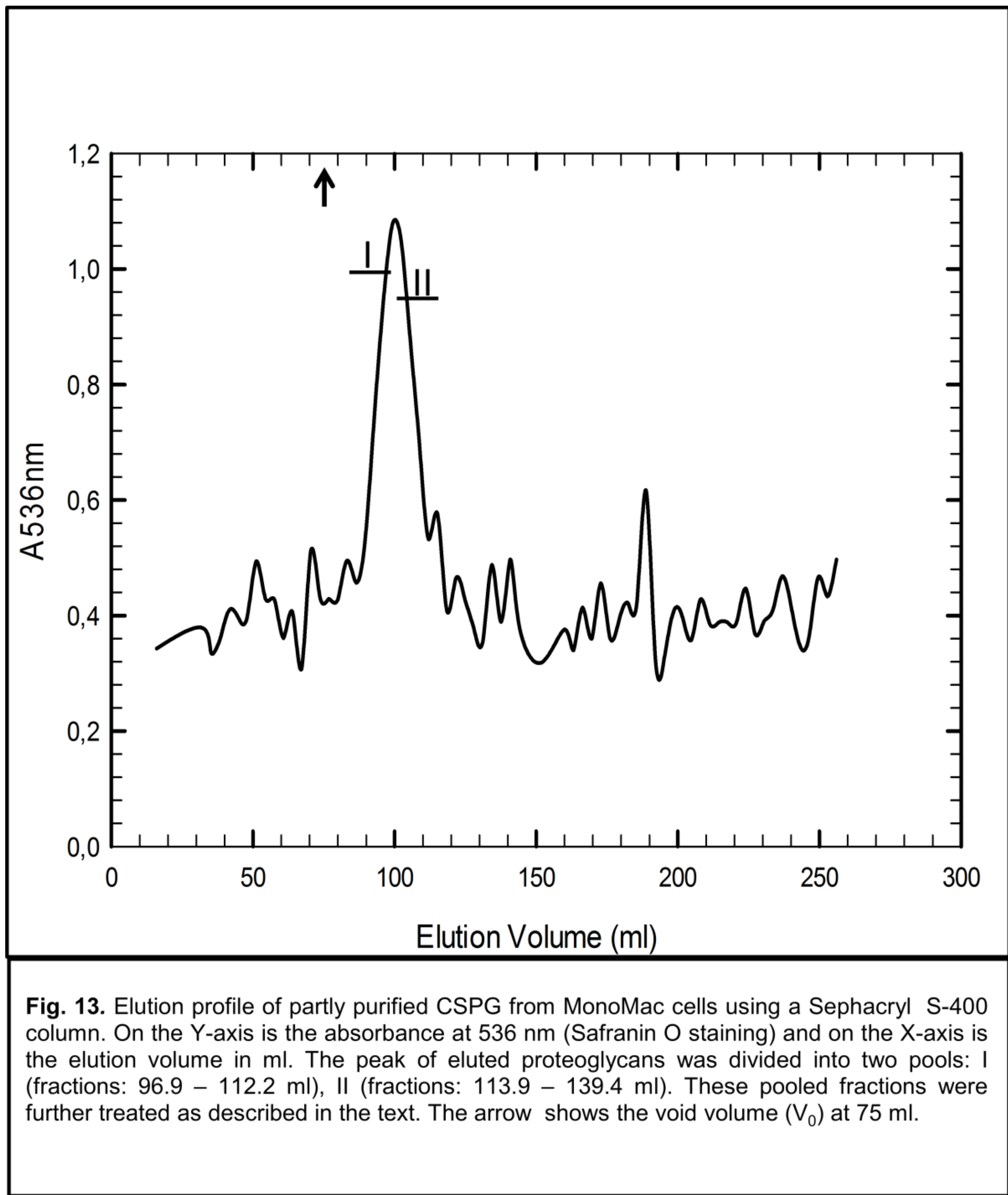

Fig. 13. Elution profile of partly purified CSPG from MonoMac cells using a Sephacryl S-400 column. On the Y-axis is the absorbance at 536 nm (Safranin O staining) and on the X-axis is the elution volume in ml. The peak of eluted proteoglycans was divided into two pools: I (fractions: 96.9 – 112.2 ml), II (fractions: 113.9 – 139.4 ml). These pooled fractions were further treated as described in the text. The arrow shows the void volume (V_0) at 75 ml.

The pools were collected from the fractions with elution volume that came after the void volume. The elution curve produced from other two experiments are also similar which shows the elution occurring after the void volume suggesting that the purified CSPGs must have a lower molecular weight than blue dextran. The fractions selected for the collection of two pools from all three runs are summarized in Table 10.

Table 10. Summary of pooled fractions from experiments 1 - 3. Two pools were collected from three different runs of gel filtration of the partly purified CSPGs from MonoMac cells. The fractions pooled are shown in figure 13 and appendix 7.5 (Fig. A6.).

Run no.	Pool	Total Volume (ml)
1	1	16
	2	18
2	1	17
	2	21
3	1	10
	2	16

The pooled fractions from the three runs were diluted 20 - 25 times so that the concentration of guanidine hydrochloride (equilibration buffer 2) was maintained at 0.3 M or less than that so that it did not affect the binding of the CSPGs to the Q-Sepharose columns. These diluted pooled fractions were applied to a Q-Sepharose ion-exchange chromatography and then desalted through Sephadex G-50 (fine) columns. The desalted materials were thereafter concentrated and the amount of CS-chains in these pools was determined by the Safranin O method. The amount of CSPGs present in both two pools from the three runs is summarized in Table 11.

Table 11. The amount and volume of the two pools of CSPGs from the three different Sephacryl S-400 gel filtrations of the partly purified CSPGs from MonoMac cells. The table shows the two final pools of the three runs, the final volumes of the purified samples, their final concentration and the total amount of CSPG.

Run no:	pool	Total volume (µl)	[CSPG] (µg/µl)	Total amount of CSPG (µg)
1	1	300	0.700	210
	2	500	0.905	452.5
2	1	300	0.944	283.2
	2	400	1.600	640
3	1	200	0.395	79
	2	250	1.908	477

These two pools from all three Sephacryl S-400 gel filtrations of the partly purified CSPGs from MonoMac cells were joined to give a final two pools and the average CSPG content was calculated with the help with Table 11 and summarized in Table 12.

Table 12. The joined of pools of purified CSPGs from the three different Sephacryl S-400 gel filtrations of the partly purified CSPGs from MonoMac cells. Pool1 from all three runs were joined, final pool1 and similar was done for other pools. The table shows the two final pools and the final volume of the purified samples after joining the pools together. The average concentration of CSPG and the total amount of the CSPG after the joining of pools are shown.

Pool	Total Volume (µl)	[CSPG] (µg/ µl)	Total amount of CSPG (µg)
1	800	0.715	512
2	1150	1.365	1570

3.3.4. Purification of CSPG from U-937 cells using Sephacryl S-400 size exclusion chromatography

During the first gel filtration experiment done with the partly purified CSPGs from U-937 cell sample, due to technical problems (clogging of filters) with the column, the sample did not pass through the column at all. Therefore, we had to stop the experiment and tried to rescue our sample from the capillary tubes connected to the column. After thorough washing of the columns and changing of filters in the sample load part of the column system, we passed through this rescued sample and collected the eluted materials. However, the CSPGs were under the detection limit.

Another gel filtration experiment was performed with the remaining of the isolated CSPG from U-937 cells. As the obtained amount of purified CSPG was very low, it was not further used.

3.4. *In vitro* reconstitution of proMMP-9/CSPG complexes

The final pools (Tables 9 and 12) of purified CSPGs from THP-1 and MonoMac cells were used for conducting *in vitro* reconstitution in order to determine if these purified CSPGs in different pools could form complexes with proMMP-9. From the four total pools from the purified CSPGs from THP-1 cells, we only selected pools II, III and IV as the amount of CSPG in the first pool was not enough for conducting the experiment. However, both pools of CSPGs from MonoMac cells were used in the *in vitro* reconstitution experiments.

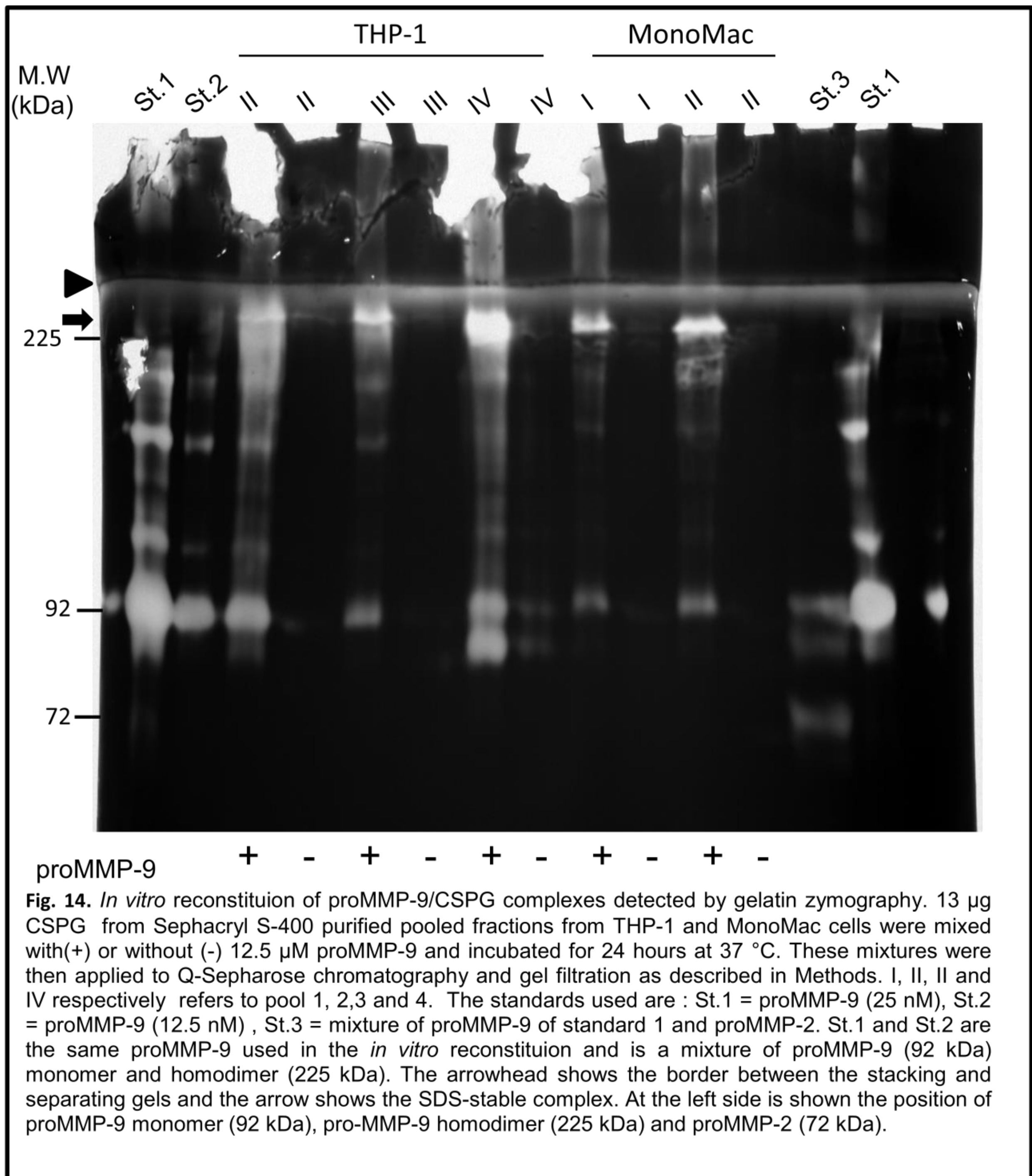

Fig. 14. *In vitro* reconstituion of proMMP-9/CSPG complexes detected by gelatin zymography. 13 µg CSPG from Sephacryl S-400 purified pooled fractions from THP-1 and MonoMac cells were mixed with(+) or without (-) 12.5 µM proMMP-9 and incubated for 24 hours at 37 °C. These mixtures were then applied to Q-Sepharose chromatography and gel filtration as described in Methods. I, II, II and IV respectively refers to pool 1, 2,3 and 4. The standards used are : St.1 = proMMP-9 (25 nM), St.2 = proMMP-9 (12.5 nM) , St.3 = mixture of proMMP-9 of standard 1 and proMMP-2. St.1 and St.2 are the same proMMP-9 used in the *in vitro* reconstituion and is a mixture of proMMP-9 (92 kDa) monomer and homodimer (225 kDa). The arrowhead shows the border between the stacking and separating gels and the arrow shows the SDS-stable complex. At the left side is shown the position of proMMP-9 monomer (92 kDa), pro-MMP-9 homodimer (225 kDa) and proMMP-2 (72 kDa).

As shown in figure 14, no proMMP-9/CSPG complexes could be observed in the purified CSPG fractions. However, mixing of the purified CSPGs from the THP-1 and MonoMac samples with proMMP-9 resulted in formation of both SDS-stable (300 kDa) and SDS-soluble (92 kDa) complexes. Larger amounts of SDS-stable and

SDS-soluble complexes were formed with CSPGs from pool IV than with the CSPGs from pools II and III (THP-1 cells). Larger amount of the SDS-soluble complex were formed pool II and IV compared to pool III (THP-1 cells). In the case of MonoMac cells, pool II formed the larger amount of the complexes than pool I.

3.5. Separation of the proteins in purified CSPG fractions from THP-1 and MonoMac cells by SDS-PAGE

SDS-PAGE was used to separate and identify proteins present in the different CSPG pools from the Sephacryl S-400 gel filtration experiments. Proteins were visualised by Coomassie staining. Pool II, III and IV from the CSPG samples of THP-1 cells and both pools from the CSPG samples of MonoMac cells were investigated. It was not enough of CSPG in pool I from the THP-1 cells for this investigation. Since the detection limit of the Imperial Coomassie blue stain is reported to be 5 ng, and based on the assumption that only 5 % of the isolated CSPGs is the protein fraction whereas, 95 % is CS-chains, 2.5 µg protein of pool II from the THP-1 cells and 5 µg protein of the other CSPG samples were applied to the SDS-gel after the CS-chains had been removed by cABC. Thus 50 and 100 µg of the CSPGs (based on the amount of CS-chains) was used and treated as described in Method (2.4.8.3) before it was applied to the SDS-gel.

Despite the half amount of CSPG in the lane with pool II (THP-1), we can see that the bands appearing at the region of 130 and 70 kDa have almost the same intensity as the respective bands from pool III. This suggests that there are more of these proteins in pool II than in pool III. We can see a strong band around 130 kDa in almost all of the lanes which is of cABC. In the two pools from the MonoMac cells, one or several bands just larger than 130 kDa can be seen. The bands appearing around the region of 70 kDa is stronger in pools II and III than in pool IV from the THP-1 cells. However, these bands are stronger in pools I and II from the MonoMac cells. In the CSPG pools from the MonoMac cells, there are several distinct bands between 50 -70 kDa. Bands in the region of 4.6 kDa appear in the two pools from the MonoMac cells. A band can be observed in the 1.7 kDa region in pool I from the THP-1 cells and both CSPG pools from the MonoMac cells. These bands might not

be proteoglycans but could be proteins that are bound in the GAG chains of the CSPGs.

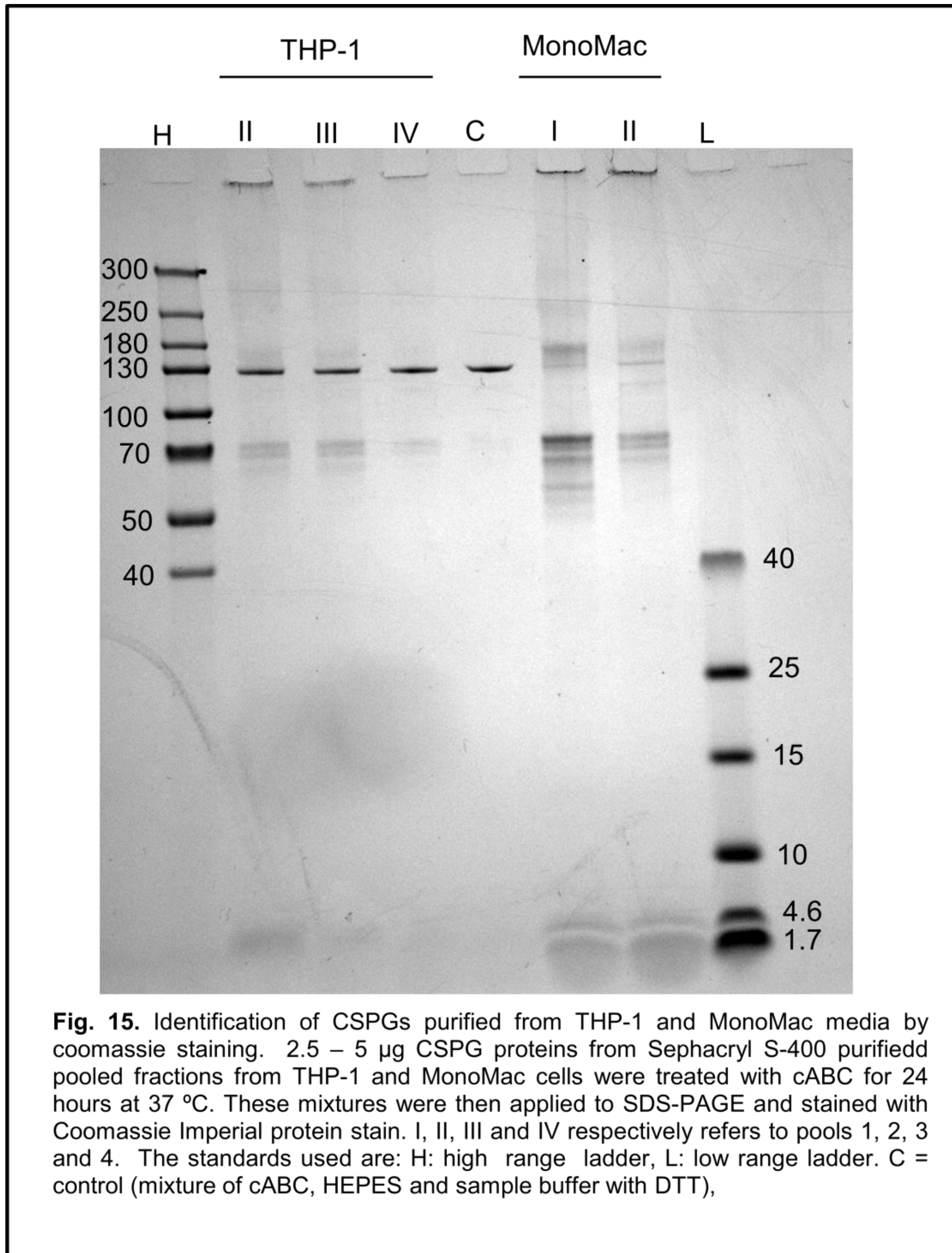

Fig. 15. Identification of CSPGs purified from THP-1 and MonoMac media by coomassie staining. 2.5 – 5 µg CSPG proteins from Sephacryl S-400 purifiedd pooled fractions from THP-1 and MonoMac cells were treated with cABC for 24 hours at 37 ºC. These mixtures were then applied to SDS-PAGE and stained with Coomassie Imperial protein stain. I, II, III and IV respectively refers to pools 1, 2, 3 and 4. The standards used are: H: high range ladder, L: low range ladder. C = control (mixture of cABC, HEPES and sample buffer with DTT),

Bands around the region of 25 kDa in pools III and IV from the THP-1 cells was observed, but these bands were obtained within the first hour of destaining and

disappeared after 24 hours of destaining. This is why we cannot see these bands in figure 15. Because of this we performed a new SDS-PAGE with pools III and IV from THP-1 cells. These two bands were cut and sent for MS-analysis for identification and is not shown here as no photo was taken of the gel. The appearance of these bands around the region of 25 kDa corresponded with the bands observed in insert C of figure 12. In the insert A of figure 12, we can also see the bands with molecular size of around 25 kDa contain serglycin. In the western blot (Fig. 12, insert A) a serglycin band around 20 kDa is also seen. This band mainly occurred after storage of the cABC treated CSPG samples and may have occurred due to proteolytic degradation, as the cABC used was not protease free. Another possibility that cannot be excluded is that the cABC has degraded more of the CS-chains. No bands were observed in the region of 25 kDa in the lanes with MonoMac pools.

Bands observed in the top of stacking gel could be the presence of large proteoglycans such as versican or perlecan. These bands are strong in the lanes with MonoMac pools although we can see these bands in the THP-1 pools too.

Various bands observed in Fig. 8 were cut out and sent to MS-analysis. The results were recently received but unfortunately all the samples showed presence of keratin which suggests that the samples were contaminated. Due to this it was not possible to obtain any reliable information about the nature of the protein in different bands.

3.6. Peptide arrays

From the previous research from our group it has been shown that FnII module and HPX domain are involved in forming of the proMMP-9/CSPG complexes [34]. Also from recent findings it has been found out that mixing of serglycin and proMMP-9 led to the formation of SDS-stable and SDS-soluble proMMP/serglycin complexes [9]. So, with this knowledge our main intention of conducting experiments with peptide arrays was to obtain detailed information of the parts in proMMP-9 FnII module and HPX domain as well as the parts in the serglycin core protein that were involved in complex formation. To be able to do this we conducted a peptide walk through the whole sequences of serglycin, the proMMP-9 FnII module and the proMMP-9 HPX

domain by ordering SPOT peptide arrays on two cellulose membranes consisting of 20-mer peptides at 2 amino acid intervals.

These membranes with the arrays were ponceau stained (as seen in appendix 7.7 Fig. A8.), cut and incubated individually with proMMP-9, His-tagged serglycin or TIMP-1 according to the type of experiment and were visualized as described in method section 2.4.9. The appearance of dark spots in the membrane confirmed binding.

Both dark and lightly stained peptides were regarded as binders. Those peptides that were not stained at all were regarded as non-binders. Our analysis of the peptide arrays were as follows: if the closest neighbours on each side of a stained peptide were unstained, this indicated that staining is an artefact or alternatively all the 20 amino acids of the peptide sequence were needed to bind to the partner protein. Another alternative is that the two most N- and C- terminal amino acids together are involved in binding. If only one or two of the amino acids in either the N- or C-terminal end of the peptide were involved in binding, at least 10 peptides in a continuous sequence should be stained. So if the stained peptides occurred in an unbroken sequence after each other, common amino acids in the stained peptides were regarded as involved in binding.

To confirm that the selected amino acids from the sequences of the stained peptides were responsible for binding we ordered a new peptide array where the amino acids assumed to be involved in binding were mutated (see appendix 7.8).

3.6.1. Peptide array based on sequences of serglycin probed with proMMP-9

From the ponceau stained membrane (appendix 7.7) containing all the peptide arrays for serglcyin, Fn-II module and HPX domain, one of the arrays containing serglycin was cut and probed with proMMP-9 as described in the method section 2.4.9. Figure 16A shows the ponceau stained array where the spots are labelled with the number of the peptide sequence shown in appendix 7.7 (Fig. A9.). Fig. 16B shows that proMMP-9 binds to several of the peptides in the serglycin sequence.

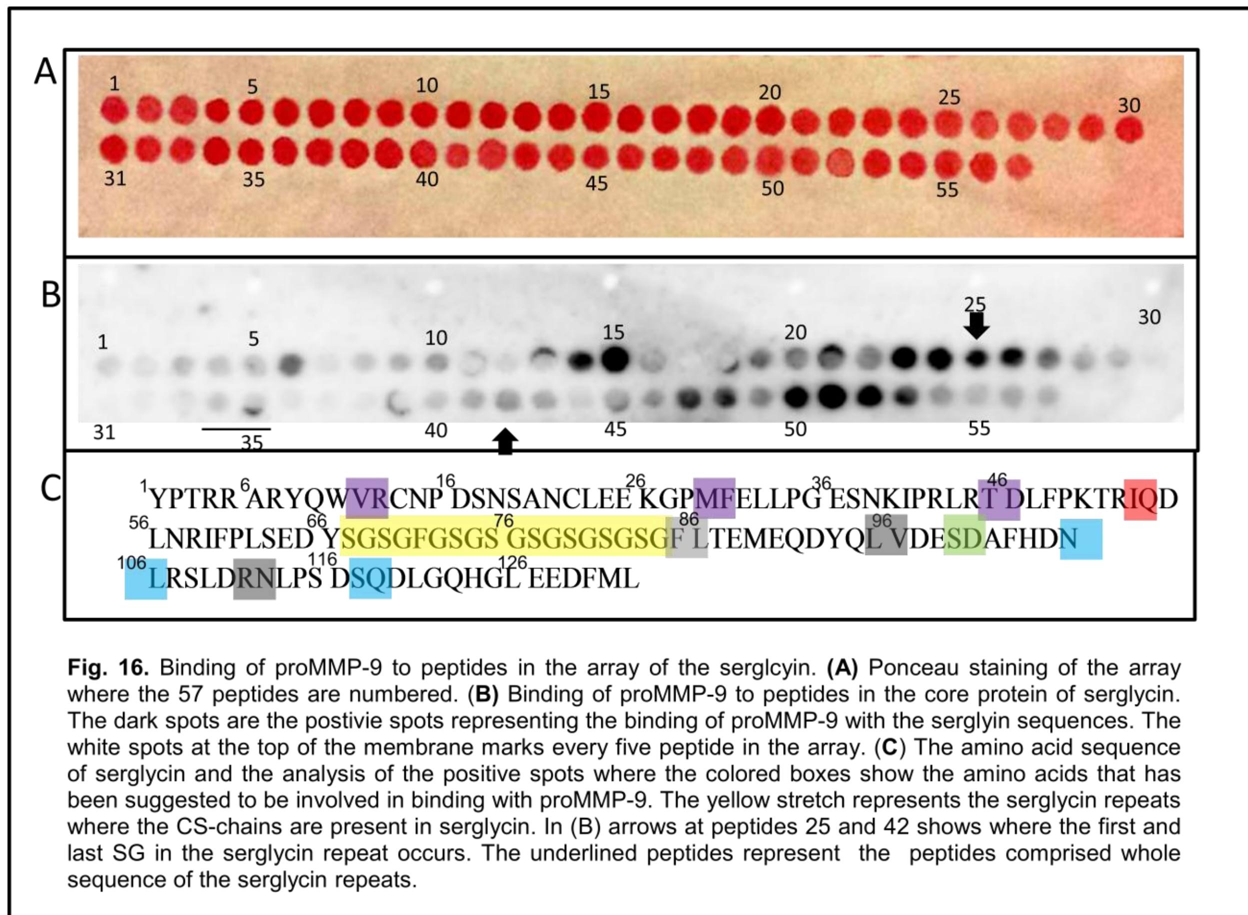

Fig. 16. Binding of proMMP-9 to peptides in the array of the serglcyin. **(A)** Ponceau staining of the array where the 57 peptides are numbered. (**B)** Binding of proMMP-9 to peptides in the core protein of serglycin. The dark spots are the postivie spots representing the binding of proMMP-9 with the serglyin sequences. The white spots at the top of the membrane marks every five peptide in the array. (**C**) The amino acid sequence of serglycin and the analysis of the positive spots where the colored boxes show the amino acids that has been suggested to be involved in binding with proMMP-9. The yellow stretch represents the serglycin repeats where the CS-chains are present in serglycin. In (B) arrows at peptides 25 and 42 shows where the first and last SG in the serglycin repeat occurs. The underlined peptides represent the peptides comprised whole sequence of the serglycin repeats.

