

2018

# Effects of a synthetic peptide derived from collagen binding domain of decorin on matrix metalloproteinase (MMP)-mediated collagen degradation

---

<https://hdl.handle.net/2144/32949>

*"Downloaded from OpenBU. Boston University's institutional repository."*

BOSTON UNIVERSITY  
HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

DISSERTATION

**EFFECTS OF A SYNTHETIC PEPTIDE DERIVED FROM COLLAGEN  
BINDING DOMAIN OF DECORIN ON MATRIX METALLOPROTEINASE  
(MMP)-MEDIATED COLLAGEN DEGRADATION**

by

**ARWA ALNOURY**

B.D.S, King Addulaziz University, 2007  
M.S., University of Buffalo, 2013

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Science in Dentistry  
In the Department of Endodontics

2018

Approved by:

First Reader \_\_\_\_\_

**Yoshiyuki Mochida, DDS, Ph.D.**

Clinical Assistant Professor, Department of Molecular and Cell Biology

Boston University, Henry M. Goldman School of Dental Medicine

Second Reader \_\_\_\_\_

**Philip C. Trackman, Ph.D.**

Professor, Department of Molecular and Cell Biology

Boston University, Henry M. Goldman School of Dental Medicine

EFFECTS OF A SYNTHETIC PEPTIDE DERIVED FROM COLLAGEN BINDING  
DOMAIN OF DECORIN ON MATRIX METALLOPROTEINASE (MMP)-  
MEDIATED COLLAGEN DEGRADATION

ARWA ALNOURY

Boston University, Henry M. Goldman School of Dental Medicine, 2018

Major Professor: Yoshiyuki Mochida, DDS, Ph.D., Clinical Assistant Professor of  
Molecular and Cell Biology

ABSTRACT

Decorin is the most characterized member of Small Leucine-Rich Repeat Proteoglycan (SLRP) family. It has been previously shown that some SLRP members including decorin bind to collagen type I. Binding of decorin to collagen was reported to inhibit collagen degradation by collagenases, suggesting that decorin might limit the access of collagenases to their collagen cleavage site. The amino acid sequence SYIRIADTNIT in decorin was reported to be the binding region to collagen type I. The objective of the current study was to investigate the effect of the synthetic SYIRIADTNIT peptide derived from the binding site of decorin (SYI peptide, hereafter) to collagen on MMP1-mediated collagen degradation in vitro.

SYI peptide and its scrambled peptide were prepared. Collagen type I samples were incubated with various concentrations of SYI peptide and degraded by MMP1. The samples were subjected to electrophoresis and Coomassie Brilliant Blue (CBB) staining. CBB- positive collagen bands were quantified to assess the extent of collagen

degradation. Biotin affinity chromatography was used to identify the collagen fragment that the biotinylated SYI peptide binds to. Two collagen degrading enzymes; MMP1 and trypsin were each used to generate collagen fragments. The activity of MMP1 to degrade a substrate different from collagen in the presence or absence of SYI peptide was measured. The intensity of non-degraded collagen in the presence and absence of SYI peptide was compared using CBB-stained SDS-PAGE. There was no difference of collagen degradation in the presence or absence of SYI peptide. Collagen fibrils were retrieved from biotin-SYI peptide/streptavidin column, suggesting that collagen fibrils bind to SYI peptide. However, when collagen was treated with MMP1 or trypsin, the collagen fragment was not retrieved from biotin-SYI peptide/streptavidin column, suggesting that biotin-SYI peptide binds to collagen fibrils, but not fragments. Although there was no statistical difference, MMP1 activity was slightly reduced when SYI peptide or scrambled peptide was used at higher concentrations.

In conclusion, there was no significant effect of SYI peptide on MMP1-mediated collagen degradation. SYI peptide did not act as a competitive substrate for MMP1.