Figure 16 shows that proMMP-9 binds to sequences in the serglycin core protein located both N- and C-terminal to the SG-repeats which bind the CS-chains. Based on the stained peptides shown in figure 16B and their amino acid sequences as shown in appendix 7.7 (Fig. A9.), the following conclusions about the amino acids in the serglycin core protein involved in binding with proMMP-9 can be drawn: in the N-terminal part of Serglcyin the first peptide that proMMP-9 seems to bind is peptide 6. The possibilities for the staining could be: pro-MMP-9 binds to the whole sequence from ^{11}VR to MF^{30} or it could be an artefact. ProMMP-9 also seem to bind to peptides 14 to 15 and based on these peptides it can be concluded that either the whole sequence from ^{29}MF to TD^{46} or just the amino acids ^{29}MF and TD^{46} are involved in binding. Peptides 18 to 27 seem to involve in binding with proMMP-9 in which I^{53} and Q^{54} are the amino acids that seem to be involved. In the C-terminal part of Serglycin, the Peptides 41 to 43 seem to be involved in binding with proMMP-9 suggesting that either the whole sequence from ^{85}FL

to SD^{100} or just the amino acids ^{85}FL and SD^{100} are involved in binding. Also, peptides 47 to 48 seems to bind with proMMP-9 and an analysis of these peptides concludes that either the whole sequence from ^{95}LV to RN^{112} or just the amino acids ^{95}LV and RN^{112} are involved in binding. The final peptides in the C-terminal part of serglycin that seem to be involved in the binding with proMMP-9 are peptides 49 to 53, suggesting that either the whole sequence from ^{105}NL to SQ^{118} or just the amino acids ^{105}NL and SQ^{118} are involved in binding. Another alternative is that proMMP-9 binds to peptides 47 to 53, suggesting that amino acids ^{105}NL to RN^{112} are involved in binding.

One extra experiment was conducted with the peptide array based on the sequence of serglycin with proMMP-9 and this experiment showed almost exactly the same results (appendix 7.7, Fig. A10.). However, there were small differences in the intensities of the signals, which could be due to the small differences in the produced batches of the peptide arrays.

To confirm the involvement of these amino acids, sequences comprising of these amino acids were mutated and another peptide array was done as described in 3.6.2.

3.6.2. Mutated peptide array based on the sequences of serglycin probed with proMMP-9

The amino acids from the peptides based on the sequence of serglycin that seemed to be involved in binding were mutated (appendix 7.8) and the membrane of the peptide array containing these mutated peptides was probed with proMMP-9 as described in the method section 2.4.9 and 2.4.10.

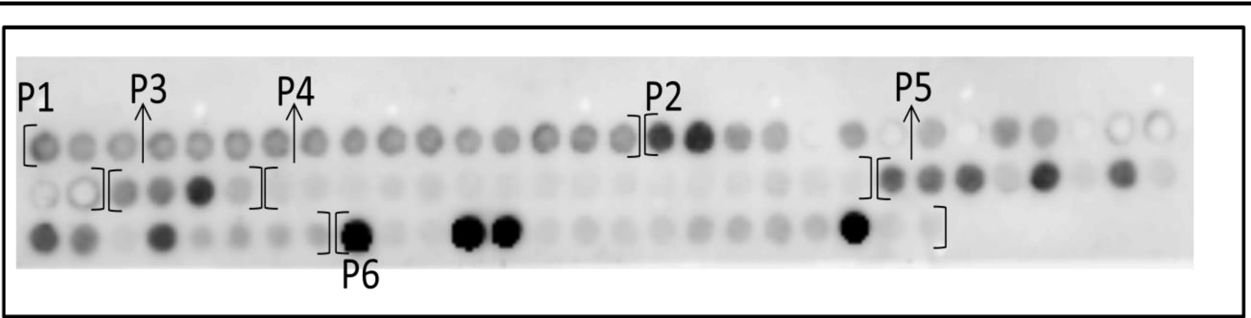

Fig. 17. Binding of MMP-9 with the mutated peptides based on the sequences of Serglycin. The original unmutated parent peptides for all the spots are; P1: ^{11}*VR*CNPDSNSA NCLEEKGP*MF* (spot 1), P2: ^{28}P*MF*ELLPGESNKIPRLR*TD*L (spot 17), P3: ^{46}DLFPKTR*IQ*DLNRIFPLSED (spot 33) , P4 : ^{85}*FL*TEMEQDYQLVDE*SD*AFHD (spot 37), P5 : ^{94}Q*LV*DESDAFHDNLRSLD*RN*L (spot 53), P6 : ^{103}HD*NL*RSLDRN LPSD*SQ*DLGQ (spot 69). The white spots at the top of the membrane marks every five peptide in the array Underlined are the amino acids in the parent sequence that are mutated in the array as described in appendix 7.8.

The ProMMP-9 seems to bind to the parent peptide and all the mutated peptide spots in first peptide (P1) which means that the most of the amino acids in this peptide must be involved in binding. In the second peptide (P2), mutation of M^{29} did not prevent the binding to MMP-9 suggesting M^{29} was not involved in binding with proMMP-9 while the mutation of F^{30}, T^{46} and D^{47} were found to be involved in binding as their mutation prevented the binding to proMMP-9. However, there is an alternative explanation of this peptide array. It is that the weak stained bands also show binding and hence only the mutation of D^{47} prevents the binding which means that only this amino acid is involved in binding. However, this seems not likely as in figure16 MMP-9 should have bound to peptides 14 to 23. In the third peptide (P3), only the double mutation of I^{53} and Q^{54} appears to prevent the binding to MMP-9. Therefore it appears that it is enough with the presence of one of these two amino acids in order to bind proMMP-9. The fourth peptide (P4) doesn't seem to bind to proMMP-9 which means that the peptides 41 - 43 as seen in figure 16C, is an artefact. In the fifth peptide (P5), the mutation of amino acids L^{95}, V^{96} and N^{112} did not prevent binding while the mutation of R^{111} did prevent the binding to proMMP-9. Thus the amino acids L^{95}, V^{96} and N^{112} were not involved in binding whereas R^{111} was involved in binding with proMMP-9. In the sixth peptide (P6), mutation of N^{105} and L^{106} were found to prevent the binding suggesting that these peptides were needed for binding to proMMP-9 whereas mutation of S^{117} and Q^{118} did

not prevent the binding suggesting these two amino acids were not involved in binding with proMMP-9.

3.6.3. Peptide array based on the sequence of serglycin probed with TIMP-1

Previously it was shown that TIMP-1 prevents the formation of the SDS-soluble proMMP-9/CSPG complex [9]. Even though it is known that TIMP-1 binds to the HPX domain of proMMP-9 [22, 34] it is possible that TIMP-1 also binds to serglycin. From the ponceau stained membrane (appendix 7.7) containing all the peptide arrays for serglcyin, Fn-II module and HPX domain, one of the arrays for serglycin was cut and was probed with TIMP-1 as described in method section 2.4.9. Figure 18A shows the ponceau stained array where the spots are labelled with the number of the peptide sequence shown in appendix 7.7 (Fig. A9.). Figure 18B shows that TIMP-1 to several peptides in the serglycin sequence.

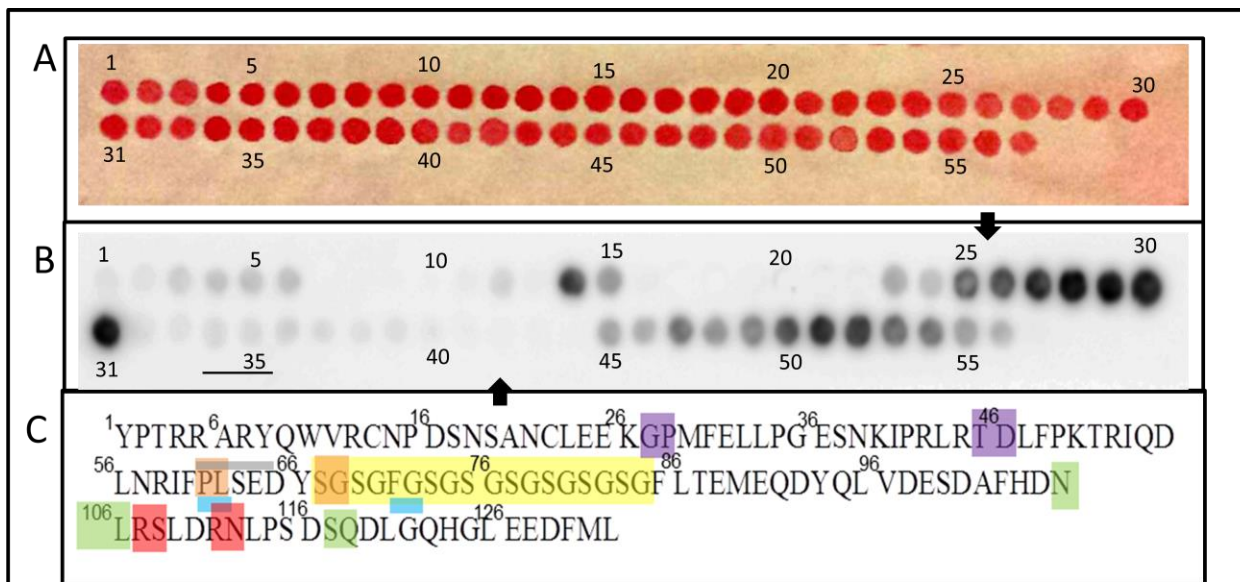

Fig. 18. Binding of TIMP-1 to peptides in the array of the serglycin core protein. **(A)** Ponceau staining of the array where the 57 peptides are numbered. (**B**) Binding of TIMP-1 to peptides in the core protein of serglycin. The dark spots are the postivie spots representing the binding of TIMP-1 with the serglyin sequences. (**C**) The amino acid sequence of serglycin and the analysis of the positive spots where the colored boxes show the amino acids that has been suggested to be involved in binding with TIMP-1. The yellow stretch represents the serglycin repeats where the CS-chains are present in serglycin. In (B) arrows at peptide 25 to arrow at peptide 42 represent the peptides containing amino acids from the serglycin repeats and the underlined peptides represent the peptides comprised of whole sequence of the serglycin repeats.

Figure18 shows that TIMP-1 binds to sequences in the serglycin core protein located both N- and C-terminal to the SG-repeats which bind the CS-chains. Based on the stained peptides shown in figure 18B and their amino acid sequences as seen in appendix 7.7 (Fig. A9.), the following conclusions about the amino acids in the serglycin core protein that is involved in binding with TIMP-1 can be drawn: in the N-terminal part of Serglycin the first peptide that proMMP-9 seems to bind is peptide 14. The possibilities for the staining could be: TIMP-1 binds to the whole sequence from ^{27}GP to TD^{46}, it binds to amino acids ^{27}GP and TD^{46}or staining of peptide 14 could be an artefact. TIMP-1 also seems to bind to peptides 25 to 31 suggesting that TIMP-1 binds to either the whole sequence from ^{61}PL to SG^{68} or just the amino acids ^{61}PL and SG^{68}. However, if the binding is to peptides 23-31, this suggests that amino acids ^{61}PLSE^{64} are involved in binding. Another possibility is that TIMP-1 only binds to peptides 27 to 31. In this case, either the whole sequence from ^{61}PL to FG ^{72} or just the amino acids ^{61}PL to FG ^{72} are involved in binding. In the C-terminal part of serglycin the peptides that seem to bind with TIMP-1 are those from 45 to 56. Thus amino acids ^{107}RSLDRN^{112} would be involved in binding where TIMP-1 bind to both ^{107}RS alone and ^{111}RN alone. Another possibility is that TIMP-1 does not bind to all these peptides but only to either the peptides 47 to 54 or 50 to 53. On the basis of analysis of the peptides 47 to 54, we can conclude that either the whole sequence from ^{107}RS to RN^{112}is involved or just the amino acids ^{107}RS and RN^{112}. If the binding is only to peptides 50 to 53, we can conclude that either the whole sequence from ^{105}NL and SQ^{118}is involved or just the amino acids ^{105}NL to SQ^{118}. In the case of TIMP-1, we have not ordered a mutation array to verify the binding observed here.

3.6.4. Peptide array based on sequences of FnII module of MMP-9 probed with His-tagged serglycin

From the ponceau stained membrane (appendix 7.7) containing all the peptide arrays for serglycin, FnII module and HPX domain, one of the arrays for FnII module was cut and was probed with His-tagged serglycin as described in the method section 2.4.9. Figure 19A shows the ponceau stained array where the spots are labelled with the number of the peptide sequence shown in appendix 7.7 (Fig. A11) and figure 19B

shows that His-tagged serglycin binds to several of the peptides in the FnII module sequence of MMP-9.

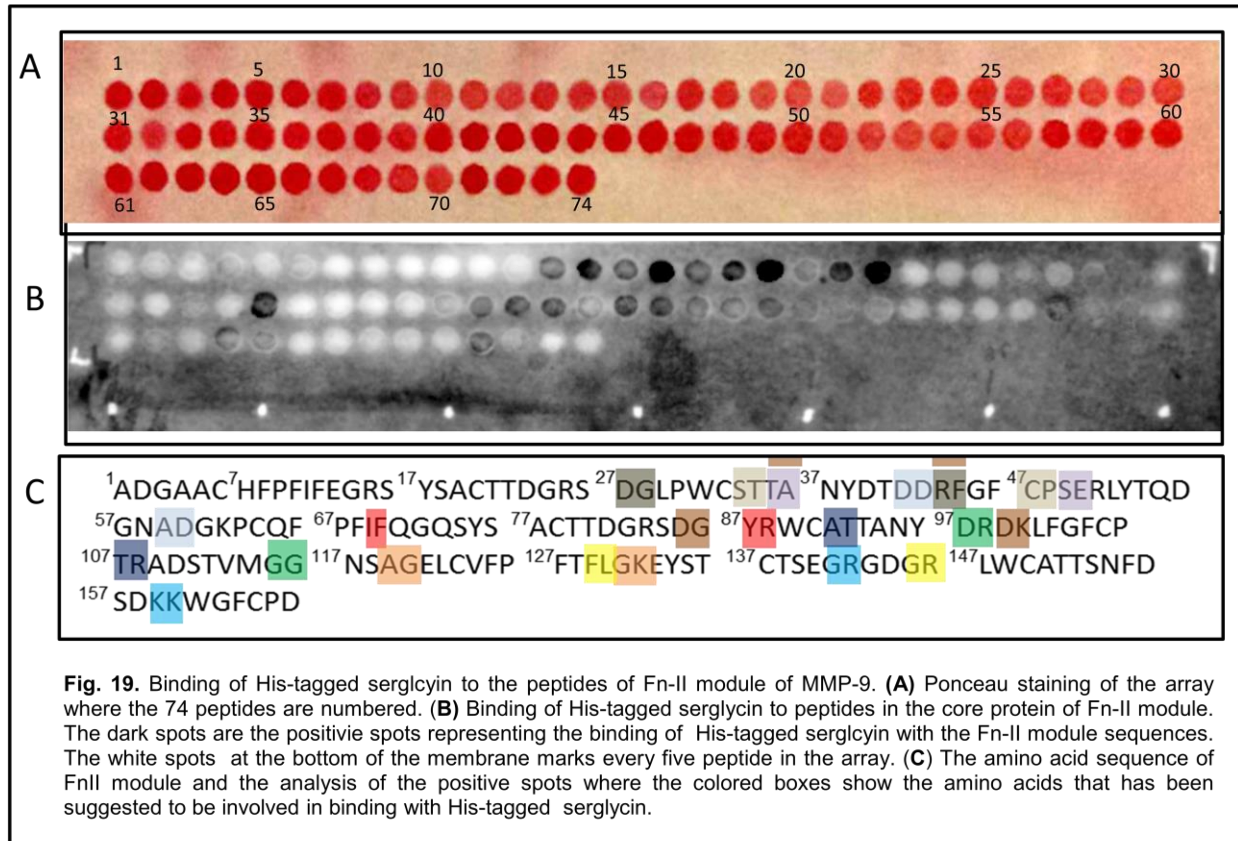

Fig. 19. Binding of His-tagged serglcyin to the peptides of Fn-II module of MMP-9. **(A)** Ponceau staining of the array where the 74 peptides are numbered. (**B)** Binding of His-tagged serglycin to peptides in the core protein of Fn-II module. The dark spots are the positivie spots representing the binding of His-tagged serglcyin with the Fn-II module sequences. The white spots at the bottom of the membrane marks every five peptide in the array. (**C**) The amino acid sequence of FnII module and the analysis of the positive spots where the colored boxes show the amino acids that has been suggested to be involved in binding with His-tagged serglycin.

Based on the stained peptides shown in figure19B and their amino acid sequences seen in appendix 7.7 (Fig. A11.), the following conclusions with respect to the amino acids in the FnII module that seem to be involved in binding with His-tagged serglycin can be drawn: as seen in figure 19B, it seems that His-tagged serglycin binds peptides 13 to 22, suggesting that amino acids R^{43} and F^{44} are involved in binding. However there are several possibilities to interpret this binding sequence as it appears that some of the spots are heavily stained while others in the sequence are only moderately to weakly stained. This is also the case for the other parts of the array. In order to make the interpretations easy to follow, Table 13 shows which peptides in the array the His-tagged serglycin is suggested to bind and the possible amino acids in these peptides that may be involved in the binding. These amino acids are also labelled in figure 19C in order to make it easy to see where in the sequence they are located.

Table 13. Peptides and amino acids in the MMP-9 FnII module that binds to His-tagged serglycin. Shown is the peptide number (sequence of peptide is presented in appendix 7.7, Fig. A11.) and the amino acids in the FnII module that is suggested to be involved in binding. The numbering and sequence of the FnII module is shown in figure 19C and binding of His-Tagged serglycin to FnII peptides are those shown in figure 19B. Shown is also the peptide used in the mutation peptide array, which amino acids in the first array the mutations are based on and which of the amino acids in the mutation array has been confirmed involved in binding. If it is enough with a single mutation to prevent binding, this is shown by the amino acid and its sequence number. In some cases only double (Do), triple (Tr) or quadruple (Qu) mutations prevent binding.

	Peptide array	Mutation peptide array	
Peptide N°	Amino acids	Peptide N°	Mutated Amino acids prevent binding
13-22	^{43}RF		
13-18	^{35}TA, ^{43}RF	P2	
13,14	^{27}DG, ^{43}RF	P1	Do, Tr, Qu
15-17	^{33}ST, ^{47}CP	P3	
16-18	^{35}TA, ^{49}SE	P4	
18,19	^{37}NY, ^{53}YT		
21	^{41}DD, ^{59}AD	P5	
21,22	^{43}RF, ^{59}AD		
35	^{69}IF, ^{87}YR	P6	^{88}R
41-50	^{99}DK		
41-49	^{97}DRDK		
41-43	^{85}DG, ^{99}DK	P7	
45,46	^{91}AT, ^{107}TR	P8	
49	^{97}DR, ^{114}GG	P9	
57-60	^{119}AG, ^{131}GK	P10	
57-59	^{117}NS, ^{131}GK		
64,65	^{129}FL, ^{145}GR	P11	^{129}F, ^{146}R
71	^{141}GR, ^{159}KK	P12	

The amino acids suggested to be involved in binding with the His-tagged serglycin is located in all three repeats of the FnII module of MMP-9. To confirm the involvement of some of these amino acids, the sequences comprising of these amino acids were mutated and another peptide array was done (see 3.6.5).

3.6.5. Mutated peptide array based on the sequences of FnII module of MMP-9 probed with His-tagged serglycin

The amino acids from the peptides based on the sequence of FnII module of MMP-9 that seemed to be involved in binding were mutated (appendix 7.8) and the membrane of the peptide array containing these mutated peptides was probed with His-tagged serglycin as described in the method section 2.4.9 and 2.4.10.

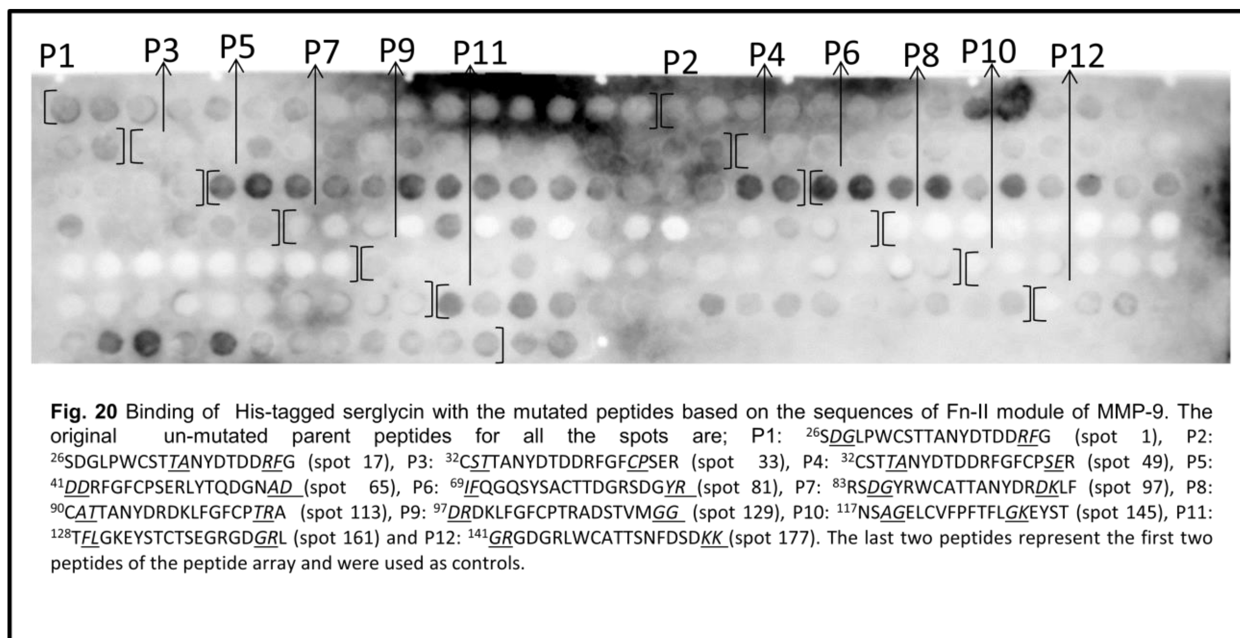

Fig. 20 Binding of His-tagged serglycin with the mutated peptides based on the sequences of Fn-II module of MMP-9. The original un-mutated parent peptides for all the spots are; P1: ^{26}SDGLPWCSTTANYDTDDRFG (spot 1), P2: ^{26}SDGLPWCSTTANYDTDDRFG (spot 17), P3: ^{32}CSTTANYDTDDRFGFCPSER (spot 33), P4: ^{32}CSTTANYDTDDRFGFCPSER (spot 49), P5: ^{41}DDRFGFCPSERLYTQDGNAD (spot 65), P6: ^{69}IFQGQSYSACTTDGRSDGYR (spot 81), P7: ^{83}RSDGYRWCATTANYDRDKLF (spot 97), P8: ^{90}CATTANYDRDKLFGFCPTRA (spot 113), P9: ^{97}DRDKLFGFCPTRADSTVMGG (spot 129), P10: ^{117}NSAGELCVFPFTFLGKEYST (spot 145), P11: ^{128}TFLGKEYSTCTSEGRGDGRL (spot 161) and P12: ^{141}GRGDGRLWCATTSNFDSDKK (spot 177). The last two peptides represent the first two peptides of the peptide array and were used as controls.

The mutation of peptide 1(P1) seemed to affect the binding to His-tagged serglycin but the effect seems to be very weak. All double mutations in P1 except D^{27} (mutated to A) and F^{44} (mutated to G) prevented the binding to His-tagged serglcyin. Although the unmutated P2 is identical to unmutated P1, a much weak staining was obtained for P2. Peptide3 (P3) and peptide 4 (P4) were not found to be involved in the binding with His-tagged serglycin. The mutation of peptide 5 (P5) did not affect the binding suggesting that amino acids ^{41}DD and AD^{60} were not essential for binding with His-tagged serglcyin. In peptide 6 (P6), only mutated R^{88} prevented the binding with MMP-9 suggesting R^{88} is involved in binding with His-tagged serglcyin. Peptide 7(P7), peptide 8 (P8), peptide 9 (P9) and peptide 10 (P10) were not found to be involved in binding which suggest that these peptides are not involved in binding with His-tagged serglcyin . In peptide 11 (P11), only F^{128} and R^{146} were found to be involved in binding as their

mutation prevented the binding to His-tagged serglycin. It seems that His-tagged serglycin don't bind to peptide 12 (P12). Table 13 summarizes which of the mutated amino acids in the FnII repeats that prevented binding to the His-tagged serglycin and hence can be anticipated to take part in the binding of the core protein of serglycin.

Note that the last two peptides are the same peptides that make up the first two spots from P1. These two spots were simply put in order to differentiate the peptide array for hemopexin domain and Fn-II module, as these two arrays along with the array for serglycin was produced in same membrane.

3.6.6. Peptide array based on sequences of HPX domain of MMP-9 probed with His-tagged serglycin

From the ponceau stained membrane (appendix 7.7) containing all the peptide arrays for serglycin, FnII module and HPX domain, one of the arrays for HPX domain was cut and was probed with His-tagged serglycin as described in the method section 2.4.9. Figure 21A shows the ponceau stained array where the spots are labelled with the number of the peptide sequence shown in appendix 7.7 (Fig. A12.) His-tagged serglcyin binds to several of the peptides in the HPX domain sequence of MMP-9 as shown in figure 21B.