## TABLE OF CONTENTS

ABSTRACT .....	iii
TABLE OF CONTENTS.....	v
LIST OF TABLES .....	vii
LIST OF FIGURES.....	viii
LIST OF ABBREVIATIONS.....	ix
INTRODUCTION.....	1
Collagen .....	1
Matrix Metalloproteinases (MMPs) .....	6
Small Leucine-Rich Repeat Proteoglycans (SLRPs) .....	9
Collagen and Decorin Interaction.....	16
HYPOTHESIS .....	17
AIMS .....	18
METHODS and Experimental Designs.....	19
MMP1-Collagen Degradation Assay.....	19
The Effect of SYI Peptide on MMP1-Mediated Collagen Degradation .....	20
Biotin Affinity Chromatography to Identify the Collagen Sequence that Binds SYI Peptide Using MMP1 and Trypsin as Collagen Degrading Enzymes.....	21

Biotin Affinity Chromatography to Identify Non-Degraded Collagen that Binds Biotin-SYI Peptide (A Control Experiment).....	21
Biotin Affinity Chromatography to Identify the Collagen Sequence that Binds Biotin-SYI Peptide Using MMP1 as a Collagen-Degrading Enzyme.....	23
Biotin Affinity Chromatography to Identify the Collagen Sequence that Binds Biotin-SYI Peptide Using Trypsin as a Collagen Degrading Enzyme.....	24
MMP1 Activity Assay .....	26
Statistical analysis.....	26
RESULTS .....	27
The Effect of SYI Peptide on MMP1-Mediated Collagen Degradation .....	27
Biotin Affinity Chromatography to Identify Collagen Sequence that Binds SYI Peptide .....	37
MMP1 Activity Assay .....	49
DISCUSSION .....	54
The Effect of SYI Peptide on MMP1-Mediated Collagen Degradation .....	54
Biotin Affinity Chromatography to Identify the Collagen Fragment that Binds to SYI Peptide .....	57
The Effect of SYI Peptide on MMP1 Activity.....	60
BIBLOGRAPHY .....	62
CURRICULUM VITAE.....	68

## LIST OF TABLES

Table 1. Classification of Mouse SLRP Members.....	11
Table 2. Average MMP1 Activity.....	53

## LIST OF FIGURES

Figure 1. Collagen biosynthesis and formation of mature collagen.....	5
Figure 2. Domain structures of the MMP family.....	7
Figure 3. Schematic diagram of decorin.....	13
Figure 4. The structure and amino acid sequence of decorin.....	15
Figure 5. MMP1 collagen degradation assay.....	29
Figure 6. The effect of the SYI peptide and the scrambled peptide at lower concentrations on MMP1-mediated collagen degradation.....	33
Figure 7. The effect of higher concentrations of SYI and its scrambled peptide on MMP1-mediated collagen degradation.....	37
Figure 8. Biotin affinity chromatography to identify the non-degraded collagen fibril that binds to SYI peptide. ....	40
Figure 9. Biotin affinity chromatography to identify the collagen fragment that binds to SYI peptide using MMP1 as a collagen-degrading enzyme.....	42
Figure 10. Degradation of collagen with trypsin.....	45
Figure 11. Biotin affinity chromatography to identify collagen fragment that binds to SYI peptide using trypsin as a collagen-degrading enzyme.....	49
Figure 12. MMP1 activity assay. ....	52

## LIST OF ABBREVIATIONS

APMA	p-Aminophenylmercuric acetate
BMP-4	Bone morphogenetic protein
CB	Cymongen bromide
CBB	Coomassie brilliant blue
DMSO	Dimethyl sulfoxide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
ECM	Extracellular matrix
GAG	Glycosaminoglycan
GGT	Galactose glucose transferase
GT	Galactose transferase
LH	Lysyl hydroxylases
LRR	Leucine-rich repeat
MMPs	Matrix metalloproteinases
NNGH	N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid
OD	Optical density
Rcf	relative centrifugal force
RER	Rough endoplasmic reticulum
SASD	Sulfosuccinimidyl-2-[p-azidosalicylamido]ethyl-1,3-dithiopropionate
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gelelectrophoresis
SLRPs	Small leucine-rich repeat proteoglycans

TGF- $\beta$  ..... Transforming growth factor beta  
TIMPs ..... Tissue inhibitor of metalloproteinases  
TLCK..... Tosyl-L-lysyl-chloromethane hydrochloride

## **INTRODUCTION**

Collagen has been widely used in the field of medicine as a biocompatible biomaterial. It is commonly used in medical devices, including growth factor or osteoinductive carriers. The carrier needs to insure controlled release of the incorporating factor over an extended period of time to achieve the optimum biological outcome. This can be achieved by manipulating the properties of the collagen and its biodegradability. The biodegradability of collagen is often modified by chemical or physical cross-linking. However, chemical cross linker reagents, such as glutaraldehyde and polyepoxy compounds, are cytotoxic (Ferreira, Gentile, Chiono, & Ciardelli, 2012). Physical treatments of collagen, such as dehydrothermal treatment, ultraviolet irradiation, gamma irradiation, and microwave irradiation, are efficient at introducing cross-links; however, collagen could be degraded by over-exposure to physical treatments. Improving the kinetic biodegradability of these collagen carriers by another biocompatible treatment may be beneficial.