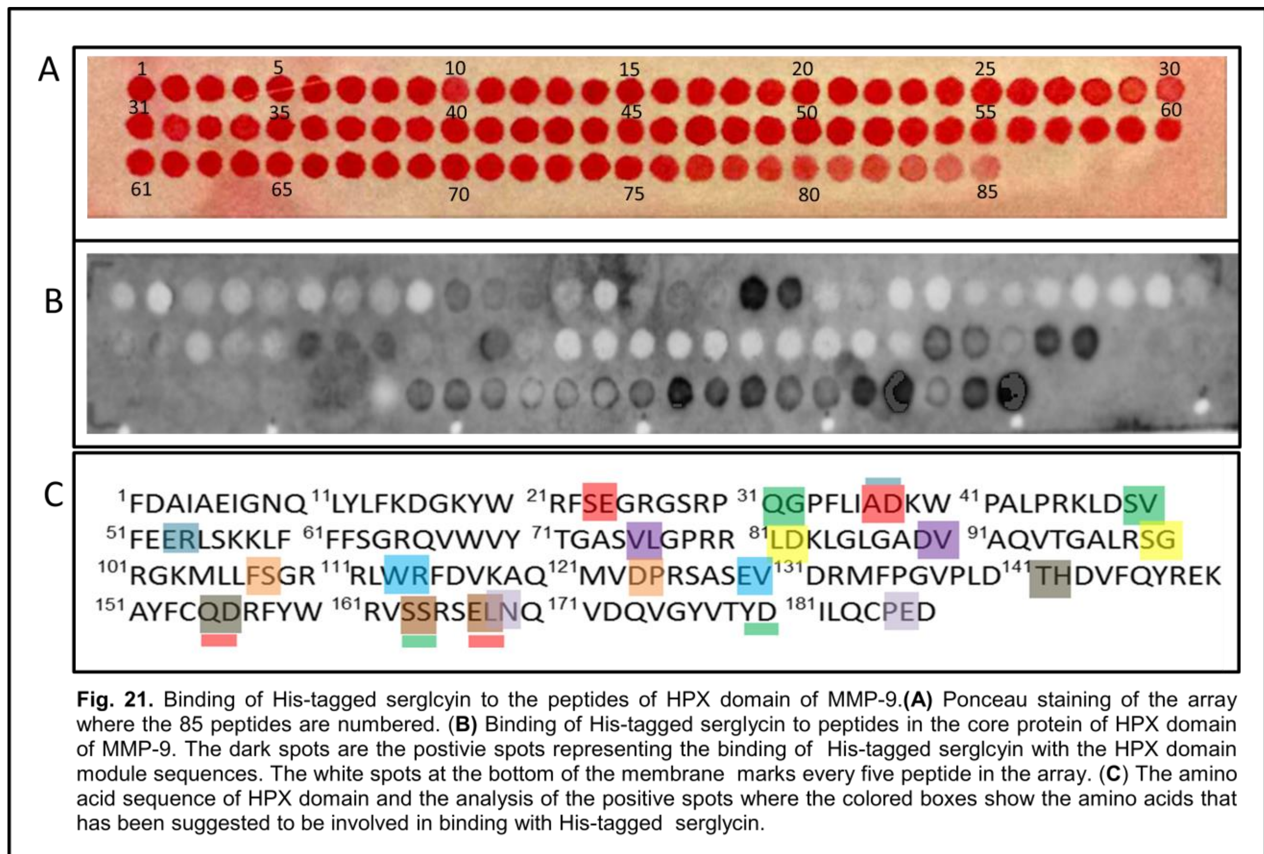

Fig. 21. Binding of His-tagged serglcyin to the peptides of HPX domain of MMP-9.**(A)** Ponceau staining of the array where the 85 peptides are numbered. (**B)** Binding of His-tagged serglycin to peptides in the core protein of HPX domain of MMP-9. The dark spots are the postivie spots representing the binding of His-tagged serglcyin with the HPX domain module sequences. The white spots at the bottom of the membrane marks every five peptide in the array. (**C**) The amino acid sequence of HPX domain and the analysis of the positive spots where the colored boxes show the amino acids that has been suggested to be involved in binding with His-tagged serglycin.

Based on the stained peptides shown in figure 21B and their amino acid sequences as seen in appendix 7.7 (Fig. A12.) the following conclusions with respect to the amino acids in the HPX domain that seem to be involved in binding with His-tagged serglycin can be drawn: the first series of peptides that seem to involve in binding are 10 to 12, suggesting that either the whole sequence from ^{23}SE to AD^{38} is involved in binding or just the amino acids ^{23}SE and AD^{38}. Peptide 16 seemed to be involved in binding, hence either the whole sequence from ^{31}QG to SV^{50} is involved or just the amino acids ^{31}QG and SV^{50}. Peptides 18 and 19 also seemed to be involved in binding and therefore, either the whole sequence from ^{38}AD to ER^{54} must be involved or just the amino acids ^{38}AD and ER^{54}. Similarly peptides 36 to 38 seem to interact with His-tagged serglycin, hence either the whole sequence from ^{75}VL to DV^{90} or just the amino acids ^{75}VL to DV^{90} are involved in the binding. Similar analysis of the binding of His-tagged serglycin to the other peptides in the array has been done. As for the binding to the FnII module, some of the spots are heavily stained while others in the sequence are only moderately to weakly stained. Thus there are several possibilities to interpret these

binding sequences. The interpretation of all the possible interactions and the amino acids involved in the HPX domain is summarized in Table 14. These amino acids are also labelled in figure 21C in order to make it easy to see where in the sequence they are located.

Table 14. Peptides and amino acids in the MMP-9 HPX domain that binds to His-tagged serglycin. Shown is the peptide number (sequence of peptide is presented in appendix 7.7, Fig. A12.) and the amino acids in the FnII module that is suggested to be involved in binding. The numbering and sequence of the HPX domain is shown in figure 21C and binding of His-Tagged serglycin to HPX domain are those shown in figure 21B. Shown is also the peptide used in the mutation peptide array, which amino acids in the first array the mutations are based on and which of the amino acids in the mutation array has been confirmed involved in binding. If it is enough with a single mutation to prevent binding, this is shown by the amino acid and its sequence number. In some cases only double (Do), triple (Tr) or quadruple (Qu) mutations prevent binding.

Peptide array		Mutation peptide array	
Peptide N^{o}	Amino acids	Peptide N^{o}	Mutated Amino acids prevent binding
10-12	^{23}SE, ^{37}AD	P1	^{24}E, ^{38}D
16	^{31}QG – SV^{50}	P2	
18,19	^{37}AD, ^{53}ER	P3	^{38}D ^{53}E (Do)
36-38	^{75}VL, ^{89}DV	P4	^{89}D
41	^{81}LD, ^{99}SG	P5	^{81}LD along with one or both ^{99}SG (Tr, Qu)
53,54	^{107}FS, ^{123}DP	P6	
56,57	^{113}WR, ^{129}EV	P7	^{113}W ^{130}V (Do); ^{113}WR (Do)
69-71	^{141}TH, ^{155}QD	P8	
76-78	^{155}QD, ^{167}EL	P9	^{167}E
75-82	^{163}SS, ^{167}EL	P10	
81,82	^{163}SS, ^{180}YD	P11	
84,85	^{168}LN, ^{185}PE	P12	

This peptide array indicates that all four parts of HPX domain of MMP-9 are involved in binding with His-tagged serglycin. To confirm the involvement of these amino acids, the

sequences comprising of these amino acids were mutated and another peptide array was performed (3.6.7).

3.6.7. Mutated peptide array based on the sequences of HPX domain of MMP-9 probed with His-tagged serglycin

The amino acids from the peptides based on the sequence of HPX domain of MMP-9 that seemed to be involved in binding were mutated (appendix 7.8) and the membrane of the peptide array containing these mutated peptides was probed with serglycin as described in the method section 2.4.9 and 2.4.10.

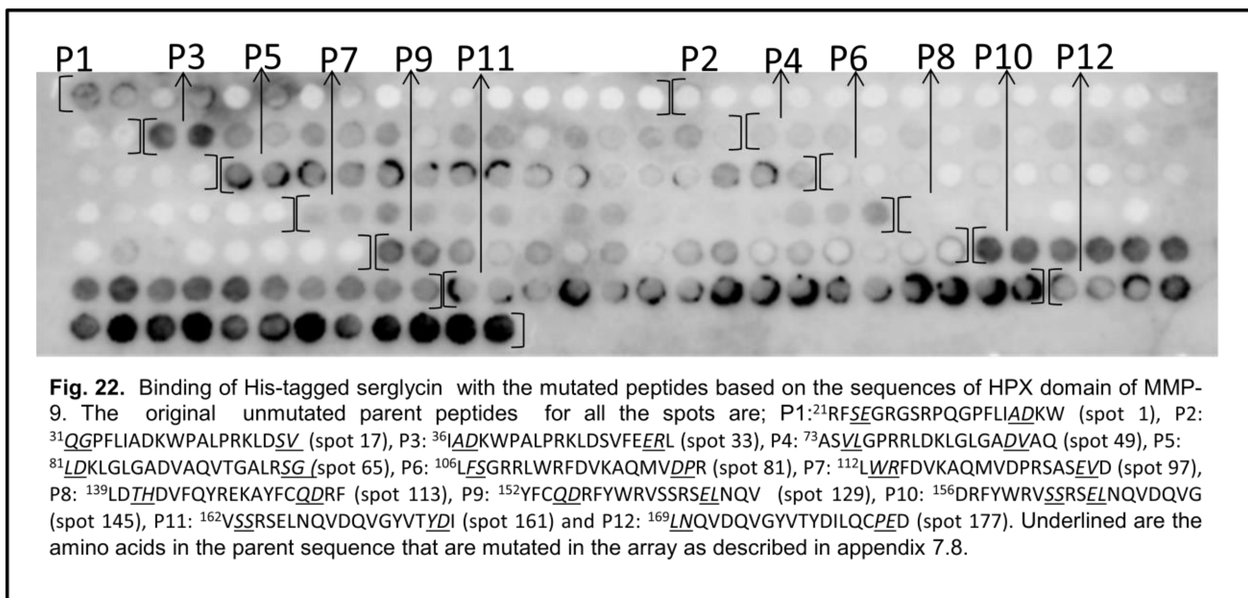

Fig. 22. Binding of His-tagged serglycin with the mutated peptides based on the sequences of HPX domain of MMP-9. The original unmutated parent peptides for all the spots are; P1: ^{21}RF*SE*GRGSRPQGPFLI*AD*KW (spot 1), P2: ^{31}*QG*PFLIADKWPALPRKLD*SV* (spot 17), P3: ^{36}I*AD*KWPALPRKLDSVFE*ER*L (spot 33), P4: ^{73}AS*VL*GPRRLDKLGLGA*DV*AQ (spot 49), P5: ^{81}*LD*KLGLGADVAQVTGALR*SG* (spot 65), P6: ^{106}L*FS*GRRLWRFDVKAQMV*DP*R (spot 81), P7: ^{112}L*WR*FDVKAQMVDPRSAS*EV*D (spot 97), P8: ^{139}LD*TH*DVFQYREKAYFC*QD*RF (spot 113), P9: ^{152}YFC*QD*RFYWRVSSRS*EL*NQV (spot 129), P10: ^{156}DRFYWRV*SS*RS*EL*NQVDQVG (spot 145), P11: ^{162}V*SS*RSELNQVDQVGYVT*YD*I (spot 161) and P12: ^{169}*LN*QVDQVGYVTYDILQC*PE*D (spot 177). Underlined are the amino acids in the parent sequence that are mutated in the array as described in appendix 7.8.

In the first peptide (P1), mutation of amino acids E^{24} and D^{38} were found to weakly prevent the binding to His-tagged serglycin suggesting that these amino acids might be needed for binding, whereas mutation of amino acids S^{23} and A^{37} had no effect on binding which suggests these amino acids are not involved in binding with His-tagged serglycin. The second peptide (P2) was not found to be involved in binding. The double mutations of amino acids D^{38} and E^{53} in peptide 3 (P3) were found to prevent the binding suggesting that these amino acids are necessary for binding with His-tagged serglycin. However mutations of amino acids A^{37} and R^{54} in P3 were not involved in binding. In peptide 4 (P4), mutation of D^{89} affected the binding suggesting this amino acid is involved in binding with His-tagged serglycin. However, we suspect that there

could be another unknown amino acid in the peptide that might be involved in the binding. In P4, mutation of amino acids V^{75} and V^{90} did not affected the binding suggesting that these amino acids were not found to be involved in the binding with His-tagged serglcyin. In peptide 5 (P5), it was found that binding disappeared only in four peptides which were either triple or quadruple mutated, i.e. the double mutation of amino acids ^{81}LD to GA along with single or double mutation of ^{99}SG to AL. When D^{81} and G^{100} are mutated independently, binding still occurred. Peptide 6(P6) was not found to be involved in binding with His-tagged serglycin. Peptide 7(P7) was found to be involved in weak binding where only the double mutations of W^{113} (mutated to A), V^{130} *(*mutated to G*)* and ^{113}*WR*(mutated to AA) were found to prevent binding to His-tagged serglycin. Peptide 8 (P8) was not found to be involved in binding with His-tagged serglycin. In peptide 9 (P9) E^{167} was found to be involved in binding, but we suspect it must be linked with another unknown amino acid in the peptide. ^{155}QD and L^{168} were not found to be involved in binding as the mutation of these peptides did not prevent binding. Possibly other amino acids in this peptide are involved in binding of His-tagged serglcyin, and could be either Y^{152} or F^{153}. The mutations in peptide 10 -12 (P10 - 12) did not affect the binding which means that the mutated amino acids are not involved in binding of His-tagged serglycin to HPX domain of MMP-9. Table 14 summarizes those mutated amino acids in the HPX domain that prevented binding to the His-tagged serglycin and hence can be anticipated to take part in the binding of the core protein of serglycin.

3.6.8. Peptide array based on the sequence of HPX domain of MMP-9 probed with TIMP-1

The C-terminal domain of TIMP-1 is known to bind to C-terminal HPX domain in proMMP-9 and form a proMMP-9/TIMP-1 heterodimer with unknown function [22, 34]. So far it is not known to which part TIMP-1 binds to the MMP-9 HPX domain. As the presence of TIMP-1 prevents the formation of the SDS-soluble proMMP-9/CSPG complex [9] it was of interest to determine if TIMP-1 and serglycin binds to identical or overlapping regions in the MMP-9 HPX domain. From the ponceau stained membrane (appendix 7.7) containing all the peptide arrays for serglcyin, FnII module and HPX

domain, one of the arrays for HPX domain was cut and was probed with TIMP-1 as described in the method section 2.4.9. Figure 23A shows the ponceau stained array where the spots are labelled with the number of the peptide sequence shown in appendix 7.7 (Fig. A12.) TIMP-1 binds to several of the peptides in the HPX domain sequence of MMP-9 as seen in figure 23B.

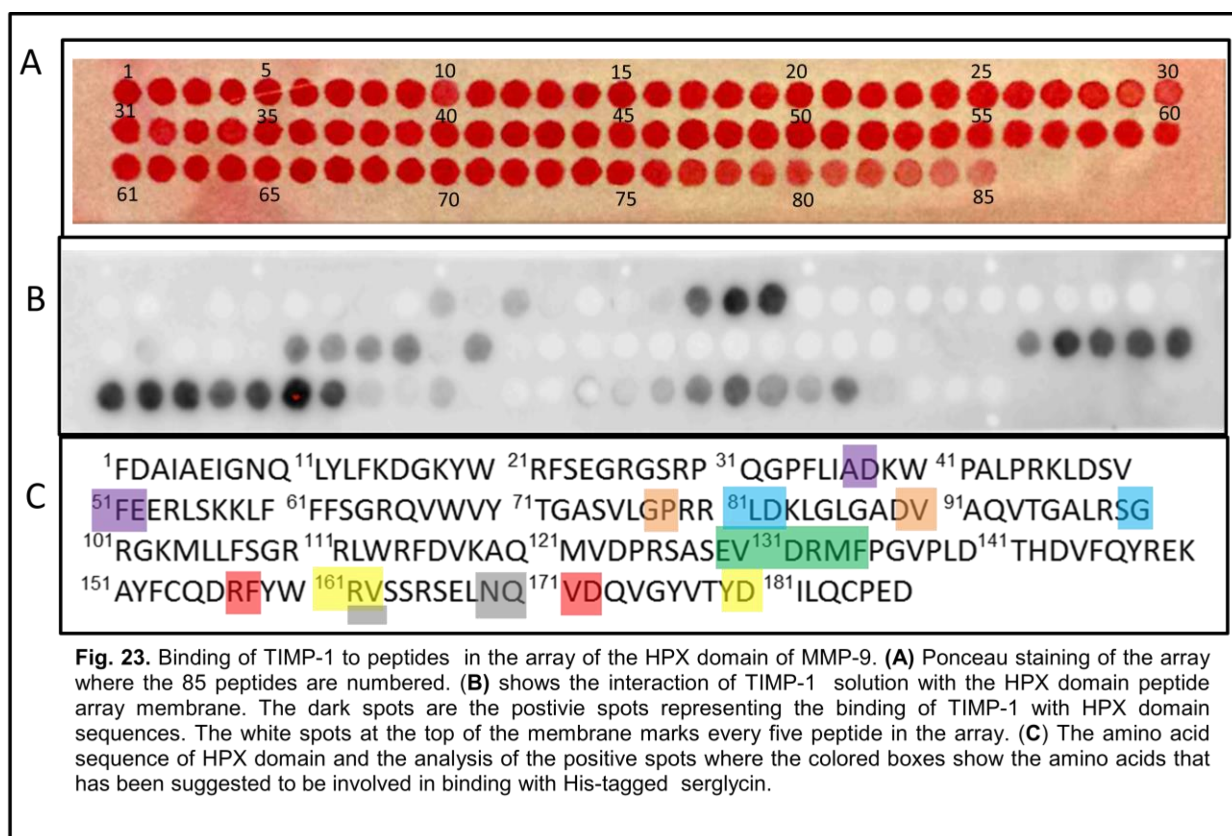

Fig. 23. Binding of TIMP-1 to peptides in the array of the HPX domain of MMP-9. **(A)** Ponceau staining of the array where the 85 peptides are numbered. (**B**) shows the interaction of TIMP-1 solution with the HPX domain peptide array membrane. The dark spots are the postivie spots representing the binding of TIMP-1 with HPX domain sequences. The white spots at the top of the membrane marks every five peptide in the array. (**C**) The amino acid sequence of HPX domain and the analysis of the positive spots where the colored boxes show the amino acids that has been suggested to be involved in binding with His-tagged serglycin.

Based on the stained peptides shown in figure 23B and their amino acid sequences as seen in appendix 7.7 (Fig. A12.), the following conclusions with respect to the amino acids in the HPX domain which seem to be involved in binding with TIMP-1 can be drawn. The first series of peptides that seem to be involved in binding are 17 to 19 and from the analysis of the sequences of these peptides it can be conclude that either the whole sequence from ^{37}AD to FE^{52} or just the amino acids ^{37}AD and FE^{52} are involved in binding with TIMP-1. Another series of peptides that seem to be involved in binding are peptide 36 to 39, suggesting that either the whole sequence from ^{77}GP to DV^{90} or just the amino acids ^{77}GP to DV^{90} are involved in binding with TIMP-1. Peptide 41 seem to be bind alone with TIMP-1 and the analysis of this peptide concludes that either the

whole sequence from ^{81}LD to SG^{100} or just the amino acids ^{81}LD and SG^{100} are involved. The whole series of peptides from 56 to 67 seems to be involved in binding and indicating that sequence ^{129}EVDRMF^{134} is involved in binding. Peptides 77 to 79 is another series of peptides that seem to involve in binding and indicating that either the sequence from ^{157}RF to VD^{172} or just the amino acids ^{157}RF and VD^{172} are involved in binding. However, if the binding is from peptide 76 to 81, then the whole sequence from ^{161}RV to NQ^{169} must be involved or just the amino acids ^{161}RV to NQ^{169}. In the case that the binding is not to the entire sequence but just to peptides 77-79, then TIMP-1 also binds peptide 81. Binding to just this peptide suggests that either the whole sequence from ^{161}RV to YD^{181} or just the amino acids ^{161}RV and YD^{181} are involved in binding. TIMP-1 also seems to bind to peptides 10, 12 and 70. The interpretation of all the possible interactions and the amino acids involved in the HPX domain is summarized in Table 15. These amino acids are also labelled in figure 23C in order to make it easy to see where in the sequence they are located.

Table 15. Peptides and amino acids in the MMP-9 HPX domain that binds to TIMP-1. Shown is the peptide number (sequence of peptide is presented in appendix 7.7, Fig. A12.) and the amino acids in the HPX domain suggested involved in binding. The numbering and sequence of the HPX domain is shown in figure 23C and binding of TIMP-1to HPX domain are those shown in figure 23B.

Peptide N^{o}	Amino acids
10	^{19}YW, ^{37}AD
12	^{23}SE, ^{41}PA
17-19	^{37}AD, ^{51}FE
36-39	^{77}GP, ^{89}DV
41	^{81}LD, ^{99}SG
56-67	^{129}EVDRMF
70	^{139}LD, ^{157}RF
76-81	^{161}RV, ^{168}NQ
77-79	^{157}RF, ^{171}VD
81	^{161}RV, ^{180}YD

3.7. *In vitro* reconstitution competition experiments with soluble peptides

The peptide arrays are composed of a small target part (20 amino acids) of a protein. When a protein binds to a peptide in a peptide array, it means that the protein can interact with that certain peptide, motif and amino acids in the target protein. However, it is difficult to know if the motifs and amino acids in these peptides are hidden in the parent protein or not, which is why it is hard to state that the interactions obtained in a peptide array actually occurs when the entire protein is present. Therefore, it is important to do additional assays. One such test is to synthesize peptides that are identical with the peptides in the peptide array that bind the partner protein and test if these can prevent the *in vitro* reconstitution of the complex. However, we need to take into consideration that in these *in vitro* reconstitution experiments, we have used the partly purified CSPGs from THP-1 cells and complexes may be also formed by other proteins than just serglycin. In such a case a peptide that prevent or inhibit the formation of the proMMP-9/serglycin complex may not affect the complex formation between proMMP-9 and another protein as the interacting regions between the different proteins may not be the same.

In vitro reconstitution experiments in the presence of peptides (D1 - D9) with sequences identical to that in the serglycin core protein, FnII and HPX of MMP-9 that seemed to be involved in binding as indicated in sections 3.6.1, 3.6.4 and 3.6.6 above. These experiments were performed in order to determine whether these peptides could prevent the formation of the SDS-stable or the SDS-soluble proMMP-9/CSPG complexes. The *in vitro* reconstitution experiments were performed as described in methods (2.4.7 and 2.4.11) and the sequences and purity of the peptides used are presented in the appendix 7.9, Table A1.

In almost every *in vitro* reconstitution competition experiment conducted, minor difficulties occurred. The main problem was during the ion-exchange chromatography, when the incubated samples were passed through the Q-Sepharose columns. Some samples blocked the column and made it difficult to elute the samples.

3.7.1. Peptides D1 and D2

Peptides D1 and D2 used in this experiment belonged to the sequence of serglycin and were mixed in different concentration with proMMP-9 and CSPG isolated from THP-1 cells. From figure 24 what we can see is that D1 does not seem to affect the formation of either SDS-stable or SDS-soluble complexes.

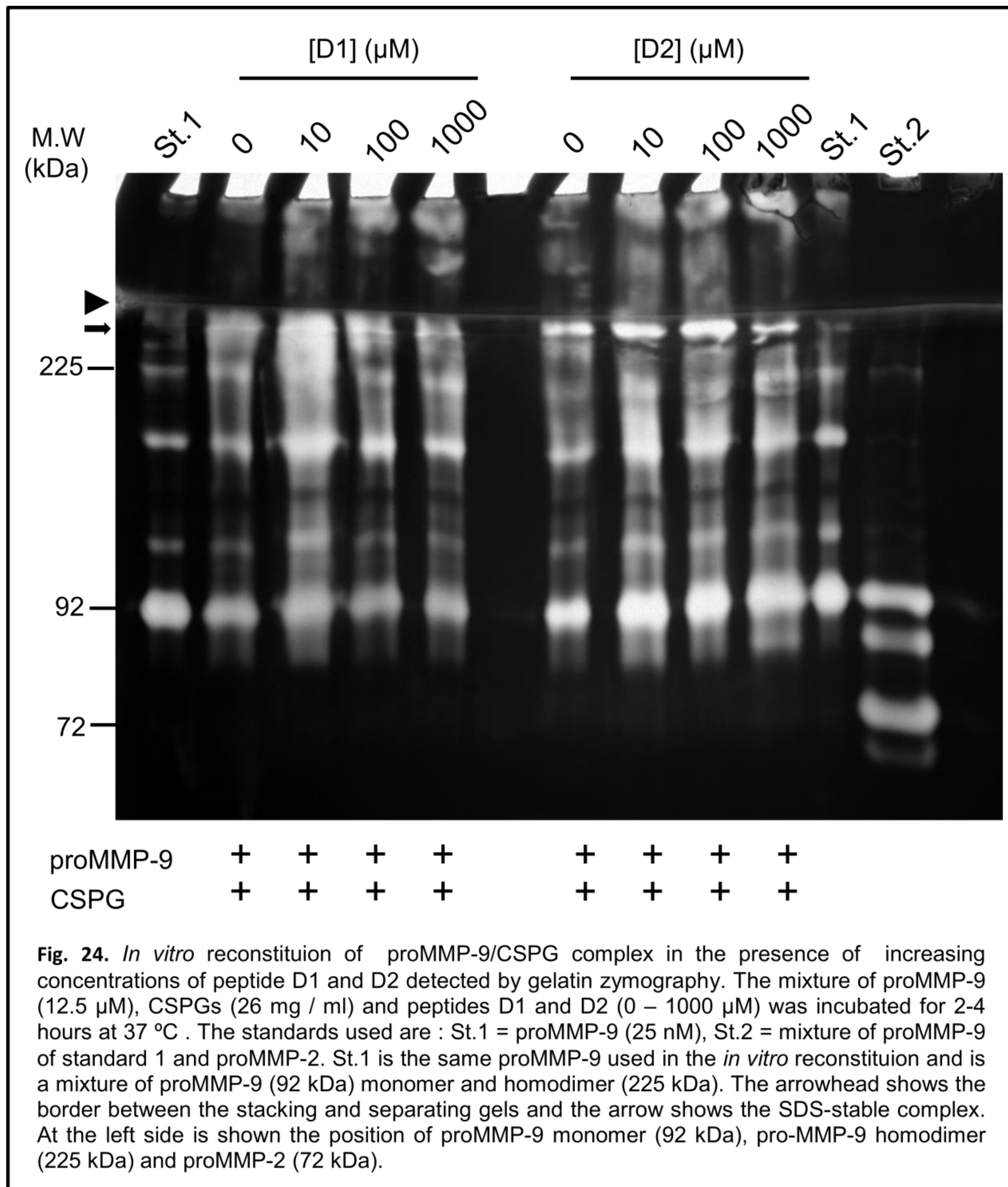

Fig. 24. *In vitro* reconstituion of proMMP-9/CSPG complex in the presence of increasing concentrations of peptide D1 and D2 detected by gelatin zymography. The mixture of proMMP-9 (12.5 µM), CSPGs (26 mg / ml) and peptides D1 and D2 (0 – 1000 µM) was incubated for 2-4 hours at 37 ºC . The standards used are : St.1 = proMMP-9 (25 nM), St.2 = mixture of proMMP-9 of standard 1 and proMMP-2. St.1 is the same proMMP-9 used in the *in vitro* reconstituion and is a mixture of proMMP-9 (92 kDa) monomer and homodimer (225 kDa). The arrowhead shows the border between the stacking and separating gels and the arrow shows the SDS-stable complex. At the left side is shown the position of proMMP-9 monomer (92 kDa), pro-MMP-9 homodimer (225 kDa) and proMMP-2 (72 kDa).

Peptide D2 seems to have an effect on both the formation of SDS-soluble and SDS-stable complexes. The formation of the 300 kDa SDS-stable complex seemed to be prevented by D2 as the size of the 300 kDa band decreased in the presence of 1000 µM D2. However, the effect of D2 on the formation of SDS-soluble complexes seemed

to have an opposite effect as the size of the 92 kDa band increased with the increasing concentrations of D2.

3.7.2. Peptide D3

Peptide D3 used in this experiment belongs to the sequence of the first repeat of the FnII module of MMP-9. It was mixed in different concentration with proMMP-9 and CSPG.

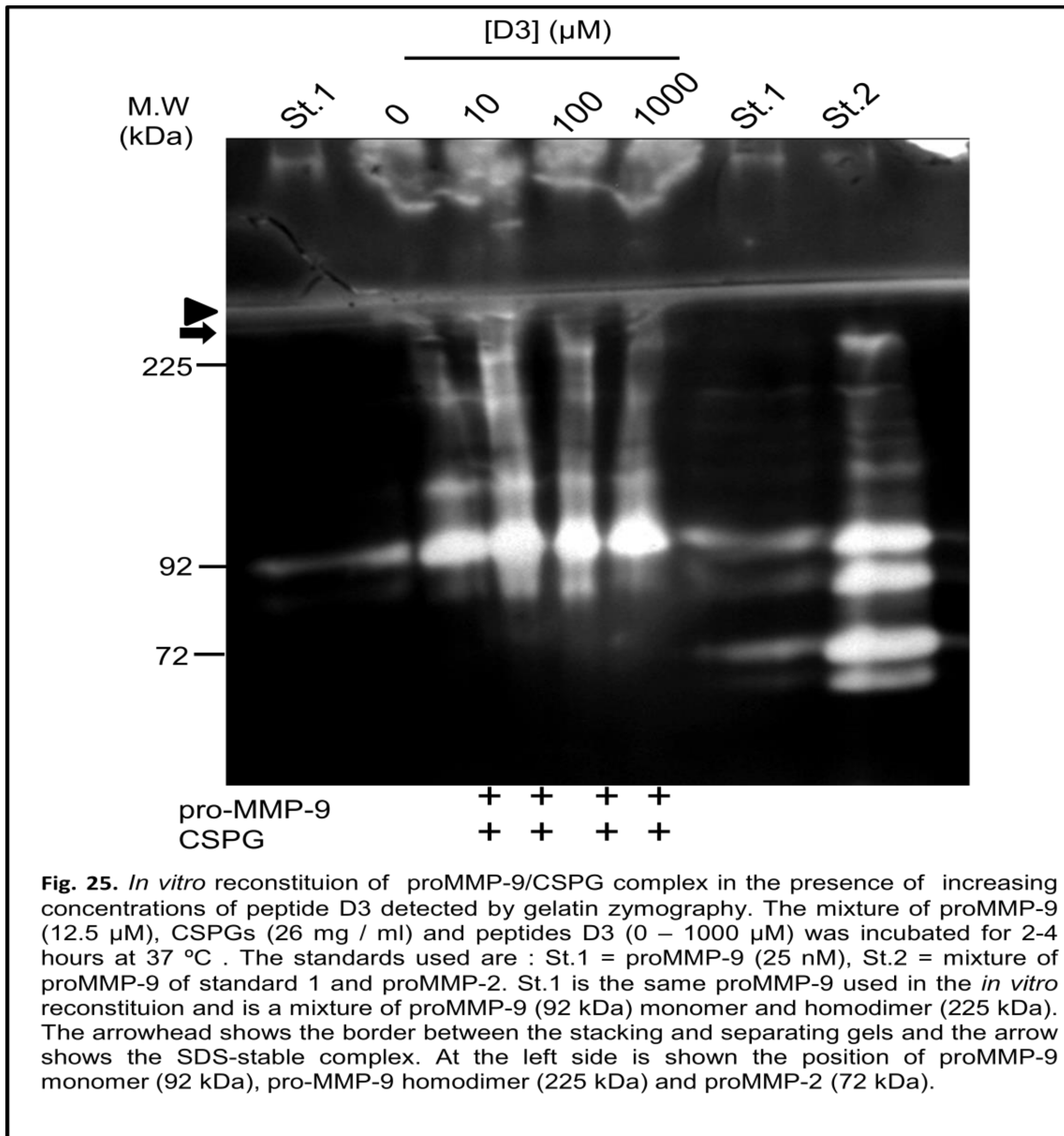

Fig. 25. *In vitro* reconstituion of proMMP-9/CSPG complex in the presence of increasing concentrations of peptide D3 detected by gelatin zymography. The mixture of proMMP-9 (12.5 μM), CSPGs (26 mg / ml) and peptides D3 (0 – 1000 μM) was incubated for 2-4 hours at 37 ºC . The standards used are : St.1 = proMMP-9 (25 nM), St.2 = mixture of proMMP-9 of standard 1 and proMMP-2. St.1 is the same proMMP-9 used in the *in vitro* reconstituion and is a mixture of proMMP-9 (92 kDa) monomer and homodimer (225 kDa). The arrowhead shows the border between the stacking and separating gels and the arrow shows the SDS-stable complex. At the left side is shown the position of proMMP-9 monomer (92 kDa), pro-MMP-9 homodimer (225 kDa) and proMMP-2 (72 kDa).

In the figure 25, we cannot confirm the formation of SDS-stable complexes, so the effect of D3 on the formation of the SDS-stable complexes cannot be concluded. We can see

that the D3 peptide don’t seems to have an effect on formation of SDS-soluble complexes, as the size of the 92 kDa band seems to be constant in the presence of 0 - 1000 µM D3.

3.7.3. Peptide D4

Peptide D4 used in this experiment belongs to the sequence of the second repeat of the FnII module of MMP-9. It was mixed in different concentration with proMMP-9 and CSPG.

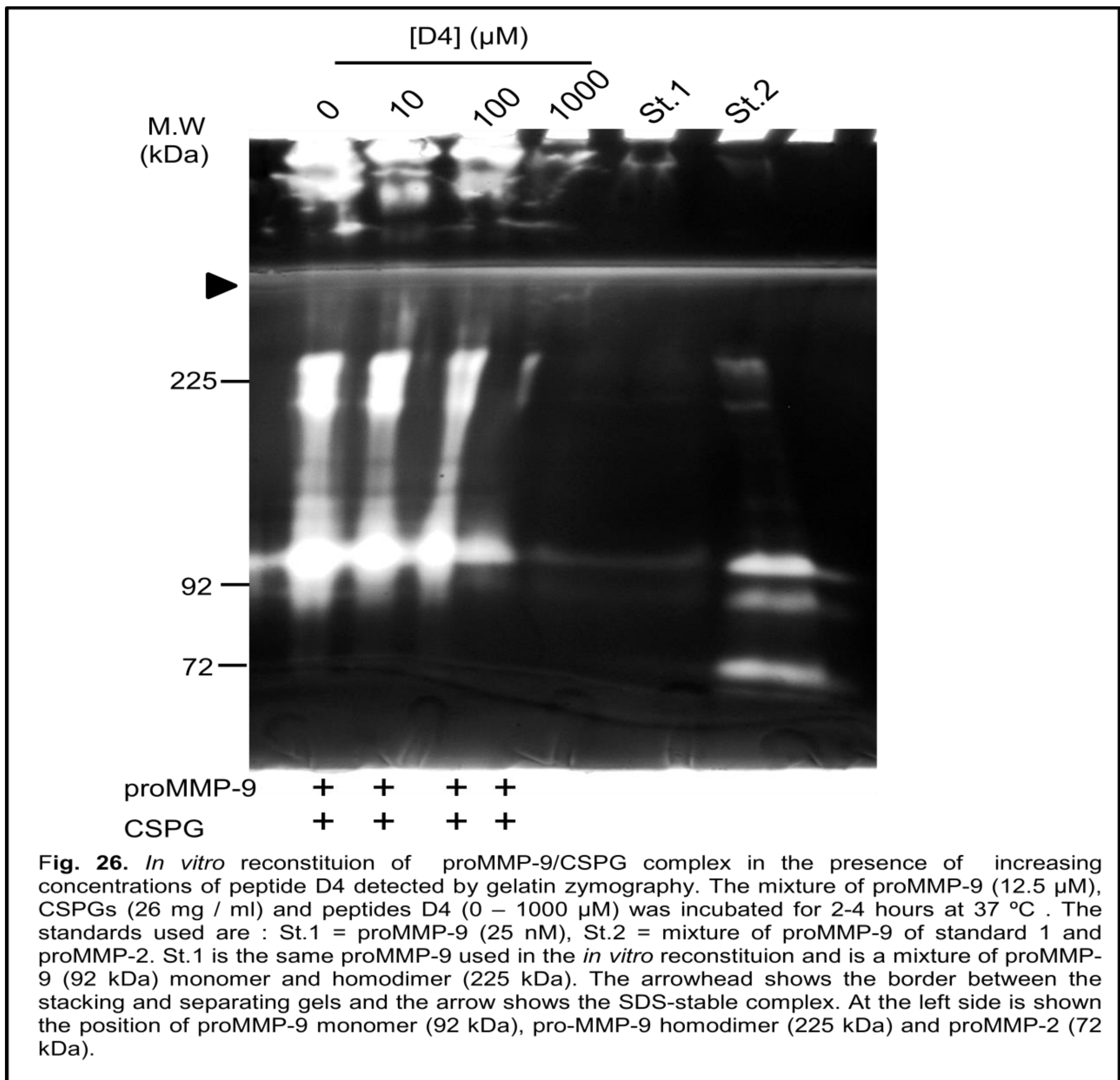

Fig. 26. *In vitro* reconstituion of proMMP-9/CSPG complex in the presence of increasing concentrations of peptide D4 detected by gelatin zymography. The mixture of proMMP-9 (12.5 µM), CSPGs (26 mg / ml) and peptides D4 (0 – 1000 µM) was incubated for 2-4 hours at 37 ºC . The standards used are : St.1 = proMMP-9 (25 nM), St.2 = mixture of proMMP-9 of standard 1 and proMMP-2. St.1 is the same proMMP-9 used in the *in vitro* reconstituion and is a mixture of proMMP-9 (92 kDa) monomer and homodimer (225 kDa). The arrowhead shows the border between the stacking and separating gels and the arrow shows the SDS-stable complex. At the left side is shown the position of proMMP-9 monomer (92 kDa), pro-MMP-9 homodimer (225 kDa) and proMMP-2 (72 kDa).

In the figure 26, and figures from other experiments with this peptide (figures not shown), it seems that D4 inhibits the formation of the SDS-soluble complexes. If we compare the monomers from samples with 0 to 1000 µM D4 peptides, the size of the 92 kDa bands seems to be successively smaller. However, we cannot decide if it has any effect on the formation of SDS-stable complexes as this has not been formed in this experiment.

3.7.4. Peptide D5 and D6

Peptide D5 used in this experiment belongs to the sequence of the end sequence of second repeat of FnII module of MMP-9 and peptide D6 belongs to the sequence in HPX domain (end of blade 1 and beginning of blade 2) of MMP-9. These peptides were mixed in different concentrations with proMMP-9 and CSPG.

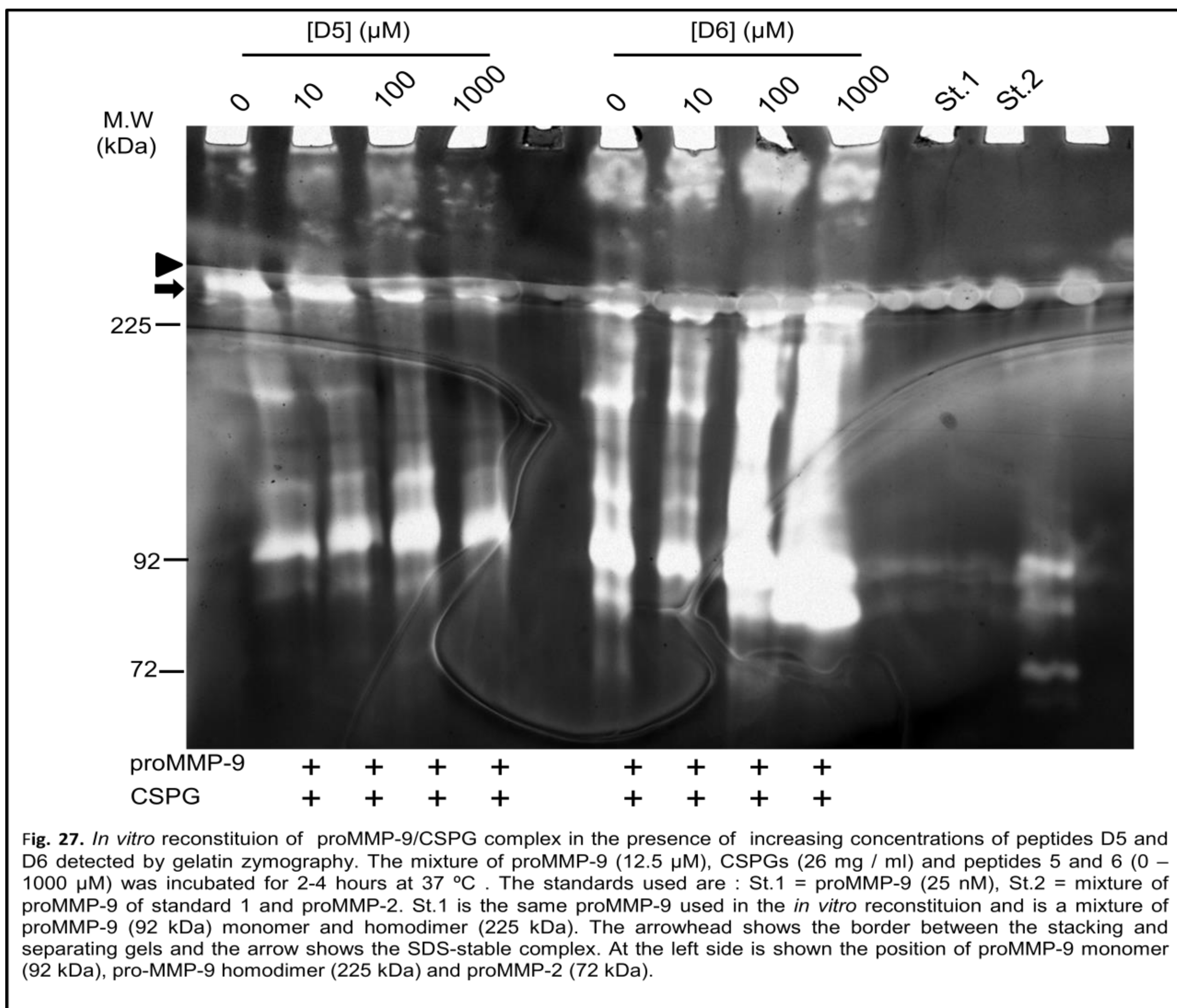

Fig. 27. *In vitro* reconstituion of proMMP-9/CSPG complex in the presence of increasing concentrations of peptides D5 and D6 detected by gelatin zymography. The mixture of proMMP-9 (12.5 µM), CSPGs (26 mg / ml) and peptides 5 and 6 (0 – 1000 µM) was incubated for 2-4 hours at 37 ºC . The standards used are : St.1 = proMMP-9 (25 nM), St.2 = mixture of proMMP-9 of standard 1 and proMMP-2. St.1 is the same proMMP-9 used in the *in vitro* reconstituion and is a mixture of proMMP-9 (92 kDa) monomer and homodimer (225 kDa). The arrowhead shows the border between the stacking and separating gels and the arrow shows the SDS-stable complex. At the left side is shown the position of proMMP-9 monomer (92 kDa), pro-MMP-9 homodimer (225 kDa) and proMMP-2 (72 kDa).

In the figure 27 we can see that D5 clearly has an effect on the formation of SDS-stable complexes. With increased concentrations of D5, there is a decrease in the amount of SDS-stable complexes formed. However, it does not seem to effect on the formation of the SDS-soluble complexes. Peptide D6 does not seem to inhibit the formation of neither the SDS-stable nor SDS-soluble complexes. In addition, the presence of 83 kDa band in the lane with 100 μM and 1000 μM of D6 occurs. This represents a processed form of MMP-9.

3.7.5. Peptide D7 and D8

Peptide 7 and 8 used in this experiment belongs to the sequences in blade 3 and blade 4 in HPX domain of MMP-9.These peptides were mixed in different concentration with proMMP-9 and CSPG.

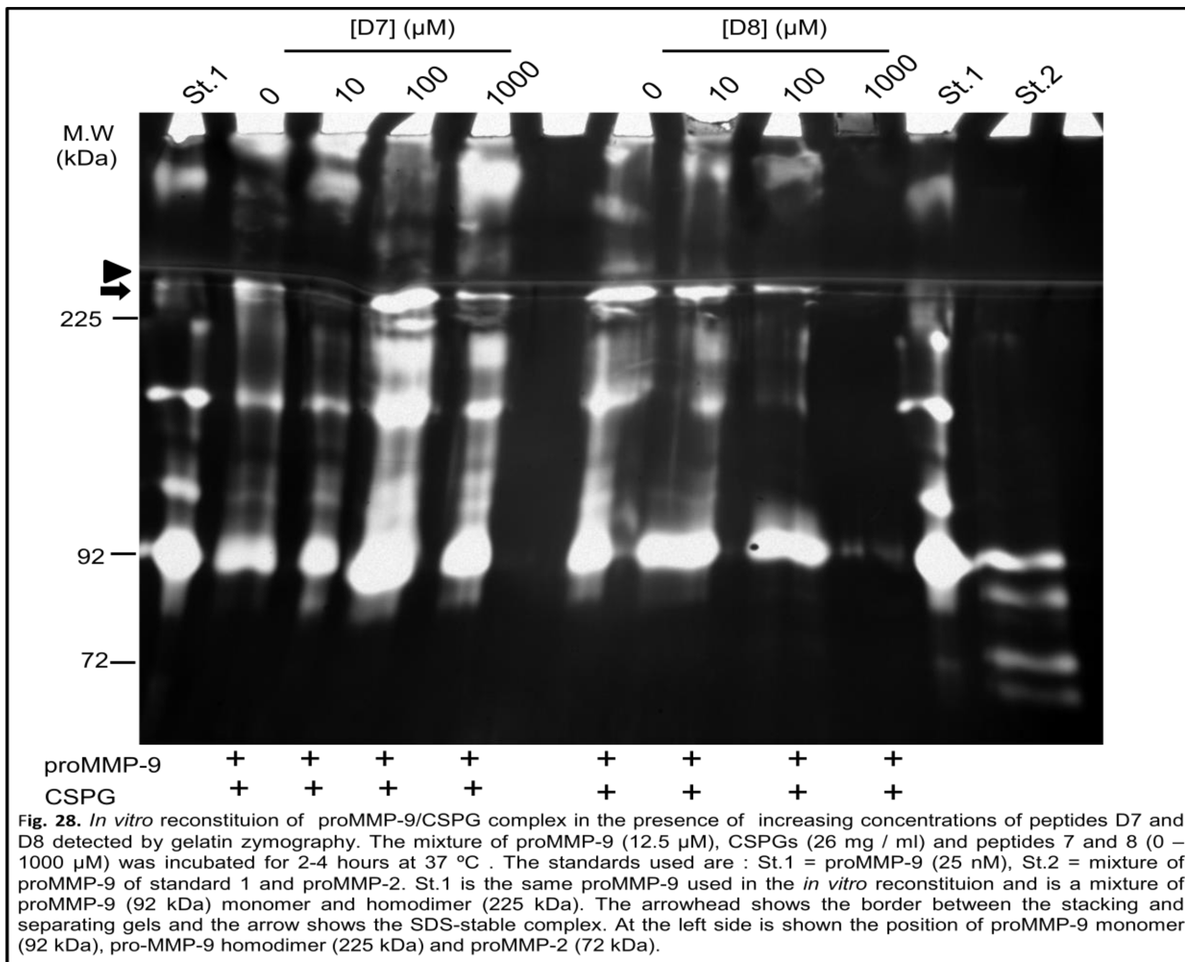

Fig. 28. *In vitro* reconstituion of proMMP-9/CSPG complex in the presence of increasing concentrations of peptides D7 and D8 detected by gelatin zymography. The mixture of proMMP-9 (12.5 μM), CSPGs (26 mg / ml) and peptides 7 and 8 (0 – 1000 μM) was incubated for 2-4 hours at 37 ºC . The standards used are : St.1 = proMMP-9 (25 nM), St.2 = mixture of proMMP-9 of standard 1 and proMMP-2. St.1 is the same proMMP-9 used in the *in vitro* reconstituion and is a mixture of proMMP-9 (92 kDa) monomer and homodimer (225 kDa). The arrowhead shows the border between the stacking and separating gels and the arrow shows the SDS-stable complex. At the left side is shown the position of proMMP-9 monomer (92 kDa), pro-MMP-9 homodimer (225 kDa) and proMMP-2 (72 kDa).

In figure 28, we can see that D7 seems to have no effect on formation of neither SDS-stable nor SDS-soluble complexes. D8 however, clearly seems to affect the formation of SDS-stable complex as the size of the 300 kDa bands seems to be decreasing when concentration of D8 is increased from 0 to 100 µM. It was a problem to obtain the sample with 1000 µM D8 from Q-Sepharose column as the sample looked clouded and it blocked the column. This is why we cannot see any bands in this lane. The formation of SDS-soluble complexes seems to be unaffected by D8.

3.7.6. Peptide D9

Peptide D9 used in this experiment belongs to the sequence of serglycin and has the same amino acid sequence as D1. This peptide was mixed in different concentration with proMMP-9 and CSPG.

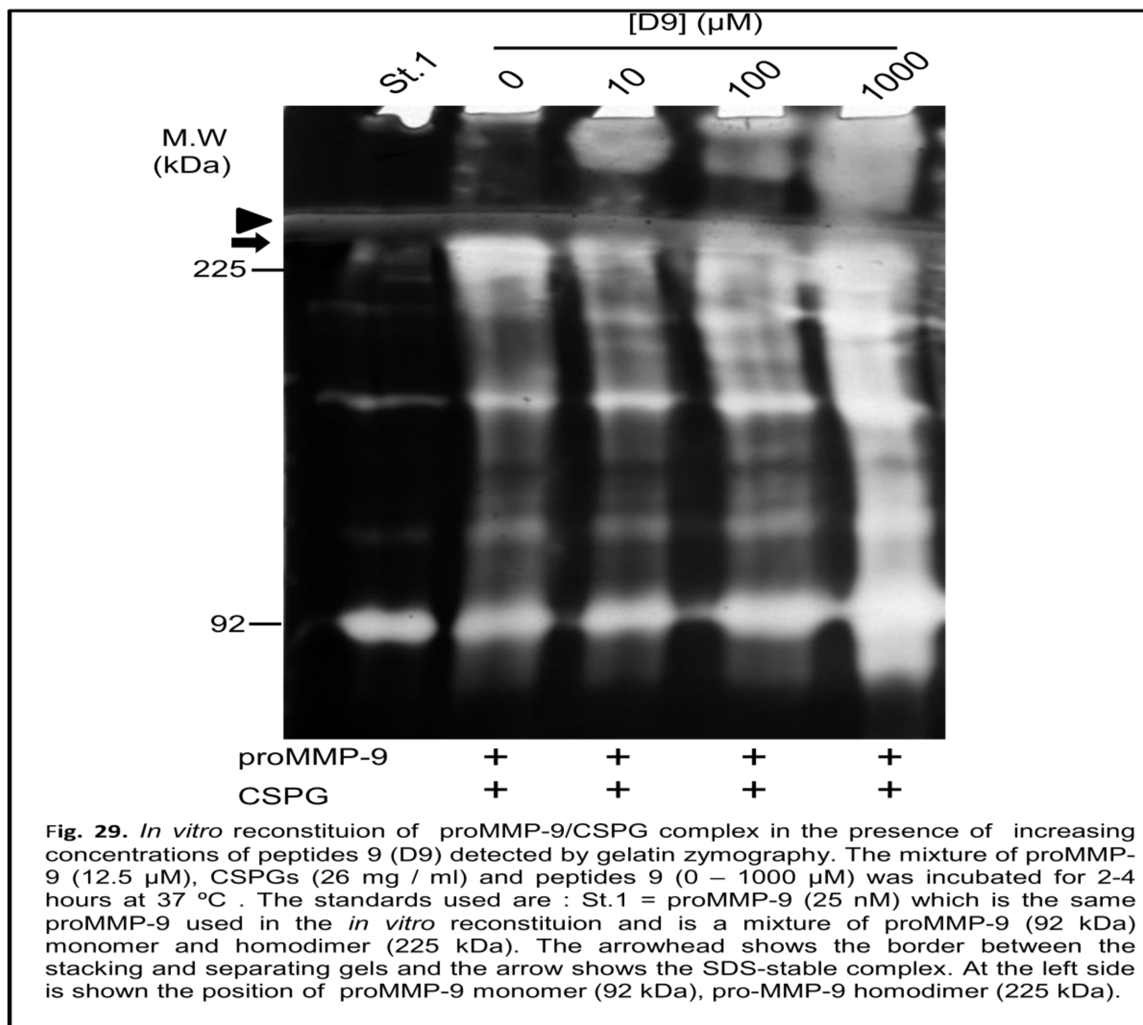

Fig. 29. *In vitro* reconstituion of proMMP-9/CSPG complex in the presence of increasing concentrations of peptides 9 (D9) detected by gelatin zymography. The mixture of proMMP-9 (12.5 µM), CSPGs (26 mg / ml) and peptides 9 (0 – 1000 µM) was incubated for 2-4 hours at 37 ºC . The standards used are : St.1 = proMMP-9 (25 nM) which is the same proMMP-9 used in the *in vitro* reconstituion and is a mixture of proMMP-9 (92 kDa) monomer and homodimer (225 kDa). The arrowhead shows the border between the stacking and separating gels and the arrow shows the SDS-stable complex. At the left side is shown the position of proMMP-9 monomer (92 kDa), pro-MMP-9 homodimer (225 kDa).

In figure 29, it appears that D9 inhibits the formation of the SDS-stable complex. However, D9 don’t seem to inhibit the formation of the SDS-soluble complex. Rather, the size of the SDS-soluble complex seemed to be increasing with the increasing concentrations of D9. There seems to be different effects of D1 and D9 in formation of SDS-stable and SDS-soluble complex.

4. Discussion

4.1. Optimization of quantitative determination of CS-chains

Two factors were important in order to obtain reliable standard curves to be used in the detection of the amount of CS-chains in the purified CSPG material. These two factors were; the dilution of the shark cartilage CS from which the standard curves were obtained and the extraction of the Safranin O from the nitrocellulose membranes. The first optimization shown in figure 9, was done with the standards diluted to 1.0 mg/ml and the low accuracy of the curve was due to the fact that none of the two factors mentioned above had been optimized. So for getting a better accuracy, the concentration of the final stock solution was decreased to 0.1 mg/ml and the total volume of the standards were also increased from 100 µl to 200 µl. This was done to minimize pipetting errors due to small volumes. This along with the continuous mixing during the extraction of Safranin O from the nitrocellulose membranes was the key steps which we found to affect the accuracy of the standard curve (compare Fig. 10. with Figs 8 and 9). The continuous mixing of the membranes was also important for optimal extraction of the Safranin O dye from the purified samples and hence an optimal quantification of the samples.

Better standard curves allowed better quantification of our unknown CSPG samples, which is why we optimized the method until the best accuracy was produced by the standards.