### **Collagen**

Collagen is the most abundant protein found in mammals. It constitutes the majority of proteins in the extracellular matrix (ECM). The fibrillar collagens, such as type I, form the structural framework of tissues and is responsible for tissue strength and integrity. Collagen type I molecules consist of two alpha-1 polypeptide chains and one alpha-2 polypeptide chain twisted into a triple helix. These polypeptide chains have a

unique amino acid sequence repeat, (Gly-X-Y)<sub>n</sub>, where X and Y are frequently the amino acids proline and hydroxyproline. This amino acid sequence allows hydrogen bonding and close packing of the three polypeptide chains along the central axis to form the collagen triple helical structure.

The pathway of collagen biosynthesis has been extensively studied for fibrillar collagen. Figure 1 summarizes the collagen biosynthesis process. Collagen gene transcription and mRNA processing within the nucleus follow the general pathway for eukaryotic secretory protein synthesis. The two types of alpha chains, alpha-1 and alpha-2, are formed during the mRNA translation process along the rough endoplasmic reticulum (RER). These peptide chains are known as procollagen peptides and have signal peptides like other secreted proteins. The signal peptides are cleaved by signal peptidase as the polypeptide chains emerge into the lumen of the ER, and the resulting polypeptide chains are known as procollagen chains (Bateman, 1996).

The precursor form of collagen, “procollagen,” undergoes different post-translational modifications. During progression through the membrane of the ER, membrane-bound enzymes, prolyl hydroxylases and lysyl hydroxylases, hydroxylate specific proline and lysine residues. Proline residues are hydroxylated by prolyl 4-hydroxylase or prolyl 3-hydroxylase in the presence of oxygen, ascorbic acid,  $\alpha$ -ketoglutarate, and Fe<sup>2+</sup>. The ratio of 4-hydroxyproline to 3-hydroxyproline is near 100:1 (Wu et al 2011; Myllyharju 2008). The presence of hydroxyproline is essential for the stability of the collagen triple helix structure at physiological temperatures (Myllyharju, 2008). Three types of lysyl hydroxylases (LH1, LH2, and LH3) catalyze the formation of