4.2. Presence of serglycin and other CSPGs in THP-1 cells

THP-1 is a human monocytic leukemic cell line which has been found to secrete various proteoglycans such as serglycin, versican, perlecan, glypican-1, CD44, thrombomodulin and syndecans-1, -2 and -3 [7-10]. From the elution graph of the fractions obtained from our gel filtration experiments with the THP-1 cells (Fig.12.) we can see that the peak is quite broad and when the CSPGs in pools III and IV were treated with cABC and were subjected to SDS-PAGE, we can observe the presence of a broad band around 26 kDa (Fig. 12. insert C) suggesting the presence of serglycin. In fact, western blotting revealed the presence of serglycin with a molecular size around 26 kDa (Fig.12. insert

A). The western blotting (Fig. 12. insert A) of these purified CSPGs shows the largest amount of serglycin in pool IV and the lowest amount in pool I. However, there are also large amounts of Serglycin in pools II and III.

As the SDS-PAGE of cABC treated material in pool III and IV (Fig. 12, insert C) show mainly one band at 26 kDa, this suggests that our THP-1 cells might contain more serglycin than any other proteoglycans. We cannot exclude that the 26 kDa band also contains other proteins than serglycin. Furthermore, since we did not use antibodies against other proteoglycans such as syndecan, perlecan or glypican we cannot be sure that these proteoglycans are not produced. When we look at figure 15 which is the coomassie stained SDS-PAGE, we could see that pool II had low amount of serglycin as no band appeared around the region of 26 kDa. However, we were able to observe bands around 26 kDa in both THP-1 pool III and IV which disappeared from the gel during the prolonged destaining process. These bands corresponded to the bands around the region of 26 kDa in SDS-PAGE silver staining (Fig. 12 insert C). The coomassie stained SDS-PAGE gels (Fig. 15.) revealed that there are also other proteins in the purified material which may be involved in the formation of the proMMP-9/CSPG complexes.

In vitro reconstitution using the CSPGs from THP-1 pools II to IV resulted in the formation of SDS-stable and SDS-soluble proMMP-9/CSPG complexes (Fig. 14.). The SDS-stable bands are those bands formed by CSPG and proMMP-9 which cannot be dissociated even in the presence of SDS, hence the name SDS-stable complexes [9]. The strongest band around 300 kDa (SDS-stable complex) appears in the reconstitution using the THP-1 pool IV, while the corresponding band was very weak using THP-1 pool II. This complex is formed most likely between serglycin and proMMP-9 as the amount of serglycin is largest in pool IV (Fig. 12, insert C). The SDS-soluble complex (as seen in Fig.14.) however appears to be strongest in pool II and IV. We can conclude from our *in vitro* reconstitution experiments using proMMP-9 along with the different THP-1 pools that proMMP-9 forms complex with serglycin as well as some other protein in the purified material.

From previous research, it has been proved that PMA stimulated THP-1 cells produce large amounts of reduction sensitive proMMP-9/CSPGs complexes. One or several CSPGs could be involved in the complex formation, including serglycin and versican. However, unstimulated THP-1 cells produced almost the same amount of CSPGs as the PMA stimulated cells, but very low amount of the proMMP-9/CSPG complexes [9]. In our experiment we used unstimulated THP-1 cells and as expected, the purified pools did not show any detectable amounts of proMMP-9/CSPG complexes (Fig. 14.). In figure 15 we can see that THP-1 pool II has almost the same amount of band at the top of the gel as THP-I pool III despite half amount of protein being used. This band is almost absent in pool IV from the THP-1 cells. Due to the molecular size of this protein, one is tempted to assume that this can be either versican or perlecan, since THP-1 cells produces mRNA for these two proteoglycans and western blotting revealed large amounts of versican in isolated CSPGs from the THP-1 cells [9]. One reason that we did not detect any versican in the purified material used here is that most of the synthesized versican may be located in pool I which was not used in the western blot as seen in figure 12 (insert B), due to the low amounts of CS chains and hence PG core protein in this fraction. The bands appearing around 70 kDa which could be shedded syndecans (dimers) or glypicans are stronger in both THP-1 pool II and III than in pool IV, and as it was loaded only the half amount of CSPGs to pool II, it must be assured that there are more of these proteins in pool II than in pool III. The broad band around 1.7 to 4.6 kDa seen in pool II could be a small protein/peptide attached to the CS-chains of the purified CSPGs. Previously it has been shown that the GAG-chains of serglycin can bind a wide variety of proteins [71]. Since this band is very weak in the other pools, it must be that this protein/peptide selectively attach to the CS chains of proteoglycans accumulated in pool II. This makes sense as the CS chains may differ in structure between different PG core proteins as well as the spacing between the CS chains may vary, which are factors that can affect the CS chains interaction with proteins. Considering only half amount of CSPGs was loaded to the lane for pool II, the bands around 150 kDa is stronger in pool II than in other pools. Bands appearing around 4 and 280 kDa is also very strong in pool II compared to pool III and IV.

4.3. Presence of unknown CSPGs in MonoMac cells

Previous researches have shown that MonoMac cells do not produce serglycin despite expressing mRNAs for serglycin. However, MonoMac cells have been shown to produce small amount of versican and expresses mRNA of glypican, perlecan or syndecan [9]. When we conducted a SDS-PAGE (coomassie staining) with cABC treated Sephacryl S-400 purified CSPGs samples from MonoMac cells we were not able to see the 26 kDa band where serglycin normally appears (Fig. 15.). However, various bands in different regions appeared. We can see bands around 120 kDa which are stronger in MonoMac pool II than in pool I. We have not been able to determine the nature of this protein, and hence whether it is a proteoglycan or a CS-associated protein, we are not sure. There are more bands in the region of 50 - 70 kDa in pool I than in II, and these bands are also stronger in pool I than in pool II. This suggests presence of various CS-associated proteins or proteoglycans. Proteins with similar sizes were observed in the CSPGs from the THP-1 cells, although weakly stained in the CSPGs from THP-1 cells. As suggested above, these bands may represent shedded syndecans or glypicans as both cell lines produces mRNA for various forms of these proteoglycans [9]. The bands in the top of the gel are slightly stronger in pool II than in pool I and could be versican or perlecan, as these cells produces mRNA for both of these proteoglycans [9].

In vitro reconstitution of proMMP-9/CSPG complexes performed using MonoMac pools I and II, revealed formation of SDS-stable and SDS-soluble complexes (Fig. 14. The SDS-stable complex occurs at the same position as that from THP-1 pools II to IV, i.e. around 300 kDa. Furthermore, the 300 kDa band is stronger in the pool II than in pool I from the MonoMac cells. In THP-1 cells, this SDS-stable complex contains at least the proteoglycan serglycin. However, as serglycin has not been detected in MonoMac cells [9], the most likely protein band detected in these cells that are stronger in pool II than in pool I is the 120 kDa protein. The bands around the region of 50 - 70 kDa appeared more strongly stained in the MonoMac pools than in the THP-1 pools (as seen in Fig.15.). If the bands around 70 kDa in THP-1 were involved in forming SDS-soluble

complexes, then the proteins that constitute these bands must be different in MonoMac cells.

4.4. Peptide array and competition experiments

The peptide array experiments provided us with clues about the complex formation between serglycin and MMP-9 and the different parts of both serglycin and MMP-9 that are involved in this process. THP-1 has been found to produce a reduction sensitive heteromer in which the proMMP-9 is linked to the core protein of one or various CSPGs [9, 64]. Recently it was shown through *in vitro* reconstitution experiments that the SDS-stable reduction sensitive complex formed between proMMP-9 and the CSPG core protein was not due to a disulphide bond between the two proteins, but rather a mixture of hydrophobic, hydrophilic and ionic interactions [9]. These *in vitro* reconstitution studies also showed that a SDS-soluble complex was formed in addition to the SDS-stable complex and that one of the CSPG core proteins involved in the complex formation was serglycin. The proMMP-9 which is linked to the CSPGs has different characteristics compared to the other forms of MMP-9 as the binding of proMMP-9 to the CSPGs has been found to alter various biochemical properties of MMP-9 [65, 67]. Also recent studies have shown that core protein of CSPGs binds both to the hemopexin domain and the FnII repeats present in the catalytic domain of MMP-9 [66, 67]. Our experiments with peptide arrays have tried to further elucidate details about the different parts of MMP-9 and the core protein in the CSPG serglycin which interacts in the complex formation.

4.4.1. Serglycin binds with MMP-9 and TIMP-1

MMP-9 has been found to bind to both C- and N- terminal region of serglycin repeats in the serglycin core protein from our experiments with the peptide arrays, which is shown in the schematic model of the proMMP-9/serglycin complex in figure 30. The different amino acids in the serglycin core protein that appears to be involved in binding with proMMP-9 based on the peptide arrays are summarized in figure 31.

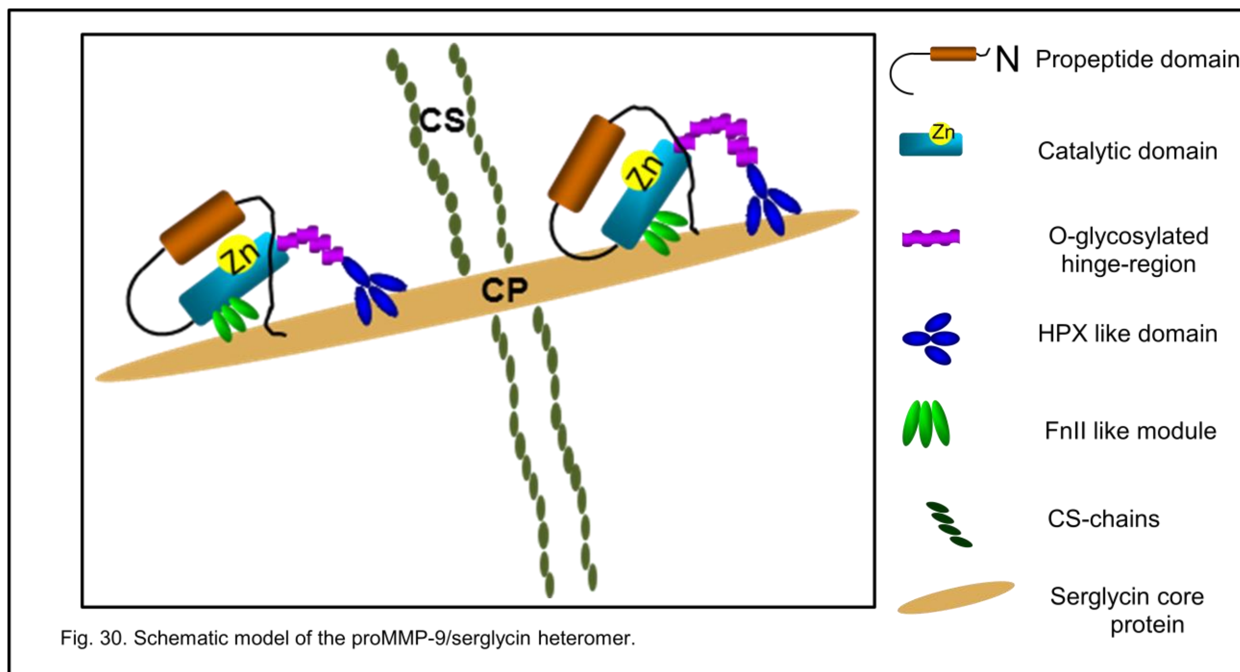

Fig. 30. Schematic model of the proMMP-9/serglycin heteromer.

Figure 30 shows the interaction between proMMP-9 and serglycin. MMP-9 binds to both terminals (C- and N- terminal) of serglycin in relation to the CS-chains. Both HPX domain and FnII module are involved in binding to serglycin but further investigations are needed to know whether HPX domain and FnII module are involved in simultaneous binding or in an independent manner. The amino acids and sequences of serglycin that are involved in binding with proMMP-9 are summarized in figure 31.

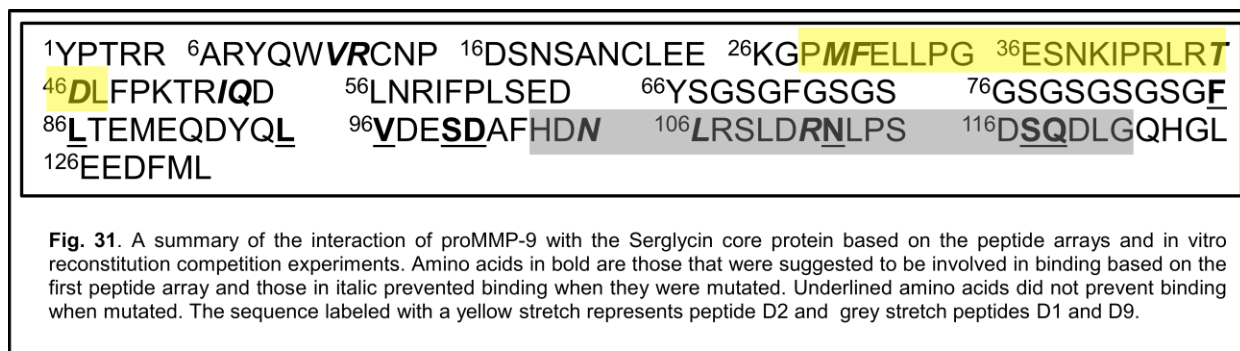

Fig. 31. A summary of the interaction of proMMP-9 with the Serglycin core protein based on the peptide arrays and in vitro reconstitution competition experiments. Amino acids in bold are those that were suggested to be involved in binding based on the first peptide array and those in italic prevented binding when they were mutated. Underlined amino acids did not prevent binding when mutated. The sequence labeled with a yellow stretch represents peptide D2 and grey stretch peptides D1 and D9.

The entire sequence from ^{11}VR to MF^{30} in serglycin is found to be involved in binding with MMP-9. The following and partly overlapping sequence was shown to contain three amino acids that are involved in binding with MMP-9 and these amino acids are F^{30}, T^{45} and D^{46}. The *in vitro* reconstitution competition experiment done with the D2 peptide

which covers these three amino acids prevent the formation of 300 kDa complex, but it did not have any effect on the formation of the SDS-soluble complex. This shows that this region N-terminal for the serglycin repeats which has the CS-chains attached is involved in the formation of the SDS-stable and reduction sensitive complex. Furthermore, it also shows the usefulness of peptide arrays in the search for regions and individual amino acids involved in the interaction between two proteins. As seen in figure 17, P3 clearly seems to bind to MMP-9 and it is represented by amino acid pair IQ^{54}. Other amino acids that were found to be involved were N^{105}, L^{106} and R^{111}. The first peptide array suggested that amino acids ^{85}FL, ^{95}LV, ^{99}SD, ^{112}N and ^{117}SQ were involved in binding with MMP-9, while the mutated peptide array showed that they were not. D1 and D9 peptide which covers amino acids 103 to 121 (Fig. 31.) seems to prevent the formation of the 300 kDa SDS-stable complex (as seen in Fig. 24 and 29), suggesting that this peptide competed with serglycin to bind with MMP-9 during the *in vitro* reconstitution competition experiments. Furthermore, it also suggests that two reduction sensitive SDS-stable complexes can be formed, one where proMMP-9 binds N-terminal to the CS-chains and the other where the enzyme binds C-terminal to the CS-chains as indicated in figure 30. Future *in vitro* reconstitution competition studies with the use of more peptides based on sequences in the serglycin core protein which has been proved to bind proMMP-9 should give more information on the complex formation and also if there is a competition or not between formation of SDS-stable and SDS-soluble complexes.

It also appears that TIMP-1 can bind to the serglycin core protein (Fig. 18.) and this also involves amino acids both C- and N-terminal to the serglycin repeats, i.e. the attachment sites for the CS chains. From previous research it has been shown that TIMP-1 inhibits the formation of SDS-soluble proMMP-9/CSPG complexes but not the SDS-stable proMMP-9/CSPG complexes [9]. Furthermore, TIMP-1 has rarely been found attached to the isolated CSPGs from THP-1 cells [9, 67]. When we compare the binding of serglycin with TIMP-1 and MMP-9 it is quite clear that there could be a competition between TIMP-1 and MMP-9 for binding sites in serglycin and the amino acid pair which seems to involve in binding to both MMP-9 and TIMP-1 are N^{105} and L^{106}. Another is the ^{27}GP to ^{45}TD sequence N-terminal to the serglycin repeats. This

suggests that SDS-soluble proMMP-9 complexes may also be formed by binding to the core protein both N- and C-terminal to the attached CS chains. In both cases it also suggests that there must be overlapping sequences with respect to the formation of the SDS-stable and SDS-soluble complexes.

4.4.2. FnII module binds with serglycin

From our experiments we have found that FnII module binds to serglycin and figure 32 shows the amino acids and sequences of the FnII module that are involved in binding with serglycin.

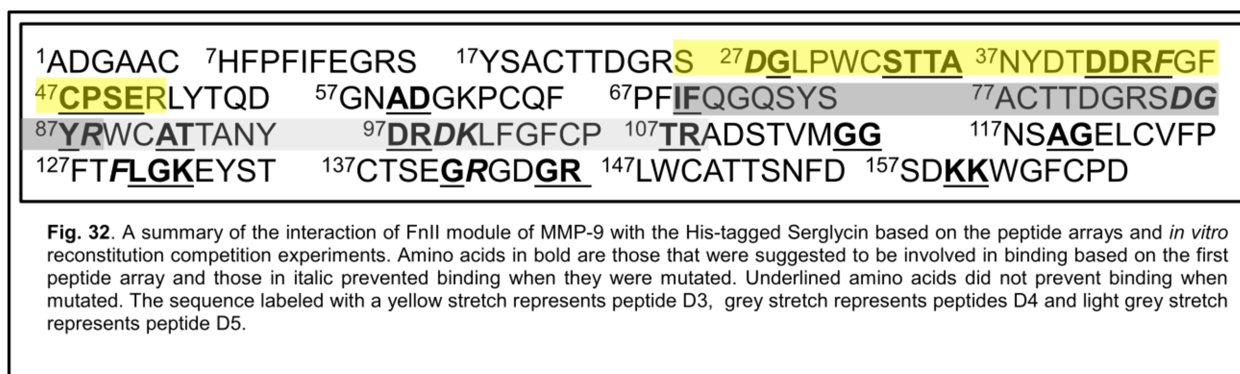

Fig. 32. A summary of the interaction of FnII module of MMP-9 with the His-tagged Serglycin based on the peptide arrays and *in vitro* reconstitution competition experiments. Amino acids in bold are those that were suggested to be involved in binding based on the first peptide array and those in italic prevented binding when they were mutated. Underlined amino acids did not prevent binding when mutated. The sequence labeled with a yellow stretch represents peptide D3, grey stretch represents peptides D4 and light grey stretch represents peptide D5.

There seem to be different binding stretches in the FnII module that is involved in binding and several amino acids in the sequence appear to act together. Figure 19 shows that peptides 13 – 22 in the sequence that stretches from amino acid R^{25} to K^{62} bind to serglycin. In this amino acid sequence, the peptides walk of two amino acids in the peptide arrays of 20 amino acids showed that approximately every second peptide bound strong while the other showed a weak binding to the His-tagged serglycin. The parent peptides in the mutated peptide array (P1, P2 and P3, P4) introduced a one amino acid peptide walk in bridging peptides 13 and 14 as well as peptides 16 and 17 in figure 19. In this region it is not possible to point to individual amino acids in the FnII domain that is involved in binding, but rather that introducing new amino acids or removing others in the sequence may either favor or disfavor binding. *In vitro* reconstitution in the presence of peptide D3 showed that this peptide did not inhibit the formation of the SDS-soluble proMMP-9/CSPG complex (Fig. 25.). Whether it affects the formation of the SDS-stable complex remains to be solved. This peptide which

covers the amino acid sequence ^{26}S to ^{51}R in the FnII module (Fig. 32.) includes regions that binds strong as well as regions that don't bind or bind very weak to the serglycin core protein. Therefore it must be concluded that this was not an appropriate peptide to use.

Unlike D3, peptides D4 and D5 which are close to each other acts as inhibitors in the *in vitro* reconstruction assay (Figs 26, 27). Thus these two neighbouring sequences (as seen in Fig. 32) in the FnII module are involved in the formation of the proMMP-9/serglycin complex. D4 is found to prevent the formation of SDS-soluble complexes while D5 is found to prevent the formation of SDS-stable complexes. Peptide D4 used in the *in vitro* reconstruction studies are identical to peptide 6 in the mutation array, which suggested that ^{88}R are involved in the binding to serglycin. Peptide D5 contained one amino acid extra W^{89} and lacked R^{108} in the parent sequence of peptide 8 in the mutation array, a peptide that did not bind to serglycin. This suggests that there are only small changes in the peptide sequence that is needed in order to induce or prevent binding.

4.4.3. HPX domain binds with serglycin and TIMP-1

Peptide D6 did not inhibit the formation of neither SDS-stable nor the SDS-soluble proMMP-9/CSPG complexes, in spite of that amino acids in the sequence were shown to be involved in binding to serglycin (Fig. 33.). One possibility is that this sequence in the MMP-9 HPX domain while is able to bind to serglycin, it is not directly involved in the formation of the stable proMMP-9/CSPG complexes. Although in figure 28 we can see that D7 does not seem to have any effect on formation of neither SDS-stable complex nor SDS-soluble complex. As was the case with peptide D6, the amino acids in D7 sequence are involved in binding of serglycin. As for the sequence of D6, the D7 sequence in the HPX domain may not be involved in the formation of the stable proMMP-9/CSPG complexes. So, the *in vitro* reconstitution competition experiment has to be repeated with other peptides that contain neighboring sequences to obtain more information of sequences involved in the complex formation.

Peptide D8 however is found to inhibit the formation of the SDS-stable complex (Fig. 28), indicating that this peptide sequence in the HXP domain (Fig. 33.) is involved in the

formation of the SDS-stable complex. In this peptide, the peptide array (Fig. 21.) shows that the peptide binds (spot 96) and the mutation array of peptide 9 (Fig. 22.) indicated that amino acid ^{167}E is involved in binding (Table 14).

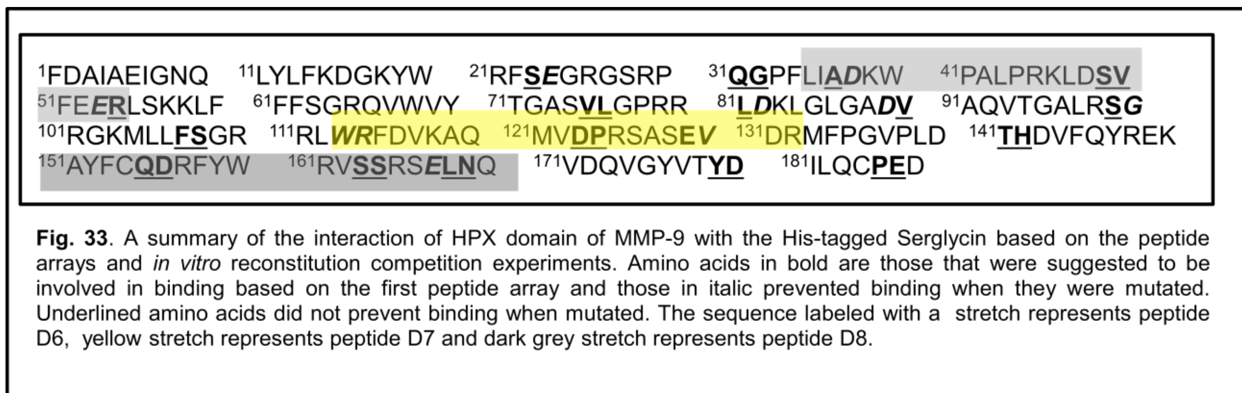

Fig. 33. A summary of the interaction of HPX domain of MMP-9 with the His-tagged Serglycin based on the peptide arrays and *in vitro* reconstitution competition experiments. Amino acids in bold are those that were suggested to be involved in binding based on the first peptide array and those in italic prevented binding when they were mutated. Underlined amino acids did not prevent binding when mutated. The sequence labeled with a stretch represents peptide D6, yellow stretch represents peptide D7 and dark grey stretch represents peptide D8.

The amino acids that was suggested to be involved in binding to serglycin based on the first peptide array and which the mutation array has confirmed to be involved are: ^{82}D, ^{53}E, ^{82}D, ^{89}D, ^{100}G, ^{113}W, ^{114}R, ^{129}E, ^{130}V and ^{167}E. Further mutation peptide arrays and peptides to *in vitro* reconstitution inhibition assays are needed in order to obtain more information about the regions and the amino acids in the HPX region that is involved in the formation of the SDS-stable and SDS-soluble proMMP-9/serglycin complexes.

TIMP-1 is known to bind to the HPX domain in proMMP-9 [34], but detailed information on which motifs and blades in the HPX that is involved lacks. It has been suggested that blade 4 is involved [48]. The peptide array (Fig. 23) shows that TIMP-1 binds to several motifs in the HPX domain in MMP-9. There are several places where it appears that TIMP-1 and serglycin binds to the same peptides and motifs. If we compare figure 21 and 23 and Tables 14 and 15, we can see that the sequence ^{37}AD to FE^{52} which seems to involve in binding with TIMP-1 overlaps with sequence ^{37}AD to ER^{54} which seems to involve in binding with serglycin. Other overlapping sequences involve the amino acid pair ^{129}EV and ^{77}GP to ^{89}DV which involves amino acid ^{89}D which has been shown in the binding to serglycin. This latter stretch may well be involved in the formation of the SDS-soluble complex while the region involving amino acids ^{37}AD to ER^{54} and ^{129}EV is less likely as peptides D6 and D7 (Figs 27 and 28) didn't inhibit the formation of the SDS-soluble complex. This shows that both serglycin and TIMP-1 could compete with

each other for binding sites in HPX domain of MMP-9 which may be involved in the formation of the SDS-soluble proMMP-9/serglycin complex.

4.5. Serglycin a substrate for MMP-9

Recent experiments done in our group by other colleagues have shown that serglycin is a substrate for MMP-9 where MMP-9 can cleave serglycin at several sites. These sites appears both C- and N- terminal to the serglycin repeats. This suggests that formation of the proMMP-9/serglycin complex is that the inactive pro-enzyme binds to its substrate, and when activated it can directly act on the substrate as suggested previously [9, 67].

From recent experiments done in our group it has been shown that peptides D4, D5 and D8 inhibits the first cleavage of the serglycin core protein, while D3, D6 and D7 don't inhibit this first cleavage. The following cleavages were however inhibited by all six peptides, D3 to D8. These experiments have further shown that serglycin is a substrate and MMP-9 probably requires exosites located in the HPX domain and the FnII repeats apart from its catalytic site for the processing of serglycin. Alternatively, these peptides bind to regions in the serglycin core protein and blocks the cleave sites. These peptides were the same peptides that had been used for the competition experiments (method section 2.4.11) and they seem to prevent the degradation of serglycin in the presence of MMP-9 .

Also, when peptides based on serglycin (D1 and D2) were mixed with MMP-9 and serglycin, the first cut in the serglycin core protein was not prevented. This suggests did not compete with serglycin for binding to MMP-9. This suggests that the parts in the serglycin core protein that corresponds to the D1 and D2 sequences don't contain the first cleavage site. Further support of this is that when these peptides were incubated with MMP-9, they were not cleaved. As both these peptides appeared to inhibit the formation of the SDS-stable complex, it seems that formation of this complex is not necessary for the initial cleavage of at least His-tagged serglycin. Future experiments with intact serglycin will show if MMP-9 can cleave its core protein and if exosites in MMP-9 are needed.

5. Conclusion

We were able to partly separate different CSPGs from THP-1 and MonoMac cells, but were unable to identify all of them. *In vitro* reconstitution of proMMP-9/CSPG complexes suggested that serglycin along with other yet unidentified proteoglycans/proteoglycan associated proteins form strong SDS-stable and SDS-soluble complexes with proMMP-9. Putative binding regions in the serglycin core protein, FnII module and the HPX domain of proMMP-9 were identified. In the serglycin core protein, both the N- and C-terminal part with respect to the attached CS chains seemed to be involved in formation of the proMMP-9/CSPG complexes. All three repeats of FnII module appeared to be involved where the second repeat showed most interactions and was involved in both the formation of the SDS-stable and the SDS-soluble complex. Similarly all four blades of HPX domain appear to be involved in forming the complex between proMMP-9 and serglycin with most interactions occurring with the fourth blade, which also was shown to be involved in the SDS-stable complex while blade 1 seemed to be involved in the formation of the SDS-soluble complex. It appears that the formation of a complex between proMMP-9 and serglycin is a proenzyme-substrate complex and when activated, the enzyme can process the serglycin core protein substrate as suggested previously [67]. Furthermore, the interacting regions in proMMP-9 may be exocites used in the processing of serglycin which preliminary data in our lab suggest.

6. Future Prospects

Still there is much to be explored about the proMMP-9/CSPG complexes and hence a wide range of studies can be performed. I will suggest just a few studies that could be performed in order to increase the knowledge of these complexes. One is to repeat is the SDS-PAGE of all the purified CSPGs from both THP-1 and MonoMac cell lines and analyze the obtained bands with M.S in order to identify the nature of the proteins present in the purified CSPGs. Based on data from MS analysis, the suggested proteins could be verified by the help of Western blotting. This of course requires that it is possible to obtain antibodies against them. It is also needed to perform purifications with the isolated and partly purified CSPGs from U-937 cells. In the case of all cells it may be a good idea to try another column for size exclusion chromatography, such as Sepharose CL-4B. *In vitro* reconstitution of proMMP-9 with other proteoglycans should also be conducted to see if they form complexes or not. If complexes are formed, this may lead to the discovery of new MMP-9 substrates and new specific exosites.

Secondly, further peptide arrays and mutated peptide arrays of the serglycin core protein as well as the FnII and HPX regions in MMP-9 should be performed in order to obtain a more precise suggestion of amino acids and amino acid motifs involved in binding. To strengthen and confirm the involvement of specific peptide stretches in the formation of the proMMP-9/serglycin complex, new soluble peptides should be synthesized and tested in *in vitro* reconstitution assays. To further confirm the involvement in specific regions in MMP-9, deletion and point mutated recombinant proMMP-9 should be constructed and tested in *in vitro* reconstitution experiments.

To obtain a 3D structure of the proMMP-9/serglycin complex would be ideal in order to understand the interaction between proMMP-9 and Serglycin. This would not only help us to understand the complex formation, but would give us a clear idea of the regions both in serglycin and MMP-9 that are in contact with one another and hence the nature of a putative exosite. This would be of great help in the generation of new MMP-9 inhibitors designed to bind to exosites in the enzyme.

As both MMP-9 and serglycin are extracellular matrix proteins involved in normal homeostasis as well as in various diseases such as cancer and neurological disorders, it would be of interest determine if the proMMP-9/serglycin complex occurs in either healthy, damaged or diseased tissue. If it occurs in diseased tissue, will this be associated with good or bad prognosis.

7. Appendix

7.1. Column Chromatography

Column chromatography is a method that fractionates proteins on the basis of protein charge, size, binding affinity or other various properties. The column holds a stationary phase which is a porous solid material with appropriate chemical properties and a mobile phase which is a buffered solution that flows through it. The sample which is a protein containing solution is applied on the top of the column that diffuses through the solid phase. The movement of the samples through the column depends upon the properties of the proteins present in the sample, the type of the column used (eg: Q-Sepharose columns, Sephadex G-50 fine columns etc) and the composition of buffers and salts in the mobile phase [80].

7.1.1. Size exclusion chromatography / Gel filtration column chromatography

Size exclusion column chromatography or gel filtration is a chromatographic technique which gathers information regarding molecular sizes of various proteins and their complexes, and is used to separate proteins according to their molecular sizes [81].

This technique is unique in a sense that the larger proteins appear from the column a lot sooner than the smaller ones as the proteins pass through the solid phase. This so called solid phase is made up of cross linked polymer beads having pores or cavities of various sizes. Since large proteins are not able to enter smaller cavities they take the shortest path around the beads and come out of the column before the smaller proteins. The smaller the protein, the smaller is the cavity of the beads that they can enter and so it takes longer time to pass through the column [82].

7.1.1.1. *Sephacryl S-400 high resolution column chromatography*

The column that was used for the purification of the proteoglycans was Sephacryl S-400 high resolution column. This column is made up of allyl dextran which is covalently cross linked with N,N'-methylene bisacrylamide to form a hydrophilic matrix and has a very high strength mechanically. The bead size in this column ranges from 25 to 75 µm. The molecular mass of the globular proteins that can be separated in this column

ranges from 20000 to 8000000 Da and of dextrans from 10000 to 2000000 Da. This column remains stable in all the biochemical aqueous buffers with pH ranging from 3 to 11. The length of column (cm) is one of the parameters that are very significant as it affects the resolution as well as the time taken to elute the samples. Another significant experimental variable which needs a consideration is the rate at which the eluent flows through the column. This has the effect on the speed at which the separation as well as the resolution that can be achieved. So, in case of large particles, the lower the flow rate, the better the resolution is [74].The column as seen in Figure A1 is connected to the chromatography system with a hydrostatic pump that controls the flow rate through the column.

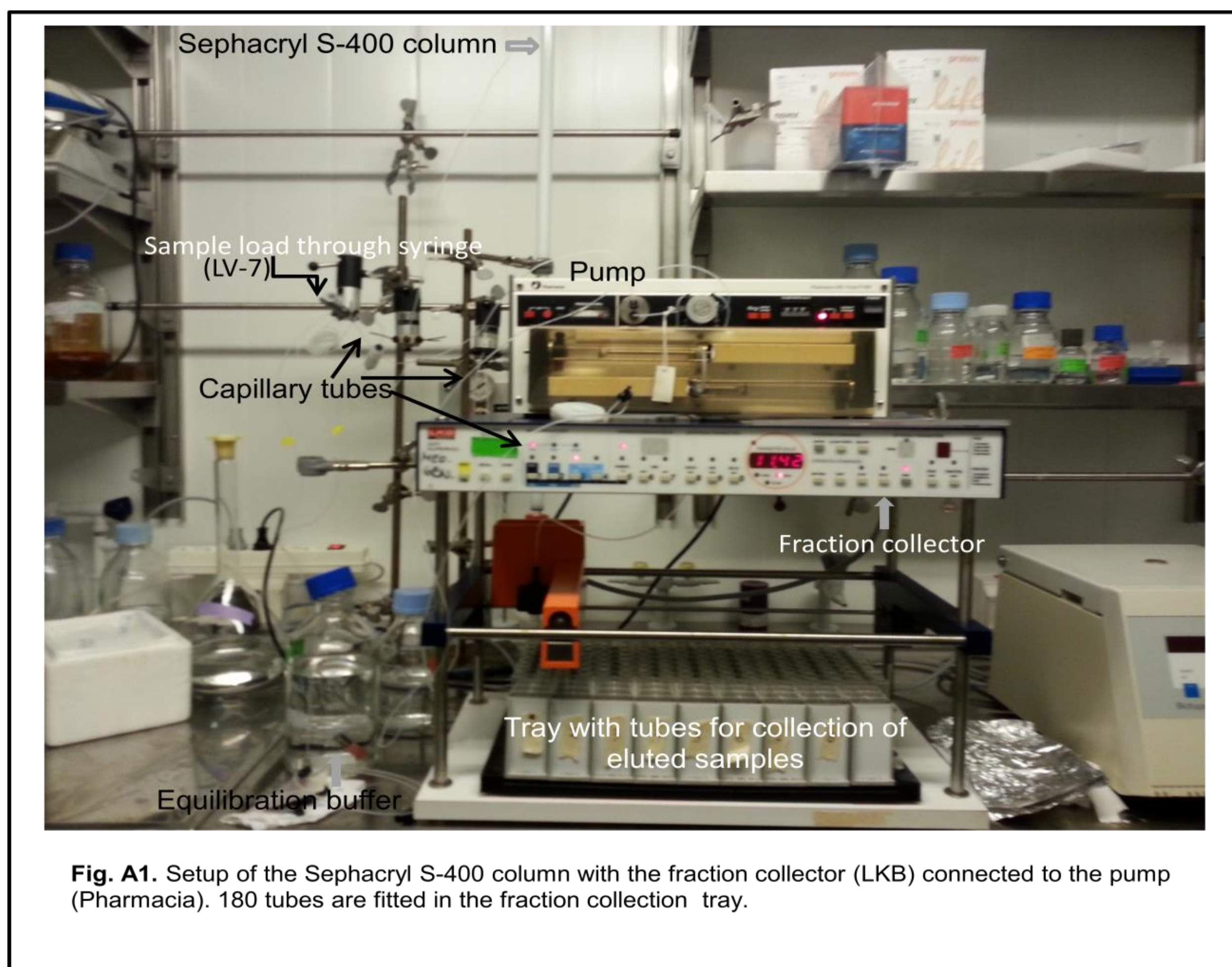

Fig. A1. Setup of the Sephacryl S-400 column with the fraction collector (LKB) connected to the pump (Pharmacia). 180 tubes are fitted in the fraction collection tray.

The mobile phase is first passed through the pump in a fine capillary tube and is connected to the valve (LV-7) where the samples are applied. The samples are applied

through syringe method where the LV-7 valve is used as syringe holder (sample load). The valve is connected to the column with a piece of fine capillary tube. The flow of liquid in this column is controlled by the difference of hydrostatic pressure by a pump which is connected into the system. The eluted materials from the column are collected into the tubes placed on a tray in the fraction collector.

7.1.2. Ion-Exchange column chromatography

Ion-exchange column chromatography is a process which allows the separation of proteins and peptides based on their charge. A protein having net negative charge has high affinity for an anion exchange matrix (Q-Sepharose gel) and similarly a positively charged protein binds to a cation exchange matrix. The net charge of proteins and peptides vary with the pH of the buffer used. Basically two steps are required for the progression for ion-exchange chromatography: the binding of the protein with the gel matrix and its elution [83].

In figure A2 we can see the setup of Q-Sepharose ion chromatography which is used for the larger samples. The columns used are made from 1ml plastic syringes cut into appropriate sizes and are fitted with small glass wool rolls as a filter. The sample passes through a fine capillary tube to the pump and is eluted in a glass flask. The pump is kept on during the whole experiment.

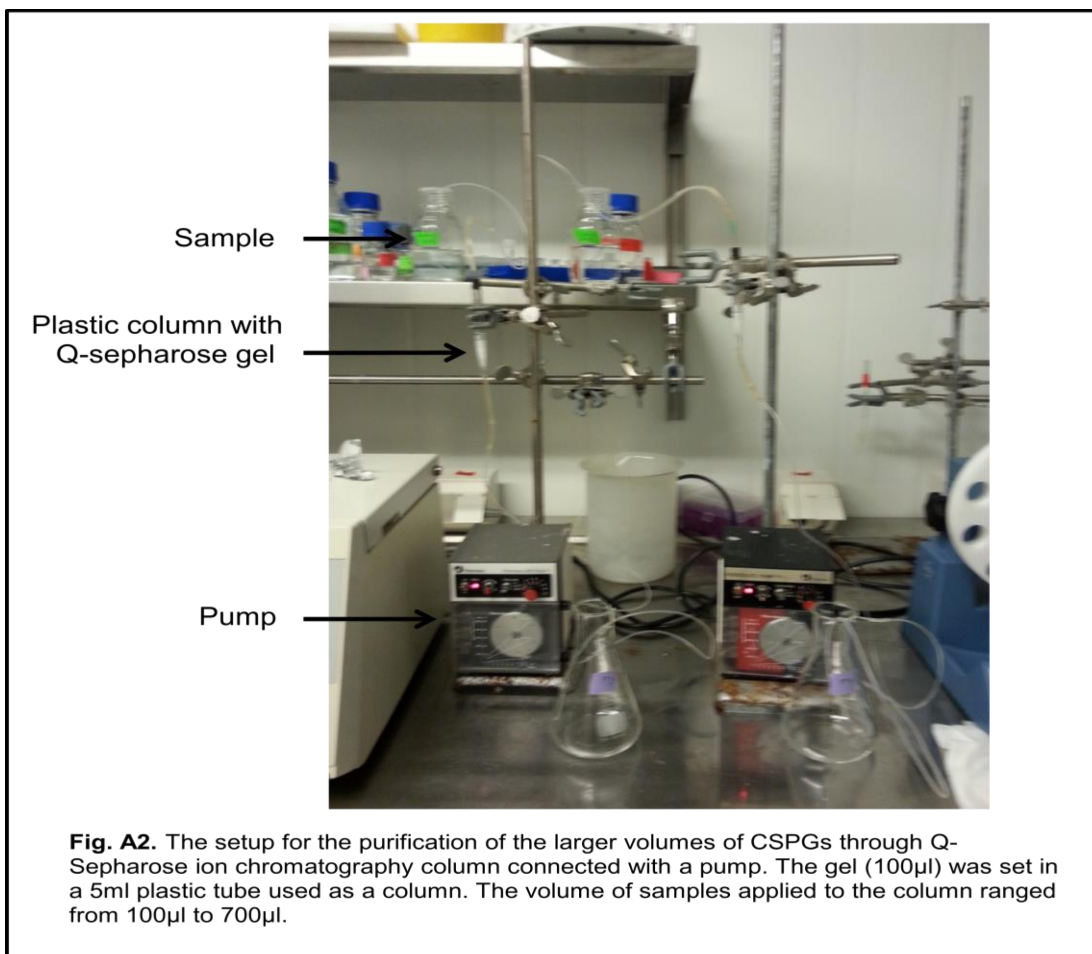

Fig. A2. The setup for the purification of the larger volumes of CSPGs through Q-Sepharose ion chromatography column connected with a pump. The gel (100µl) was set in a 5ml plastic tube used as a column. The volume of samples applied to the column ranged from 100µl to 700µl.

7.2. Slot blot apparatus used in the Safranin O method

Safranin O test was used for the quantification of CS-chains. To do this slot blotting was done so that our possible CS-chains from samples can be quantified. Slot blotting provides a precise quantification solid phase assay for large number of samples. The samples were added in the slots of the slot blot wells as seen in figure A3. The slot blot apparatus is attached to the vacuum pump through a pipe and is used to draw out the solution from the sample so that only the precipitates formed after adding our samples to the Safranin O solution are left in the nitrocellulose membrane. These precipitates are later used for quantification of the samples.

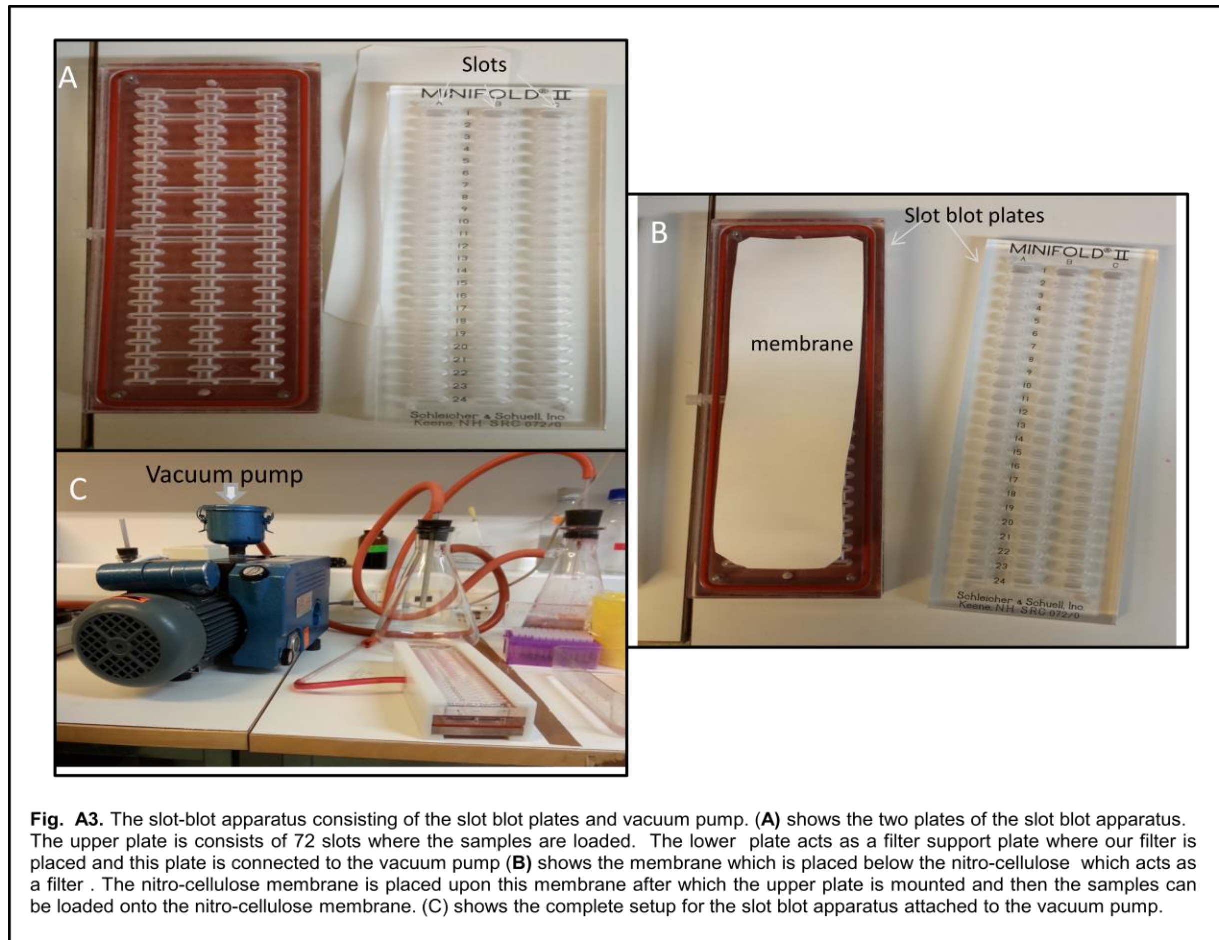

Fig. A3. The slot-blot apparatus consisting of the slot blot plates and vacuum pump. (**A)** shows the two plates of the slot blot apparatus. The upper plate is consists of 72 slots where the samples are loaded. The lower plate acts as a filter support plate where our filter is placed and this plate is connected to the vacuum pump (**B)** shows the membrane which is placed below the nitro-cellulose which acts as a filter . The nitro-cellulose membrane is placed upon this membrane after which the upper plate is mounted and then the samples can be loaded onto the nitro-cellulose membrane. (C) shows the complete setup for the slot blot apparatus attached to the vacuum pump.

7.3. Western Blotting

Western blot is a technique used for separation and identification of specific proteins from a complex mixture of proteins that are extracted from cells. There are three main elements that comprise this technique, which are: separation by size, transfer to a solid support and the usage of proper primary and secondary antibody to mark the target protein for visualization. The separation of proteins from a mixture of proteins occurs through gel electrophoresis and is based on the molecular weights of the proteins. These separated proteins are then transferred to a membrane (solid support). The membrane is then blocked in a blocking buffer made from non fat milk powder. This step is done to prevent the unspecific binding of primary antibodies to the membrane. After the blocking of the membrane it is incubated with primary antibodies specific to the proteins which are diluted in the blocking buffer. Washing of gel occurs after this step which is done for the removal of the unbound antibodies.

Thereafter, the membrane is incubated in blocking buffer containing diluted HRP-conjugated secondary antibodies. This is done for the visualization of our proteins bound to their specific primary antibodies. The membrane was again washed so that unbound secondary antibodies were washed off. The membrane is treated with substrate so that the HRP conjugated antibodies bound to our proteins can be detected under immunofluorescence. The thicker and intense the bands were the higher the amount of the protein present in the samples [84].

7.4. SDS-PAGE electrophoresis instruments

The SDS-PAGE was used to separate and analyze proteins according to their molecular size. After the electrophoresis, the gels could either be stained for protein (coomassie staining or silver staining) or the proteins in the gel could be transferred to membrane for Western blotting analysis. The same setup and program was used for all these techniques using the electrophoresis chamber attached to the voltage power supply via the electrodes (Fig. A4.).

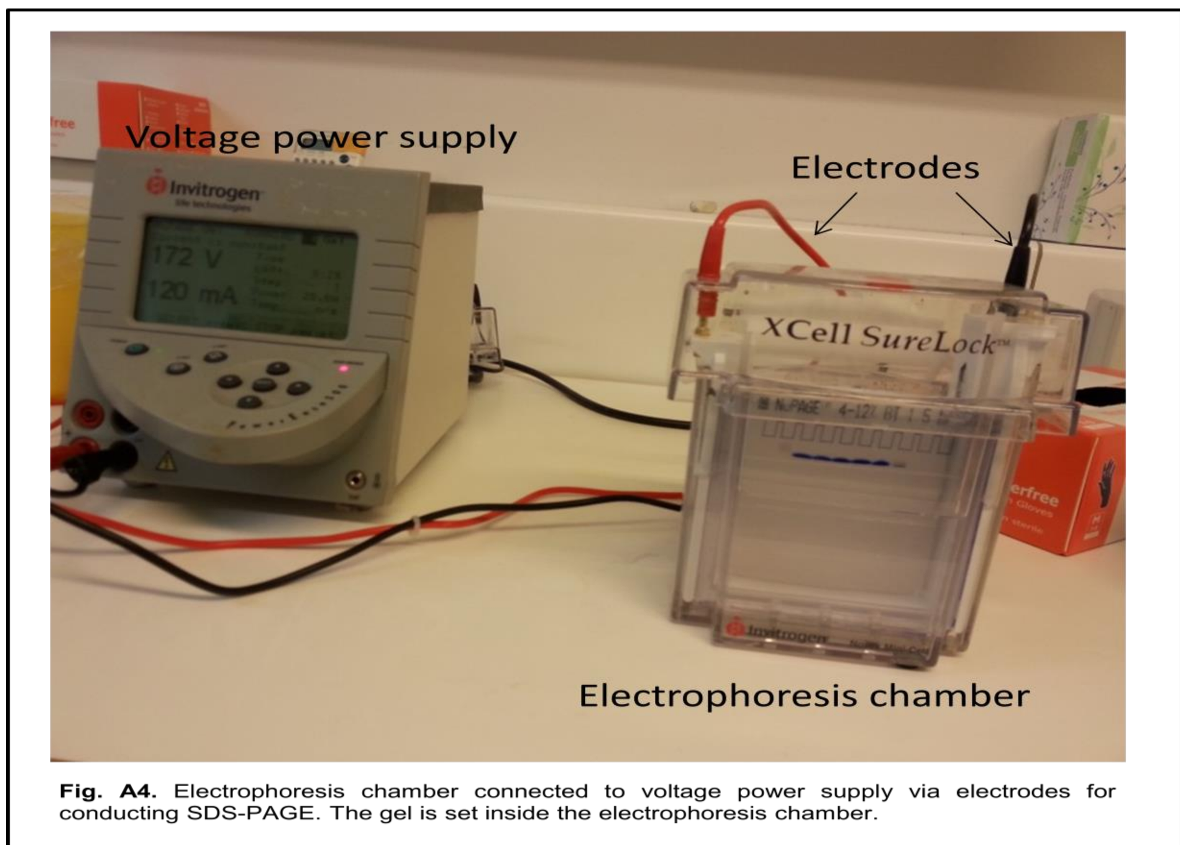

Fig. A4. Electrophoresis chamber connected to voltage power supply via electrodes for conducting SDS-PAGE. The gel is set inside the electrophoresis chamber.

7.5. Purification of partly purified CSPGs from THP-1 and MonoMac cells

For the gel filtration of the partly purified CSPGs from THP-1 cells we had conducted two experiments. The second experiment is shown in figure A5. And for the gel filtration of the partly purified CSPGs from MonoMac cells we had conducted three experiments, the second experiment is shown in figure A6.

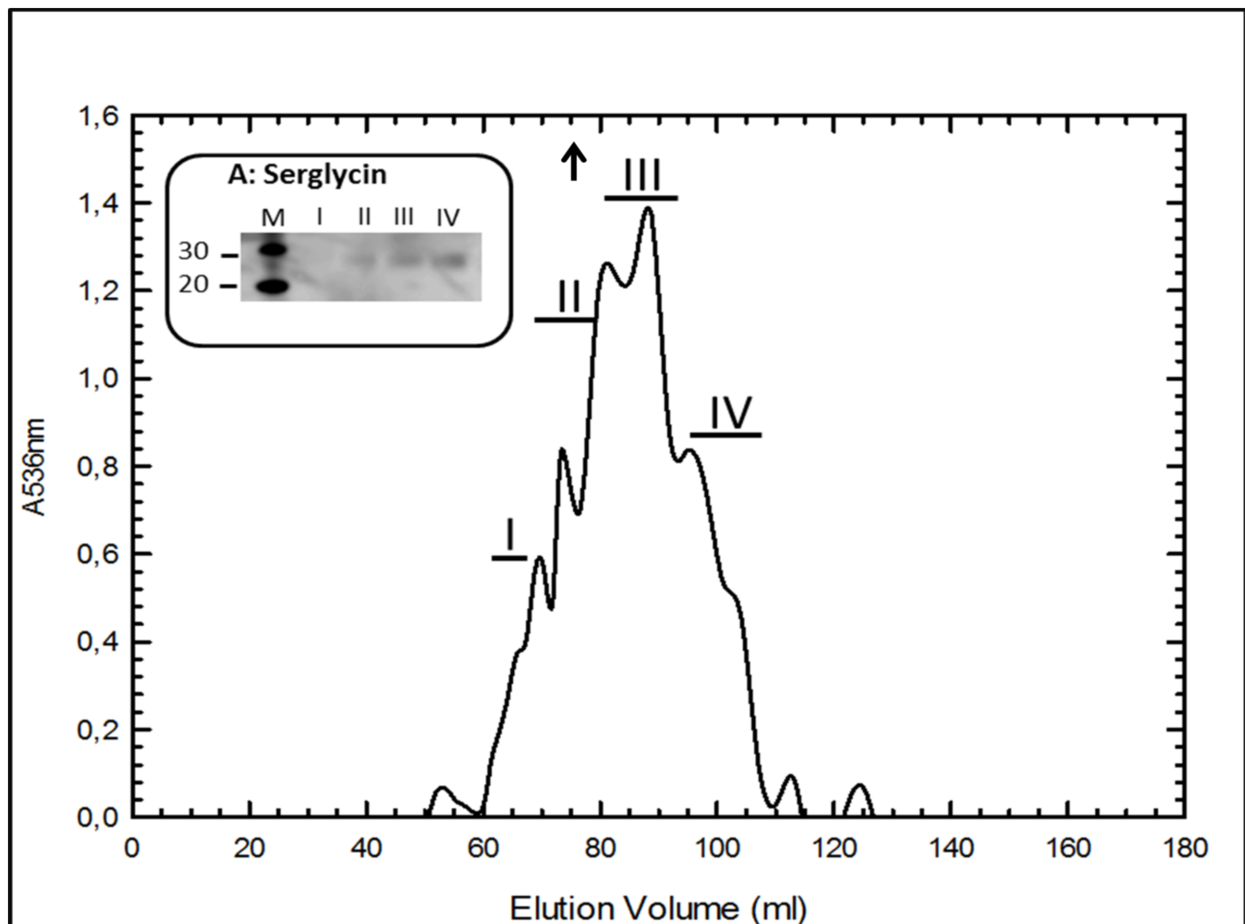

Fig. A5. Elution profile of partly purified CSPG from THP-1 cells using a Sephacryl S-400 column. On the Y-axis is the absorbance at 536 nm (Safranin O staining) and on the X-axis is the elution volume in ml and the arrow shows the void volume (V_0) at 70.4 ml. The peak of eluted proteoglycans was divided into four pools: I (fractions: 61.4 – 67.1 ml), II (fractions: 68.8 -79.2 ml), III (fractions: 81.1 – 93.8 ml) and IV (fractions: 94.9 - 108 ml). These pooled fractions were further treated as described in the text. cABC treated fractions were subjected to SDS-PAGE for characterization. Gels were subjected to Western blotting using antibodies against serglycin (A). CSPG loaded per lanes were 3 ug (A). Standard used in the insert is: M = Magic Marker and arrow = void volume (V_0) at 75ml. The molecular sizes of magic marker and the high and low range ladders are shown in kDa.