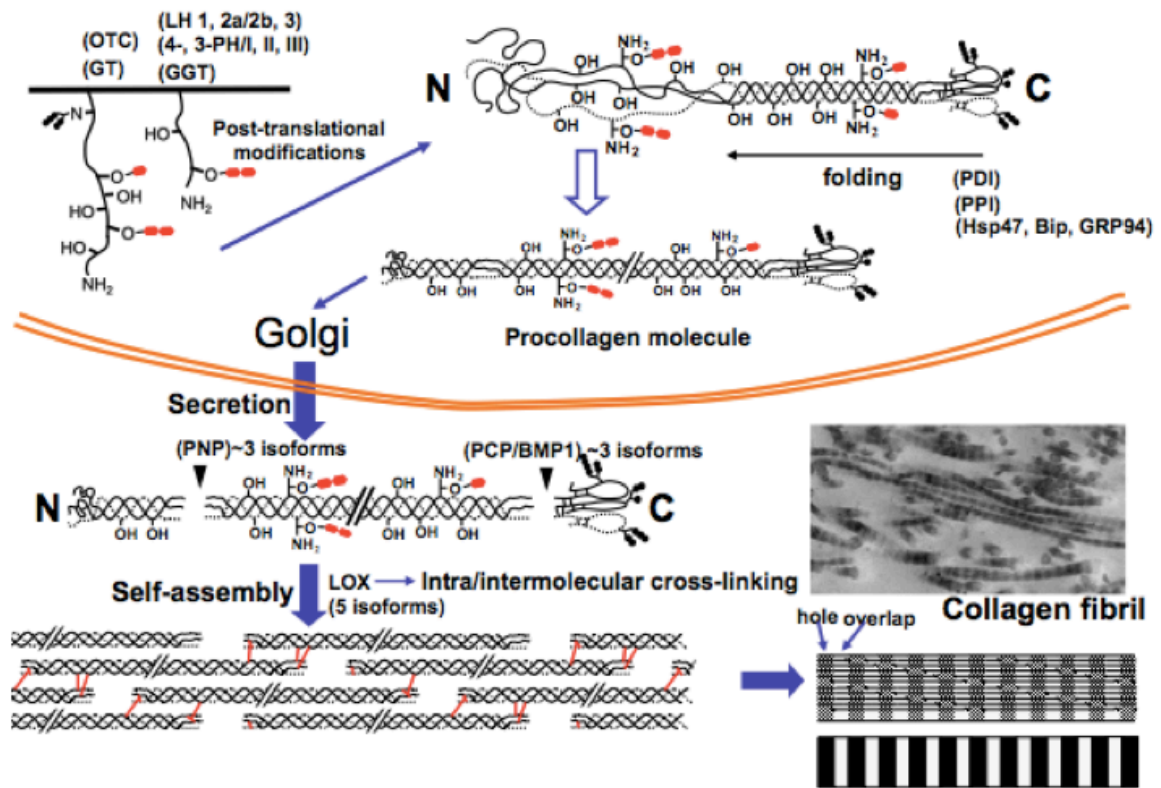
hydroxylysine. LH1 primarily hydroxylates lysine residues in the helical domains of fibrillar and non-fibrillar collagen. LH2 was reported to be associated with lysine hydroxylation in the telopeptide domains of type I collagen molecules. LH3 has both lysine hydroxylation and sugar transferase activity; however, its LH activity may be related to type IV and V, but not type I collagen (Yamauchi & Sricholpech, 2012). Some of the hydroxylysine residues are further modified by O-linked glycosylation. This reaction is catalyzed by galactose transferase (GT) and galactose glucose transferase (GGT) activities of LH3 and produces galactosylhydroxylysine and glucosylgalactosylhydroxylysine (Yamauchi & Sricholpech, 2012).

After hydroxylation and glycosylation of the pro-alpha chains, the three collagen molecules assemble to form the triple helix procollagen molecules, beginning from the carboxyl terminal end. This process is assisted by the formation of disulfide bonds between the cysteine residues in both carboxyl and amino terminal extensions of the polypeptide collagen molecules. Then, the procollagen molecules are packaged in the secretory vesicles, which fuse with the cell membrane, causing the release of procollagen molecules to the extracellular space (Bateman, 1996; Yamauchi & Sricholpech, 2012).

In the ECM, the procollagen molecules are subjected to further post-translational modifications that are of greater importance, including proteolysis, fiber formation, and crosslinking. First, both C- and N- terminal extensions of the procollagen molecules are removed by procollagen N-proteinase and procollagen C-proteinase, releasing triple helical tropocollagen. Second, the interstitial collagen molecules orient themselves specifically to form collagen fibers (Last & Reiser, 1984). Third, intra- and inter-

molecular covalent crosslinks of collagen chains are formed, which is initiated by lysyl oxidase, a copper-dependent enzyme. Lysyl oxidase oxidatively deaminates the amino group of certain lysine and hydroxylysine residues, which initiates a series of condensation reactions to form covalent intra- and inter-molecular cross-links. These reactions result in the stable covalent cross-links that are crucial for the tensile strength of the collagen fibers (Scott, 1988; Yamauchi & Sricholpech, 2012).

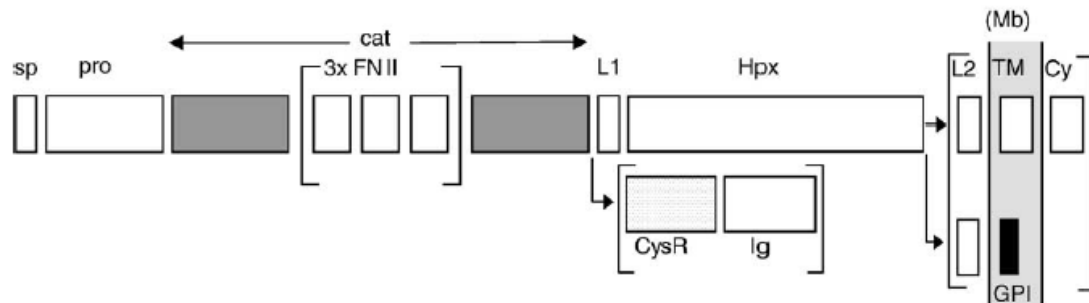
The collagen fibrils have a repeating banding pattern along the fiber axis when visualized by transmission electron microscopy, designated a-e bands in the collagen D-period (Scott, 1988). This pattern results from packing the collagen molecules into a staggered overlap arrangement; the molecules are packed side by side with adjacent molecules staggered along their axis by a fixed distance. According to a simple model, bands d and e are located at the gap between the ends of the collagen molecules and alternate with band b, located in the overlap zone, while bands a and c are at the step to the gap zone.



**Figure 1. Collagen biosynthesis and formation of mature collagen.** Collagen type I alpha chains undergo post-translational modifications. Specific proline and lysine residues are hydroxylated by prolyl hydroxylases (PH) and lysyl hydroxylases (LH), respectively. Some of the hydroxylysine residues are further modified by glycosylation. This reaction is catalyzed by galactose transferase (GT) and galactose glucose transferase (GGT) activities of LH3. Then, the three alpha chains are folded into triple helical structure. The triple helical procollagen molecules are secreted to the ECM and then both N- and C-terminal extensions are cleaved by procollagen N-proteinase and procollagen C-proteinase to form mature type I collagen. The interstitial collagen molecules orient themselves specifically to form collagen fibers. The collagen molecules are further stabilized by the formation of intra- and inter-molecular covalent crosslinks, which is initiated by the lysyl oxidase enzyme (LOX) (Kaku & Yamauchi, 2014).

## **Matrix Metalloproteinases (MMPs)**

MMPs are the major enzymes hydrolyzing ECM proteins. There are twenty three MMPs in humans (H Nagase & Woessner, 1999; Hideaki Nagase, Visse, & Murphy, 2006), which play central roles in morphogenesis, wound healing, tissue repair, and remodeling in response to injury. The typical structure of MMPs consists of a pro-domain, a catalytic domain, and a hemopexin domain. The pro-domain contains the cysteine switch motif (PRCXXPD, where P is proline, R is arginine, C is cysteine, D is aspartate, and X is any amino acid), and the catalytic domain contains the zinc binding motif (HEXXHXXGXXH, where H is histidine, E is glutamic acid, G is glycine, and X is any amino acid) (Bode, Gomis-Rüth, & Stöckler, 1993). These motifs are common structural signatures of MMPs, where the cysteine residue of the pro-domain coordinates to three histidine residues in the zinc binding motif of the catalytic domain. This cysteine-zinc coordination maintains the enzyme in an inactive state until removal of the pro-domain by proteolysis or destabilization renders the enzyme active. The catalytic domain also contains a conserved methionine, forming the “Met-turn” eight residues after the zinc binding motif. Located C-terminal to the Met-turn, the hemopexin domain facilitates substrate recognition (Hideaki Nagase et al., 2006; Paladini et al., 2013) (Figure 2).



**Figure 2. Domain structures of the MMP family.** sp, signal peptide; pro, pro-domain; cat, catalytic domain, FNII, fibronectin type II motif; L1, linker 1; Hpx, hemopexin domain; L2, linker 2; Mb, plasma membrane; TM, transmembrane domain; Cy, cytoplasmic tail; CysR, cysteine rich; Ig, immunoglobulin domain; GPI, glycosylphosphatidylinositol anchor (Hideaki Nagase et al., 2006).

MMPs are synthesized as pre-proenzymes and secreted from the cells as pro-MMP after removal of the signal peptide. Therefore, activation of MMPs is an important regulatory step of their activity. Removal of the propeptide by tissue and plasma proteinases, opportunistic bacterial proteinase, or other active MMPs renders the enzyme active (H. Nagase, Enghild, Suzuki, & Salvesen, 1990; Hideaki Nagase et al., 2006). Other pro-MMPs are activated intracellularly by proprotein convertases and secreted or cell surface-bound as active enzymes (Hideaki Nagase, 2001; Remacle, 2003).