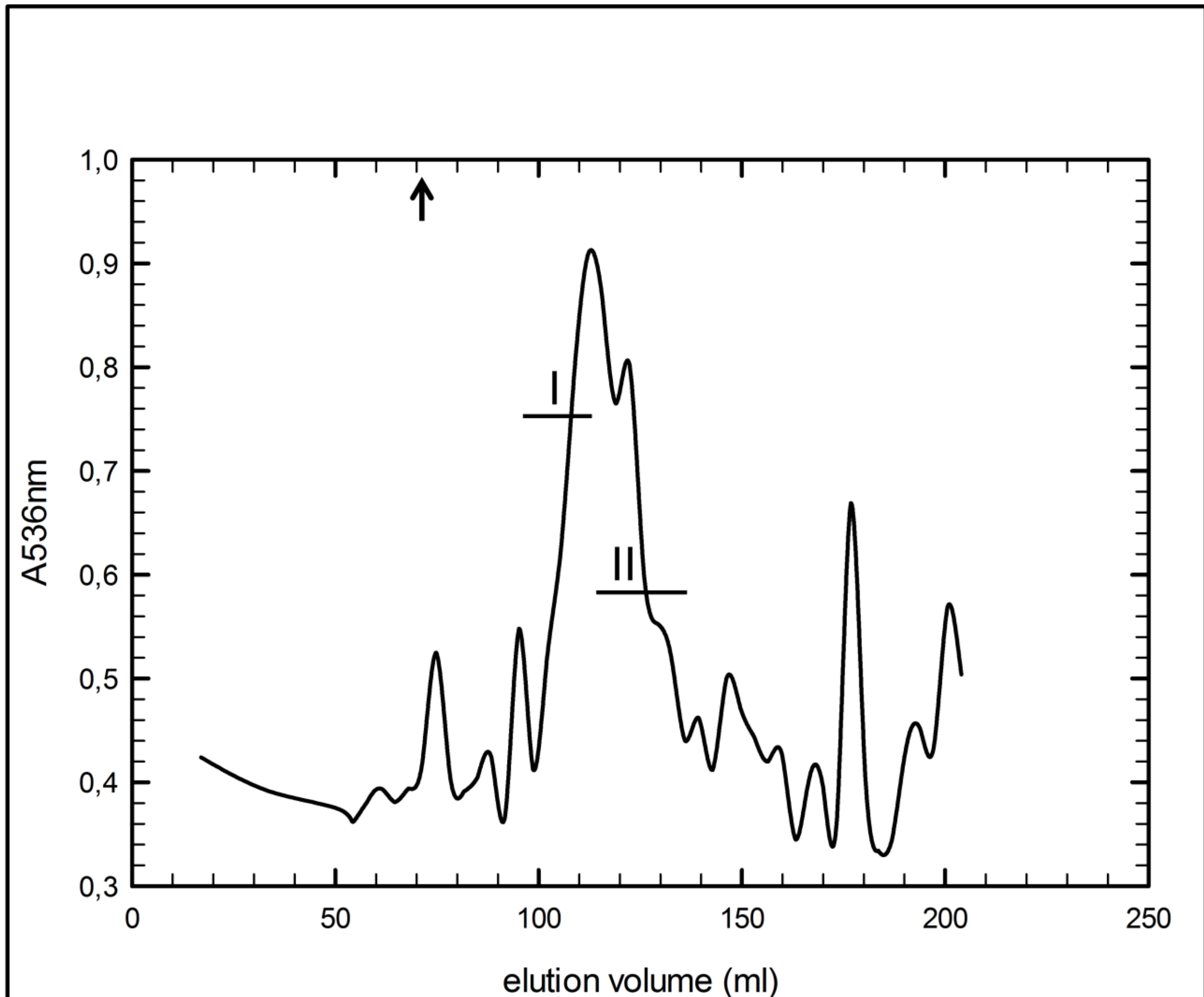

Fig. A6. Elution profile of partly purified CSPG from MonoMac cells using a Sephacryl S-400 column. On the Y-axis is the absorbance at 536 nm (Safranin O staining) and on the X-axis is the elution volume in ml and the arrow shows the void volume (V_0) at 70.4 ml. The peak of eluted proteoglycans was divided into two pools: I (fractions: 96.9 – 112.2 ml), II (fractions: 113.9 – 139.4 ml). These pooled fractions were further treated as described in the text. The arrow shows the void volume (V_0) at 75 ml.

7.6. Mass spectrometry

Mass spectrometry is a tool of measurement of molecular weight as well as the mass-to-charge ratios of molecules. There are three fundamental parts in any mass spectrometer: ionization source, the mass analyzer and the detector. The ionization

source is the first part of spectrometer that ionizes the target materials. This is done by electron impact that leads to opening up of covalent bonds in the target molecules leading to generation of high amount of fragment ions. These ions are then accelerated, so all the ions have the same kinetic energy. Then there is a separation and sorting out of these ions on the basis of their mass-to-charge ratio by help of mass analyzers. There are various types of mass analyzers such as: quadrupole, quadrupole ion trap, linear ion trap, orbitrap etc as seen in the figure A7. After these ions are analyzed by these analysers, the beam of these ions passing through the machine is detected electronically. Depending upon the type of experiment from which the data are formed, data analysis differ in Mass spectrometry [85].

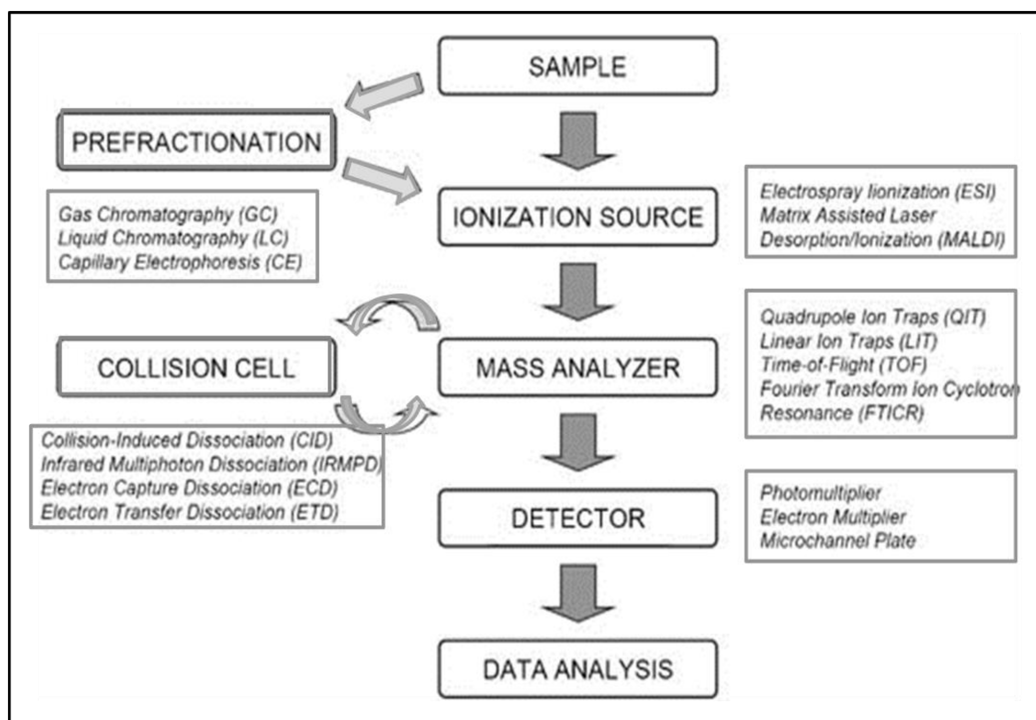

Fig.A7 Flow chart for mass spectrometry (modified from [85]). The sample is passed through ionization source and is analyzed in mass analyzer. Then it is detected electronically and the data is analyzed according to the type of spectrometry.

7.7. Peptide array membranes

Two peptide array membranes were ordered. They consisted of a total of two arrays based on the sequence of FnII module, three arrays based on the sequence of HPX domain and six arrays based on the sequence of serglycin as seen in figure A8.

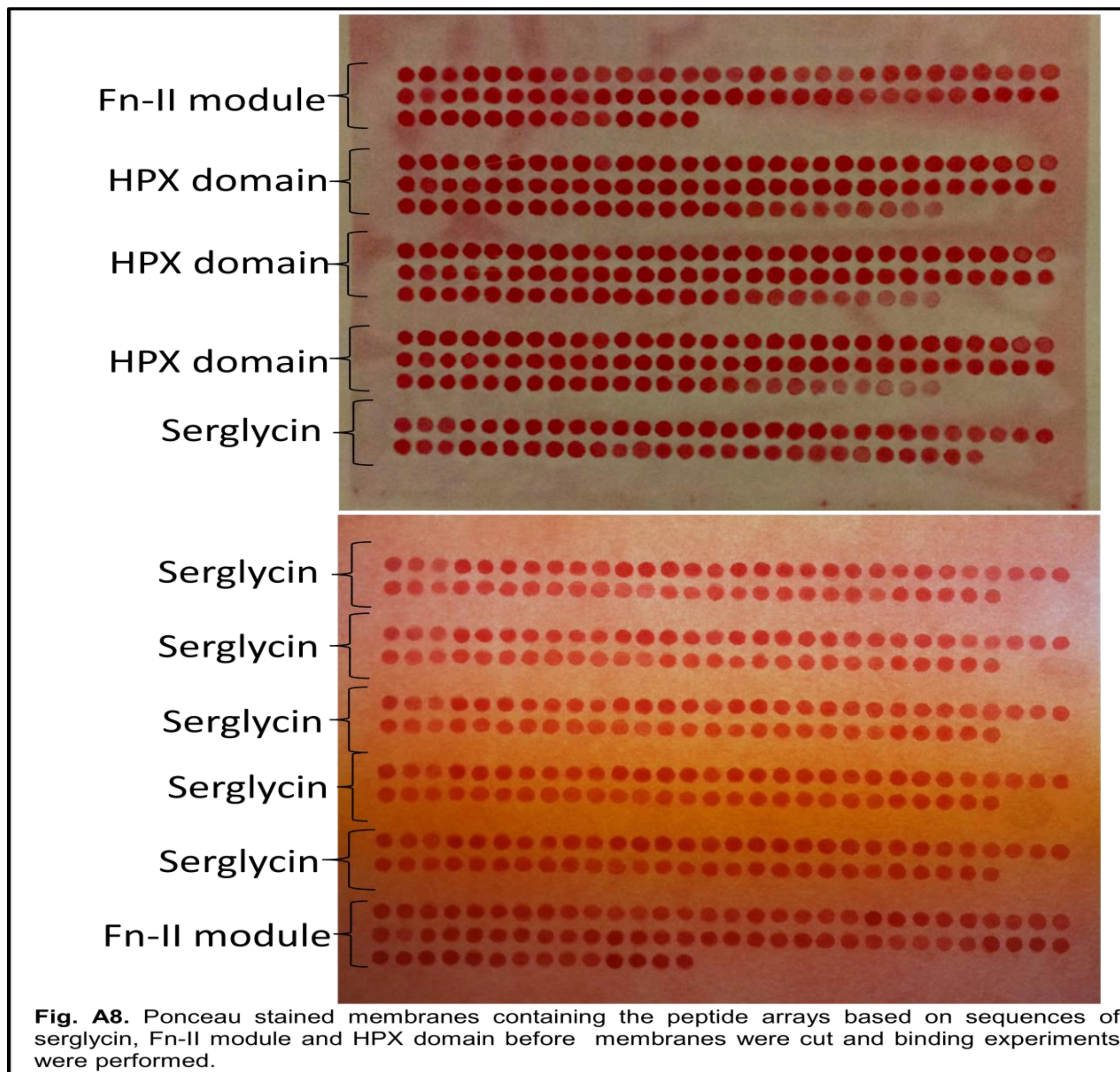

Fig. A8. Ponceau stained membranes containing the peptide arrays based on sequences of serglycin, Fn-II module and HPX domain before membranes were cut and binding experiments were performed.

The information regarding the composition of 20 amino acids in each peptide spot in these peptide array membranes (Fig. A8.) are shown in figures A9, A11 and A12

Nr.	Mol.Weight	Sequence
1	2440.7	Y-P-T-R-R-A-R-Y-Q-W-V-R-C-N-P-D-S-N-S-A
2	2397.6	T-R-R-A-R-Y-Q-W-V-R-C-N-P-D-S-N-S-A-N-C
3	2382.6	R-A-R-Y-Q-W-V-R-C-N-P-D-S-N-S-A-N-C-L-E
4	2412.6	R-Y-Q-W-V-R-C-N-P-D-S-N-S-A-N-C-L-E-E-K
5	2247.4	Q-W-V-R-C-N-P-D-S-N-S-A-N-C-L-E-E-K-G-P
6	2211.5	V-R-C-N-P-D-S-N-S-A-N-C-L-E-E-K-G-P-M-F
7	2198.5	C-N-P-D-S-N-S-A-N-C-L-E-E-K-G-P-M-F-E-L
8	2191.6	P-D-S-N-S-A-N-C-L-E-E-K-G-P-M-F-E-L-L-P
9	2165.6	S-N-S-A-N-C-L-E-E-K-G-P-M-F-E-L-L-P-G-E
10	2165.6	S-A-N-C-L-E-E-K-G-P-M-F-E-L-L-P-G-E-S-N
11	2248.8	N-C-L-E-E-K-G-P-M-F-E-L-L-P-G-E-S-N-K-I
12	2284.9	L-E-E-K-G-P-M-F-E-L-L-P-G-E-S-N-K-I-P-R
13	2312	E-K-G-P-M-F-E-L-L-P-G-E-S-N-K-I-P-R-L-R
14	2270.9	G-P-M-F-E-L-L-P-G-E-S-N-K-I-P-R-L-R-T-D
15	2377.1	M-F-E-L-L-P-G-E-S-N-K-I-P-R-L-R-T-D-L-F
16	2324	E-L-L-P-G-E-S-N-K-I-P-R-L-R-T-D-L-F-P-K
17	2339	L-P-G-E-S-N-K-I-P-R-L-R-T-D-L-F-P-K-T-R
18	2370	G-E-S-N-K-I-P-R-L-R-T-D-L-F-P-K-T-R-I-Q
19	2412.1	S-N-K-I-P-R-L-R-T-D-L-F-P-K-T-R-I-Q-D-L
20	2481.2	K-I-P-R-L-R-T-D-L-F-P-K-T-R-I-Q-D-L-N-R
21	2500.2	P-R-L-R-T-D-L-F-P-K-T-R-I-Q-D-L-N-R-I-F
22	2457.2	L-R-T-D-L-F-P-K-T-R-I-Q-D-L-N-R-I-F-P-L
23	2404	T-D-L-F-P-K-T-R-I-Q-D-L-N-R-I-F-P-L-S-E
24	2466.1	L-F-P-K-T-R-I-Q-D-L-N-R-I-F-P-L-S-E-D-Y
25	2349.9	P-K-T-R-I-Q-D-L-N-R-I-F-P-L-S-E-D-Y-S-G
26	2268.8	T-R-I-Q-D-L-N-R-I-F-P-L-S-E-D-Y-S-G-S-G
27	2215.8	I-Q-D-L-N-R-I-F-P-L-S-E-D-Y-S-G-S-G-F-G
28	2118.7	D-L-N-R-I-F-P-L-S-E-D-Y-S-G-S-G-F-G-S-G
29	2034.6	N-R-I-F-P-L-S-E-D-Y-S-G-S-G-F-G-S-G-S-G
30	1908.5	I-F-P-L-S-E-D-Y-S-G-S-G-F-G-S-G-S-G-S-G
31	1792.3	P-L-S-E-D-Y-S-G-S-G-F-G-S-G-S-G-S-G-S-G
32	1726.2	S-E-D-Y-S-G-S-G-F-G-S-G-S-G-S-G-S-G-S-G
33	1654.2	D-Y-S-G-S-G-F-G-S-G-S-G-S-G-S-G-S-G-S-G
34	1636.3	S-G-S-G-F-G-S-G-S-G-S-G-S-G-S-G-S-G-F-L
35	1722.3	S-G-F-G-S-G-S-G-S-G-S-G-S-G-S-G-F-L-T-E
36	1838.4	F-G-S-G-S-G-S-G-S-G-S-G-S-G-F-L-T-E-M-E
37	1877.3	S-G-S-G-S-G-S-G-S-G-S-G-F-L-T-E-M-E-Q-D
38	2024.4	S-G-S-G-S-G-S-G-S-G-F-L-T-E-M-E-Q-D-Y-Q
39	2092.5	S-G-S-G-S-G-S-G-F-L-T-E-M-E-Q-D-Y-Q-L-V
40	2192.5	S-G-S-G-S-G-F-L-T-E-M-E-Q-D-Y-Q-L-V-D-E
41	2250.5	S-G-S-G-F-L-T-E-M-E-Q-D-Y-Q-L-V-D-E-S-D
42	2324.6	S-G-F-L-T-E-M-E-Q-D-Y-Q-L-V-D-E-S-D-A-F
43	2432.6	F-L-T-E-M-E-Q-D-Y-Q-L-V-D-E-S-D-A-F-H-D
44	2399.5	T-E-M-E-Q-D-Y-Q-L-V-D-E-S-D-A-F-H-D-N-L
45	2412.6	M-E-Q-D-Y-Q-L-V-D-E-S-D-A-F-H-D-N-L-R-S
46	2380.6	Q-D-Y-Q-L-V-D-E-S-D-A-F-H-D-N-L-R-S-L-D
47	2407.7	Y-Q-L-V-D-E-S-D-A-F-H-D-N-L-R-S-L-D-R-N
48	2326.7	L-V-D-E-S-D-A-F-H-D-N-L-R-S-L-D-R-N-L-P
49	2316.6	D-E-S-D-A-F-H-D-N-L-R-S-L-D-R-N-L-P-S-D
50	2287.6	S-D-A-F-H-D-N-L-R-S-L-D-R-N-L-P-S-D-S-Q
51	2313.7	A-F-H-D-N-L-R-S-L-D-R-N-L-P-S-D-S-Q-D-L
52	2280.6	H-D-N-L-R-S-L-D-R-N-L-P-S-D-S-Q-D-L-G-Q
53	2222.6	N-L-R-S-L-D-R-N-L-P-S-D-S-Q-D-L-G-Q-H-G
54	2237.6	R-S-L-D-R-N-L-P-S-D-S-Q-D-L-G-Q-H-G-L-E
55	2238.5	L-D-R-N-L-P-S-D-S-Q-D-L-G-Q-H-G-L-E-E-D
56	2288.6	R-N-L-P-S-D-S-Q-D-L-G-Q-H-G-L-E-E-D-F-M
57	2245.6	N-L-P-S-D-S-Q-D-L-G-Q-H-G-L-E-E-D-F-M-L

Fig. A9. The list consisting of the the sequences based on serglycin. The 57 sequences represent the 57 peptide spots in the peptide array membranes (Fig. A8.). The first column shows the peptide numbers, the second column shows the molecular weight of each peptide and the third column shows the sequences of each peptide comprising of 20 amino acids each. The entire serglycin sequence in figure 16C (result section).

Figure A10 shows the second peptide array experiment conducted for the peptide array based on serglycin probed with proMMP-9.

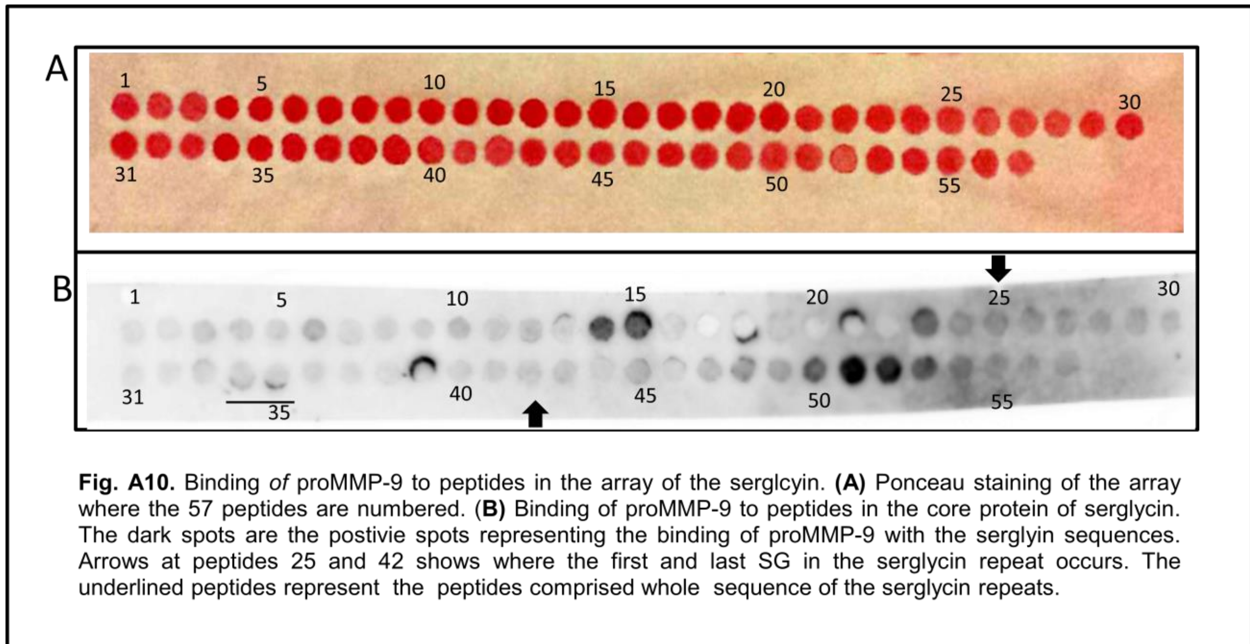

Fig. A10. Binding *of* proMMP-9 to peptides in the array of the serglcyin. **(A)** Ponceau staining of the array where the 57 peptides are numbered. (**B)** Binding of proMMP-9 to peptides in the core protein of serglycin. The dark spots are the postivie spots representing the binding of proMMP-9 with the serglyin sequences. Arrows at peptides 25 and 42 shows where the first and last SG in the serglycin repeat occurs. The underlined peptides represent the peptides comprised whole sequence of the serglycin repeats.

Figure A10.A shows the ponceau stained array where the spots are labelled with the number of the peptide sequence as shown in appendix 7.7 (Fig. A9.). Figure A10.B shows that proMMP-9 binds to several of the peptides in the serglycin sequence.