In the laboratory setting, many MMPs are readily activated by treatment with sulfhydryl reagents, such as heavy metals, iodoacetamide, and mercury compounds (Galazka, Windsor, Birkedal-Hansen, & Engler, 1996). Activation of pro-MMP is believed to occur by dissociation of the sulfhydryl group of the cysteine from the zinc site and its replacement with a water molecule, as predicted by the cysteine switch model.

Pro-MMP can also be activated by detergents or chaotropic agents, which induce conformational changes in the polypeptide chain (Birkedal-Hansen & Taylor, 1982; Galazka et al., 1996; Stricklin, Jeffrey, Roswit, & Eisen, 1983). Pro-MMP can also be activated by limited cleavage of the propeptide by proteolytic enzymes, such as trypsin or chymotrypsin (Galazka et al., 1996; Okada, Gonoji, Nakanishi, Nagase, & Hayakawa, 1990; Stricklin et al., 1983). Moreover, MMP activities are regulated by two major types of endogenous inhibitors,  $\alpha_2$ -macroglobulin and tissue inhibitor of metalloproteinases (TIMPs).  $\alpha_2$ -Macroglobulin primarily regulates MMP activities in the fluid phase and inhibits most MMPs by entrapping the proteinase within the macroglobulin, after which the complex is cleared by endocytosis. TIMPs inhibit MMPs by binding to their active sites, chelating the catalytic zinc atom and rendering the enzyme inactive (Hideaki Nagase et al., 2006). Additionally, several MMP inhibitors have been discovered and investigated during the past years, such as Marimastat and sulfonamide-based hydroxamic acid derivatives (Bertini et al., 2004; Hanessian, Moitessier, Gauchet, & Viau, 2001; Jeng, Chou, & Parker, 1998).

MMPs are grouped into subfamilies based on domain organization and substrate preference, such as collagenases, gelatinases, stromelysins, matrilysins, and others. Collagenases are characterized by their abilities to cleave triple-helical interstitial collagen. This MMP subfamily includes MMP1 (interstitial collagenase/collagenase 1), MMP8 (neutrophil collagenase/collagenase 2), and MMP13 (collagenase 3). Each collagenase exhibits a preference for different collagen types. Based on kinetic studies of degradation of different collagen types from different species origins, human MMP1

prefers to digest human collagen types I and III rather than type II, whereas MMP13 degrades collagen type II more readily than MMP1 or MMP8 (Hasty, Jeffrey, Hibbs, & Welgus, 1987; Welgus, Jeffrey, & Eisen, 1981b). MMP1 degradation of a particular collagen type may depend on species (Hasty et al., 1987; Welgus et al., 1981b).

Collagenases catalyze the cleavage of interstitial collagen types I, II, and III at the peptide bond between Gly775 and Ile776 or Gly775 and Lys776 (Welgus, Jeffrey, & Eisen, 1981a). This cleavage results in the generation of the characteristic 3/4 and 1/4 fragments. These fragments denature into gelatin at physiologic temperature. Then, further breakdown of collagen is catalyzed by less specific proteinases (Van Doren, 2015).

### **Small Leucine-Rich Repeat Proteoglycans (SLRPs)**

Proteoglycans are composed of covalently linked diverse chains called glycosaminoglycans (GAGs) attached to serine residues of a protein core (Schaefer & Schaefer, 2010). GAGs are long unbranched polysaccharides of repeating units of disaccharide. The disaccharide unit mostly consists of N-acetylglucosamine or N-acetylgalactosamine attached to a uronic sugar or galactose (Esko, Kimata, & Lindahl, 2009). Owing to their polarity, GAGs enable proteoglycans to bind water and extend in conformation, conferring their hydrating and shock absorbing functions. In addition to GAGs, most proteoglycans also have N- and/or O-linked oligosaccharides (Kjuein & Lindahl, 1991). Proteoglycans are categorized by their relative size and the type of their GAG chains. The large proteoglycan family includes twenty-five members (Iozzo &

Schaefer, 2015). For example, aggrecan and perlecan are relatively large proteoglycans with multi-domain assemblies. On the other hand, certain proteoglycans with relatively smaller protein cores are considered members of the small leucine-rich repeat proteoglycans (SLRPs).