Nr.	Mol. Weight	Sequence
1	2149.6	A-D-G-A-A-C-H-F-P-F-I-F-E-G-R-S-Y-S-A-C
2	2165.6	G-A-A-C-H-F-P-F-I-F-E-G-R-S-Y-S-A-C-T-T
3	2209.6	A-C-H-F-P-F-I-F-E-G-R-S-Y-S-A-C-T-T-D-G
4	2278.7	H-F-P-F-I-F-E-G-R-S-Y-S-A-C-T-T-D-G-R-S
5	2166.6	P-F-I-F-E-G-R-S-Y-S-A-C-T-T-D-G-R-S-D-G
6	2132.6	I-F-E-G-R-S-Y-S-A-C-T-T-D-G-R-S-D-G-L-P
7	2161.5	E-G-R-S-Y-S-A-C-T-T-D-G-R-S-D-G-L-P-W-C
8	2163.5	R-S-Y-S-A-C-T-T-D-G-R-S-D-G-L-P-W-C-S-T
9	2092.4	Y-S-A-C-T-T-D-G-R-S-D-G-L-P-W-C-S-T-T-A
10	2119.4	A-C-T-T-D-G-R-S-D-G-L-P-W-C-S-T-T-A-N-Y
11	2161.4	T-T-D-G-R-S-D-G-L-P-W-C-S-T-T-A-N-Y-D-T
12	2189.4	D-G-R-S-D-G-L-P-W-C-S-T-T-A-N-Y-D-T-D-D
13	2320.6	R-S-D-G-L-P-W-C-S-T-T-A-N-Y-D-T-D-D-R-F
14	2281.6	D-G-L-P-W-C-S-T-T-A-N-Y-D-T-D-D-R-F-G-F
15	2309.6	L-P-W-C-S-T-T-A-N-Y-D-T-D-D-R-F-G-F-C-P
16	2315.5	W-C-S-T-T-A-N-Y-D-T-D-D-R-F-G-F-C-P-S-E
17	2295.6	S-T-T-A-N-Y-D-T-D-D-R-F-G-F-C-P-S-E-R-L
18	2371.7	T-A-N-Y-D-T-D-D-R-F-G-F-C-P-S-E-R-L-Y-T
19	2442.7	N-Y-D-T-D-D-R-F-G-F-C-P-S-E-R-L-Y-T-Q-D
20	2336.6	D-T-D-D-R-F-G-F-C-P-S-E-R-L-Y-T-Q-D-G-N
21	2306.6	D-D-R-F-G-F-C-P-S-E-R-L-Y-T-Q-D-G-N-A-D
22	2261.7	R-F-G-F-C-P-S-E-R-L-Y-T-Q-D-G-N-A-D-G-K
23	2158.5	G-F-C-P-S-E-R-L-Y-T-Q-D-G-N-A-D-G-K-P-C
24	2229.5	C-P-S-E-R-L-Y-T-Q-D-G-N-A-D-G-K-P-C-Q-F
25	2273.6	S-E-R-L-Y-T-Q-D-G-N-A-D-G-K-P-C-Q-F-P-F
26	2317.8	R-L-Y-T-Q-D-G-N-A-D-G-K-P-C-Q-F-P-F-I-F
27	2233.6	Y-T-Q-D-G-N-A-D-G-K-P-C-Q-F-P-F-I-F-Q-G
28	2184.5	Q-D-G-N-A-D-G-K-P-C-Q-F-P-F-I-F-Q-G-Q-S
29	2191.6	G-N-A-D-G-K-P-C-Q-F-P-F-I-F-Q-G-Q-S-Y-S
30	2194.6	A-D-G-K-P-C-Q-F-P-F-I-F-Q-G-Q-S-Y-S-A-C
31	2210.6	G-K-P-C-Q-F-P-F-I-F-Q-G-Q-S-Y-S-A-C-T-T
32	2197.5	P-C-Q-F-P-F-I-F-Q-G-Q-S-Y-S-A-C-T-T-D-G
33	2240.6	Q-F-P-F-I-F-Q-G-Q-S-Y-S-A-C-T-T-D-G-R-S
34	2137.5	P-F-I-F-Q-G-Q-S-Y-S-A-C-T-T-D-G-R-S-D-G
35	2212.6	I-F-Q-G-Q-S-Y-S-A-C-T-T-D-G-R-S-D-G-Y-R
36	2241.5	Q-G-Q-S-Y-S-A-C-T-T-D-G-R-S-D-G-Y-R-W-C
37	2228.5	Q-S-Y-S-A-C-T-T-D-G-R-S-D-G-Y-R-W-C-A-T
38	2185.5	Y-S-A-C-T-T-D-G-R-S-D-G-Y-R-W-C-A-T-T-A
39	2212.5	A-C-T-T-D-G-R-S-D-G-Y-R-W-C-A-T-T-A-N-Y
40	2309.6	T-T-D-G-R-S-D-G-Y-R-W-C-A-T-T-A-N-Y-D-R
41	2350.7	D-G-R-S-D-G-Y-R-W-C-A-T-T-A-N-Y-D-R-D-K
42	2438.9	R-S-D-G-Y-R-W-C-A-T-T-A-N-Y-D-R-D-K-L-F
43	2399.9	D-G-Y-R-W-C-A-T-T-A-N-Y-D-R-D-K-L-F-G-F
44	2427.9	Y-R-W-C-A-T-T-A-N-Y-D-R-D-K-L-F-G-F-C-P
45	2365.8	W-C-A-T-T-A-N-Y-D-R-D-K-L-F-G-F-C-P-T-R

46	2262.7	A-T-T-A-N-Y-D-R-D-K-L-F-G-F-C-P-T-R-A-D
47	2278.7	T-A-N-Y-D-R-D-K-L-F-G-F-C-P-T-R-A-D-S-T
48	2336.8	N-Y-D-R-D-K-L-F-G-F-C-P-T-R-A-D-S-T-V-M
49	2173.7	D-R-D-K-L-F-G-F-C-P-T-R-A-D-S-T-V-M-G-G
50	2103.6	D-K-L-F-G-F-C-P-T-R-A-D-S-T-V-M-G-G-N-S
51	1988.5	L-F-G-F-C-P-T-R-A-D-S-T-V-M-G-G-N-S-A-G
52	1970.4	G-F-C-P-T-R-A-D-S-T-V-M-G-G-N-S-A-G-E-L
53	1968.3	C-P-T-R-A-D-S-T-V-M-G-G-N-S-A-G-E-L-C-V
54	2012.4	T-R-A-D-S-T-V-M-G-G-N-S-A-G-E-L-C-V-F-P
55	2003.4	A-D-S-T-V-M-G-G-N-S-A-G-E-L-C-V-F-P-F-T
56	2077.6	S-T-V-M-G-G-N-S-A-G-E-L-C-V-F-P-F-T-F-L
57	2074.7	V-M-G-G-N-S-A-G-E-L-C-V-F-P-F-T-F-L-G-K
58	2136.7	G-G-N-S-A-G-E-L-C-V-F-P-F-T-F-L-G-K-E-Y
59	2210.7	N-S-A-G-E-L-C-V-F-P-F-T-F-L-G-K-E-Y-S-T
60	2213.7	A-G-E-L-C-V-F-P-F-T-F-L-G-K-E-Y-S-T-C-T
61	2301.7	E-L-C-V-F-P-F-T-F-L-G-K-E-Y-S-T-C-T-S-E
62	2272.7	C-V-F-P-F-T-F-L-G-K-E-Y-S-T-C-T-S-E-G-R
63	2242.7	F-P-F-T-F-L-G-K-E-Y-S-T-C-T-S-E-G-R-G-D
64	2211.7	F-T-F-L-G-K-E-Y-S-T-C-T-S-E-G-R-G-D-G-R
65	2262.8	F-L-G-K-E-Y-S-T-C-T-S-E-G-R-G-D-G-R-L-W
66	2176.6	G-K-E-Y-S-T-C-T-S-E-G-R-G-D-G-R-L-W-C-A
67	2193.5	E-Y-S-T-C-T-S-E-G-R-G-D-G-R-L-W-C-A-T-T
68	2102.4	S-T-C-T-S-E-G-R-G-D-G-R-L-W-C-A-T-T-S-N
69	2176.5	C-T-S-E-G-R-G-D-G-R-L-W-C-A-T-T-S-N-F-D
70	2174.5	S-E-G-R-G-D-G-R-L-W-C-A-T-T-S-N-F-D-S-D
71	2214.7	G-R-G-D-G-R-L-W-C-A-T-T-S-N-F-D-S-D-K-K
72	2244.7	G-D-G-R-L-W-C-A-T-T-S-N-F-D-S-D-K-K-W-G
73	2322.8	G-R-L-W-C-A-T-T-S-N-F-D-S-D-K-K-W-G-F-C
74	2321.7	L-W-C-A-T-T-S-N-F-D-S-D-K-K-W-G-F-C-P-D

Fig. A11. The list consisting of the the sequences based on FnII module of MMP-9. The 74 sequences represent the 74 peptide spots in the peptide array membranes (Fig. A8.). The first column shows the peptide number, the second column shows the molecular weight of each peptide and the third column shows the sequences of each peptide comprising of 20 amino acids each. The entire sequence of FnII module can be seen in figure 19C (result section).

Nr.	Mol.Weight	Sequence
1	2392.1	F-D-A-I-A-E-I-G-N-Q-L-Y-L-F-K-D-G-K-Y-W
2	2433.2	A-I-A-E-I-G-N-Q-L-Y-L-F-K-D-G-K-Y-W-R-F
3	2465.1	A-E-I-G-N-Q-L-Y-L-F-K-D-G-K-Y-W-R-F-S-E
4	2478.2	I-G-N-Q-L-Y-L-F-K-D-G-K-Y-W-R-F-S-E-G-R
5	2452.1	N-Q-L-Y-L-F-K-D-G-K-Y-W-R-F-S-E-G-R-G-S
6	2463.2	L-Y-L-F-K-D-G-K-Y-W-R-F-S-E-G-R-G-S-R-P
7	2372	L-F-K-D-G-K-Y-W-R-F-S-E-G-R-G-S-R-P-Q-G
8	2355.9	K-D-G-K-Y-W-R-F-S-E-G-R-G-S-R-P-Q-G-P-F
9	2339	G-K-Y-W-R-F-S-E-G-R-G-S-R-P-Q-G-P-F-L-I
10	2339.9	Y-W-R-F-S-E-G-R-G-S-R-P-Q-G-P-F-L-I-A-D
11	2304.9	R-F-S-E-G-R-G-S-R-P-Q-G-P-F-L-I-A-D-K-W
12	2169.7	S-E-G-R-G-S-R-P-Q-G-P-F-L-I-A-D-K-W-P-A
13	2163.8	G-R-G-S-R-P-Q-G-P-F-L-I-A-D-K-W-P-A-L-P
14	2234.9	G-S-R-P-Q-G-P-F-L-I-A-D-K-W-P-A-L-P-R-K
15	2319	R-P-Q-G-P-F-L-I-A-D-K-W-P-A-L-P-R-K-L-D
16	2251.9	Q-G-P-F-L-I-A-D-K-W-P-A-L-P-R-K-L-D-S-V
17	2343	P-F-L-I-A-D-K-W-P-A-L-P-R-K-L-D-S-V-F-E
18	2384	L-I-A-D-K-W-P-A-L-P-R-K-L-D-S-V-F-E-E-R
19	2357.9	A-D-K-W-P-A-L-P-R-K-L-D-S-V-F-E-E-R-L-S
20	2428.1	K-W-P-A-L-P-R-K-L-D-S-V-F-E-E-R-L-S-K-K
21	2374.1	P-A-L-P-R-K-L-D-S-V-F-E-E-R-L-S-K-K-L-F
22	2500.3	L-P-R-K-L-D-S-V-F-E-E-R-L-S-K-K-L-F-F-F
23	2434.2	R-K-L-D-S-V-F-E-E-R-L-S-K-K-L-F-F-F-S-G
24	2434.1	L-D-S-V-F-E-E-R-L-S-K-K-L-F-F-F-S-G-R-Q
25	2491.1	S-V-F-E-E-R-L-S-K-K-L-F-F-F-S-G-R-Q-V-W
26	2567.2	F-E-E-R-L-S-K-K-L-F-F-F-S-G-R-Q-V-W-V-Y
27	2449.1	E-R-L-S-K-K-L-F-F-F-S-G-R-Q-V-W-V-Y-T-G
28	2322	L-S-K-K-L-F-F-F-S-G-R-Q-V-W-V-Y-T-G-A-S
29	2334	K-K-L-F-F-F-S-G-R-Q-V-W-V-Y-T-G-A-S-V-L
30	2231.8	L-F-F-F-S-G-R-Q-V-W-V-Y-T-G-A-S-V-L-G-P
31	2283.8	F-F-S-G-R-Q-V-W-V-Y-T-G-A-S-V-L-G-P-R-R
32	2217.7	S-G-R-Q-V-W-V-Y-T-G-A-S-V-L-G-P-R-R-L-D
33	2314.9	R-Q-V-W-V-Y-T-G-A-S-V-L-G-P-R-R-L-D-K-L
34	2200.9	V-W-V-Y-T-G-A-S-V-L-G-P-R-R-L-D-K-L-G-L
35	2043.8	V-Y-T-G-A-S-V-L-G-P-R-R-L-D-K-L-G-L-G-A
36	1995.7	T-G-A-S-V-L-G-P-R-R-L-D-K-L-G-L-G-A-D-V
37	2036.7	A-S-V-L-G-P-R-R-L-D-K-L-G-L-G-A-D-V-A-Q

38	2078.7	V-L-G-P-R R-L-D-K-L-G-L-G-A-D-V-A-Q-V-T
39	1994.6	G-P-R-R-L-D-K-L-G-L-G-A-D-V-A-Q-V-T-G-A
40	2109.8	R-R-L-D-K-L-G-L-G-A-D-V-A-Q-V-T-G-A-L-R
41	1941.6	L-D-K-L-G-L-G-A-D-V-A-Q-V-T-G-A-L-R-S-G
42	1926.6	K-L-G-L-G-A-D-V-A-Q-V-T-G-A-L-R-S-G-R-G
43	1944.6	G-L-G-A-D-V-A-Q-V-T-G-A-L-R-S-G-R-G-K-M
44	2000.7	G-A-D-V-A-Q-V-T-G-A-L-R-S-G-R-G-K-M-L-L
45	2106.8	D-V-A-Q-V-T-G-A-L-R-S-G-R-G-K-M-L-L-F-S
46	2105.9	A-Q-V-T-G-A-L-R-S-G-R-G-K-M-L-L-F-S-G-R
47	2176.1	V-T-G-A-L-R-S-G-R-G-K-M-L-L-F-S-G-R-R-L
48	2318.3	G-A-L-R-S-G-R-G-K-M-L-L-F-S-G-R-R-L-W-R
49	2452.4	L-R-S-G-R-G-K-M-L-L-F-S-G-R-R-L-W-R-F-D
50	2410.3	S-G-R-G-K-M-L-L-F-S-G-R-R-L-W-R-F-D-V-K
51	2465.3	R-G-K-M-L-L-F-S-G-R-R-L-W-R-F-D-V-K-A-Q
52	2482.3	K-M-L-L-F-S-G-R-R-L-W-R-F-D-V-K-A-Q-M-V
53	2435.1	L-L-F-S-G-R-R-L-W-R-F-D-V-K-A-Q-M-V-D-P
54	2452	F-S-G-R-R-L-W-R-F-D-V-K-A-Q-M-V-D-P-R-S
55	2375.9	G-R-R-L-W-R-F-D-V-K-A-Q-M-V-D-P-R-S-A-S
56	2390.8	R-L-W-R-F-D-V-K-A-Q-M-V-D-P-R-S-A-S-E-V
57	2392.7	W-R-F-D-V-K-A-Q-M-V-D-P-R-S-A-S-E-V-D-R
58	2328.7	F-D-V-K-A-Q-M-V-D-P-R-S-A-S-E-V-D-R-M-F
59	2220.6	V-K-A-Q-M-V-D-P-R-S-A-S-E-V-D-R-M-F-P-G
60	2189.5	A-Q-M-V-D-P-R-S-A-S-E-V-D-R-M-F-P-G-V-P
61	2218.6	M-V-D-P-R-S-A-S-E-V-D-R-M-F-P-G-V-P-L-D
62	2226.5	D-P-R-S-A-S-E-V-D-R-M-F-P-G-V-P-L-D-T-H
63	2228.5	R-S-A-S-E-V-D-R-M-F-P-G-V-P-L-D-T-H-D-V
64	2260.5	A-S-E-V-D-R-M-F-P-G-V-P-L-D-T-H-D-V-F-Q
65	2421.7	E-V-D-R-M-F-P-G-V-P-L-D-T-H-D-V-F-Q-Y-R
66	2450.8	D-R-M-F-P-G-V-P-L-D-T-H-D-V-F-Q-Y-R-E-K
67	2413.8	M-F-P-G-V-P-L-D-T-H-D-V-F-Q-Y-R-E-K-A-Y

68	2385.7	P-G-V-P-L-D-T-H-D-V-F-Q-Y-R-E-K-A-Y-F-C
69	2474.7	V-P-L-D-T-H-D-V-F-Q-Y-R-E-K-A-Y-F-C-Q-D
70	2581.9	L-D-T-H-D-V-F-Q-Y-R-E-K-A-Y-F-C-Q-D-R-F
71	2703	T-H-D-V-F-Q-Y-R-E-K-A-Y-F-C-Q-D-R-F-Y-W
72	2720.1	D-V-F-Q-Y-R-E-K-A-Y-F-C-Q-D-R-F-Y-W-R-V
73	2680.1	F-Q-Y-R-E-K-A-Y-F-C-Q-D-R-F-Y-W-R-V-S-S
74	2648.1	Y-R-E-K-A-Y-F-C-Q-D-R-F-Y-W-R-V-S-S-R-S
75	2571	E-K-A-Y-F-C-Q-D-R-F-Y-W-R-V-S-S-R-S-E-L
76	2555.9	A-Y-F-C-Q-D-R-F-Y-W-R-V-S-S-R-S-E-L-N-Q
77	2535.8	F-C-Q-D-R-F-Y-W-R-V-S-S-R-S-E-L-N-Q-V-D
78	2512.7	Q-D-R-F-Y-W-R-V-S-S-R-S-E-L-N-Q-V-D-Q-V
79	2489.8	R-F-Y-W-R-V-S-S-R-S-E-L-N-Q-V-D-Q-V-G-Y
80	2386.6	Y-W-R-V-S-S-R-S-E-L-N-Q-V-D-Q-V-G-Y-V-T
81	2315.5	R-V-S-S-R-S-E-L-N-Q-V-D-Q-V-G-Y-V-T-Y-D
82	2286.6	S-S-R-S-E-L-N-Q-V-D-Q-V-G-Y-V-T-Y-D-I-L
83	2343.6	R-S-E-L-N-Q-V-D-Q-V-G-Y-V-T-Y-D-I-L-Q-C
84	2326.5	E-L-N-Q-V-D-Q-V-G-Y-V-T-Y-D-I-L-Q-C-P-E
85	2312.5	L-N-Q-V-D-Q-V-G-Y-V-T-Y-D-I-L-Q-C-P-E-D

Fig. A12. The list consisting of the sequences based on HPX domain of MMP-9. The 85 sequences represent the 85 peptide spots in the peptide array membranes (Fig. A8). The first column shows the peptide number, the second column shows the molecular weight of each peptide and the third column shows the sequences of each peptide comprising of 20 amino acids each. The enitre sequence for HPX domain can be seen in figure 21C (result section).

7.8. Mutation scans for the MMP-9 Fn-II module, HPX domain and Serglycin

The amino acids selected for mutation were based on the results from the first peptide array and they were mutated as follows: Polar and charged amino acids such as serine (S), histidine (H), asparagine (N), glutamine (Q), tyrosine (Y), tryphtophan (W), aspartate (D), glutamate (E), arginine (R), cysteine (C) and lycine (K) were mutated to alanine (A) since it has a small hydrophobic side chain and is an uncharged amino acid.

Amino acid such as threonine (T) is a polar and it was changed into valine (V) because it is hydrophobic, similar in size to threonine and both are branched amino acids.

Amino acids which are hydrophobic such as Valine (V), alanine (A), isoleucine (I), methionine (M), leucine (L), proline (P) and phenylalanine (F) were mutated to glycine (G) as it lacks a side chain. Glycine (G) was mutated to leucine (L) which has a large hydrophobic side chain.

Serglycin sequence:

^{1}YPTRR ^{6}ARYQWVRCNP ^{16}DSNSANCLEE ^{26}KGPMFELLPG ^{36}ESNKIPRLRT ^{46}DLFPKTRIQD ^{56}LNRIFPLSED ^{66}YSGSGFGSGS ^{76}GSGSGSGSGF ^{86}LTEMEQDYQL ^{96}VDESDAFHDN ^{106}LRSLDRNLPS ^{116}DSQDLGQHGL ^{126}EEDFML

Eg: ^{11}*VR*CNPDSNSA NCLEEKGP*MF* (GA - GA)

This is the first sequence which is thought to be involved in binding, with the major amino acids (V,R, M and F) which were thought to be involved in binding. We then did a mixture of single and double mutations with these four amino acids (G instead of V and M and A instead of R and F). This is how the 20-mer sequence looked like, where the labeled amino acids are the mutated ones.

Original: *VR*CNPDSNSA NCLEEKGP*MF*

Mut. 1: ***G****R*CNPDSNSA NCLEEKGP*MF*

Mut. 2: *V****A***CNPDSNSA NCLEEKGP*MF*

Mut. 3: *VR*CNPDSNSA NCLEEKGP***G****F*

Mut. 4: *VR*CNPDSNSA NCLEEKGP*M****A***

Mut. 5: ***G****R*CNPDSNSA NCLEEKGP***G****F*

Mut. 6: ***G****R*CNPDSNSA NCLEEKGP*M****A***

Mut. 7: *V**A*CNPDSNSA NCLEEKGP*G**F*

Mut. 8: *V**A*CNPDSNSA NCLEEKGP*M**F*

Mut. 9: *G**A*CNPDSNSA NCLEEKGP*MF*

Mut. 10: *G**A*CNPDSNSA NCLEEKGP*G**F*

Mut. 11: *G**A*CNPDSNSA NCLEEKGP*M**A*

Mut. 12: *G**A*CNPDSNSA NCLEEKGP*G**A*

Mut. 13: *VR*CNPDSNSA NCLEEKGP*G**A*

Mut. 14: *G**R*CNPDSNSA NCLEEKGP*G**A*

Mut. 15: *V**A*CNPDSNSA NCLEEKGP*G**A*

Now in the peptide array of serglycin mutation, these 16 sequences represents the first 16 spots and all these spots comprising as peptide 1.

The pattern of mutation was similar for the other selected sequences when four amino acids are mutated and this also generated 16 sequences where the first peptide always is the unmutated parent peptide. The rest of the serglycin mutated peptides are as follows:

Peptide 2: ^{28}P*MF*ELLPGESNKIPRLR*TD*L (where M,F,T and D were mutated into G,A,V and A respectively).

Peptide 3: ^{46}DLFPKTR*IQ*DLNRIFPLSED (where I and Q were mutated into G and A respectively). This resulted in four peptides.

Peptide 4: ^{85}*FL*TEMEQDYQLVDE*SD*AFHD (where F,L,S and D were mutated into A,G, A and A respectively).

Peptide 5: ^{94}Q*LV*DESDAFHDNLRSLD*RN*L (where L,V,R and N were changed into G,G,A and A respectively).

Peptide 6: ^{103}HD*NL*RSLDRN LPSD*SQ*DLGQ (where N,L,S and Q were changed into A,G,A and A respectively).

Similarly with the selected sequences of FnII module:

Full sequence of FnII module

^{1}ADGAAC ^{7}HFPFIFEGRS ^{17}YSACTTDGRS ^{27}DGLPWCSTTA ^{37}NYDTDDRFGF
^{47}CPSERLYTQD ^{57}GNADGKPCQF ^{67}PFIFQGQSYS ^{77}ACTTDGRSDG
^{87}YRWCATTANY ^{97}DRDKLFGFCP ^{107}TRADSTVMGG ^{117}NSAGELCVFP
^{127}FTFLGKEYST ^{137}CTSEGRGDGR ^{147}LWCATTSNFD ^{157}SDKKWGFCPD

The peptides are:

Peptide 1: ^{26}S*DG*LPWCSTTANYDTDD*RF*G (where D,G, R and F were mutated into A,L,A and G respectively).

Peptide 2: ^{26}SDGLPWCST*TA*NYDTDD*RF*G (where T, A, R and F were mutated into V,G,A and A respectively).

Peptide 3: ^{32}C*ST*TANYDTDDRFGF*CP*SER (where S, T, C and P were mutated into A,V,A and G respectively).

Peptide 4: ^{32}CST*TA*NYDTDDRFGFCP*SE*R (where T, A,S and E were mutated into V,G,A and A respectively).

Peptide 5: ^{41}*DD*RFGFCPSERLYTQDGN*AD* (where D, D, A and D were changed into A,A,G and A respectively).

Peptide 6: ^{69}*IF*QGQSYSACTTDGRSDG*YR* (where I, F, Y and R were mutated into G,A,A and A respectively).

Peptide 7: ^{83}RS*DG*YRWCATTANYDR*DK*LF (where D, G and K were mutated into A, L and A respectively).

Peptide 8: ^{90}C*AT*TANYDRDKLFGFCP*TR*A (where A, T and R were mutated into G, V and A respectively).

Peptide 9: ^{97}*DR*DKLFGFCPTRADSTVM*GG* (where D, R, and G were mutated into A, A and L respectively).

Peptide 10: ^{117}NS*AG*ELCVFPFTFL*GK*EYST (where A, G and K were mutated into G,L and A respectively).

Peptide 11: ^{128}T*FL*GKEYSTCTSEGRGD*GR*L (where F, L, G and R were mutated into A,G,L and A respectively).

Peptide 12: ^{141}*GR*GDGRLWCATTSNFDSD*KK* (where G, R and K were mutated into L,A and A respectively).

Similarly with the selected sequences of HPX-domain:

Full sequence of HPX domain:

^{1}FDAIAEIGNQ ^{11}LYLFKDGKYW ^{21}RFSEGRGSRP ^{31}QGPFLIADKW
^{41}PALPRKLDSV ^{51}FEERLSKKLF ^{61}FFSGRQVWVY ^{71}TGASVLGPRR
^{81}LDKLGLGADV ^{91}AQVTGALRSG ^{101}RGKMLLFSGR ^{111}RLWRFDVKAQ
^{121}MVDPRSASEV ^{131}DRMFPGVPLD ^{141}THDVFQYREK ^{151}AYFCQDRFYW
^{161}RVSSRSELNQ ^{171}VDQVGYVTYD ^{181}ILQCPED

The peptides are:

Peptide 1: ^{21}RF*SE*GRGSRPQGPFLI*AD*KW (where S, E, A and D were mutated into A,A,G and A respectively).

Peptide 2: ^{31}*QG*PFLIADKWPALPRKLD*SV* (where Q, G, S and V were mutated into A,L,A and A respectively).

Peptide 3: ^{36}I*AD*KWPALPRKLDSVFE*ER*L (where A, D, E and R were mutated into G,A,A and A respectively).

Peptide 4: ^{73}AS*VL*GPRRLDKLGLGA*DV*AQ (where V, L, D and V were mutated into G,G,A and G respectively).

Peptide 5: ^{81}*LD*KLGLGADVAQVTGALR*SG* (where L, D, S and G were mutated into G,A,A and L respectively).

Peptide 6: ^{106}L*FS*GRRLWRFDVKAQMV*DP*R (where F, S, D and P were mutated into A,A,A and G respectively).

Peptide 7: ^{112}L*WR*FDVKAQMVDPRSAS*EV*D (where W, R, E and V were mutated into A,A,A and G respectively).

Peptide 8: ^{139}LD*TH*DVFQYREKAYFC*QD*RF (where T, H, Q and D were mutated into V,A,A and A respectively).

Peptide 9: ^{152}YFC*QD*RFYWRVSSRS*EL*NQV (where Q, D,E and L were mutated into A,A,A and G).

Peptide 10: [156]DRFYWRV*SS*RS*EL*NQVDQVG (where S, E and L were mutated into A,A and G respectively).

Peptide 11: [162]V*SS*RSELNQVDQVGYVT*YD*I (where S, Y and D were mutated into A,A and A respectively).

Peptide 12: [169]*LN*QVDQVGYVTYDILQC*PE*D (where L, N, P and E were mutated into G,A,G and A respectively).

7.9. List of peptides for *in vitro* reconstitution competition experiments

The peptides used for the *in vitro* competition experiments are listed in Table A1 which also gives the information of the sequences of the peptides, their molecular masses, their total quantity in milli gram and their purity. Two peptides are based on binding sequences in serglycin (SG) and three peptides each from binding sequences in the MMP-9 FnII module and HPX domain, respectively.

Table. A1 Information regarding the peptides ordered for conducting *in vitro* reconstitution competititon experiments.

Peptide	Name	Sequence	Molecular Mass	Quantity (mg)	Purity (%) from HPLC
D1	SG-peptide 1	^{103}HDNLRSLDRNLPSDSQDLG	2192.565	6.6	95
D2	SG-peptide 2	^{28}PMFELLPGESNKIPRLRTDL	2366.2775	5.6	85
D3	FnII peptide 1	^{26}SDGLPWCSTTANYDTDDRFGFCPSER	2980.2387	3.0	75
D4	FnII peptide 2	^{69}IFQGQSYSACTTDGRSDGYR	2251.9911	1.9	60-65
D5	FnII peptide 3	^{89}WCATTANYDRDKLFGFCPTR	2405.1040	2.1	70
D6	HPX-peptide 1	^{35}LIADKWPALPRKLDSVFEER	2423.3320	2.2	55-60
D7	HPX-peptide 2	^{113}WRFDVKAQMVDPRSASEVDR	2432.2014	2.6	50
D8	HPX-peptide 3	^{151}AYFCQDRFYWRVSSRSELNQ	2595.2072	3.5	85
D9	SG-peptide 1	^{103}HDNLRSLDRNLPSDSQDLG	2192	1	88

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