SLRPs are a family of proteins characterized by a core protein with multiple leucine-rich repeat (LRR) structural motifs covalently linked to glycosaminoglycan side chains and flanked by cysteine-rich clusters on the N- and C- termini (Geng, McQuillan, & Roughley, 2006; Merline, Schaefer, & Schaefer, 2009). Each LRR consists of 20-29 amino acids, commonly 24 residues, with a conserved eleven amino acid hallmark sequence of LxxLxLxxNxL, where L is leucine, or can be substituted by isoleucine, valine, and other hydrophobic amino acids, and x is any amino acid (McEwan, Scott, Bishop, & Bella, 2006). At the N-terminus, all SLRPs have many cysteine residues with class-conserved internal spacing (Chen & Birk, 2013). Another feature of SLRPs is the presence of a long laterally extending repeat at the C-terminus of certain classes, called 'ear repeat' (Chen & Birk, 2013).

SLRPs are now classified into five distinct families based on conservation and homology at genomic and protein levels and the presence of a characteristic Cys-rich cluster at the N-terminus of the core protein (Iozzo & Schaefer, 2015; Mochida et al., 2011; Tillgren, Ho, Önnarfjord, & Kalamajski, 2015) (Table 1). The SLRPs are closely related, but they differ in expression and acquired functions (Kalamajski & Oldberg, 2010).

Class I	Decorin Biglycan Asporin
Class II	Lumican Fibromodulin PRELP Keratocan Osteomodulin
Class III	Opticin Epiphycan Osteoglycin
Class IV	Chondroadrerin Chondroadherin-like (CHADL) Nyctalopin
	Nephrocan
	Podocan
	Podocan-like
	TSK

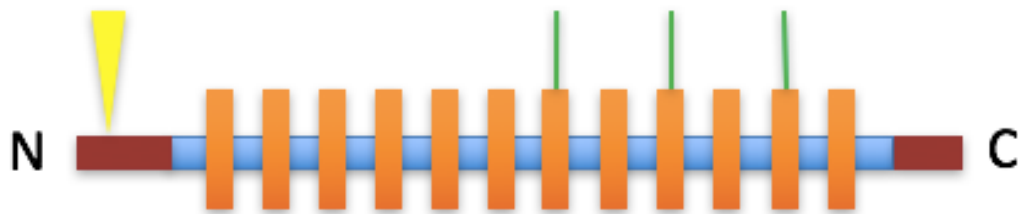
**Table 1. Classification of Mouse SLRP Members.** PRELP, proline/arginine-rich end leucine-rich repeat protein; TSK, tsukushi. Adopted from (Mochida et al., 2011; Tillgren et al., 2015).

One of the molecular functions of SLRPs is to modify the deposition and arrangement of collagen fibrils through their interaction in the ECM (Sylvester & Ratner, 2017). SLRPs also interact with different components of the ECM, leading to modulation of cellular functions. For example, decorin, biglycan, asporin, and fibromodulin bind to TGF- $\beta$  (Hildebrand et al., 1994). Also, biglycan is capable of binding to BMP-4 and modulates its activity (Nakajima et al., 2007).

Binding to collagen is a prominent feature of some SLRPs. Decorin is the most extensively studied member of the SLRP family and is well documented to bind to collagen type I (Svensson, Oldberg, & Heinegård, 2001; Vogel, Paulsson, & Heinegård, 1984). Moreover, as listed in Table 1, biglycan, asporin, fibromodulin, lumican, osteoglycin, and podocan were also reported to bind to collagen type I (Chen & Birk, 2013; Hedbom & Heinegard, 1989; Kalamajski & Oldberg, 2010; Rada, Cornuet, & Hassell, 1993).

Decorin is the most characterized member of the SLRPs. It belongs to class I of the SLRPs, with a defined N-terminal cysteine sequence (CX<sub>3</sub>CXCX<sub>6</sub>C, where C is cysteine, and X is any amino acid) that forms two disulfide bonds (Ameye & Young, 2002). The mature protein is highly conserved across species. Its core protein is around 45 kDa in size and is composed of twelve LRRs (numbered I-XII), each containing an average of twenty-four amino acid residues. The LRR region is flanked by disulfide-bonded terminal sequences from both sides. Close to the N-terminus, decorin is linked to a single GAG chain, chondroitin or dermatan sulfate, whereas the central domain contains three

attachment sites for N-linked oligosaccharides (Weber, Harrison, & Iozzo, 1996) (Figure 3).

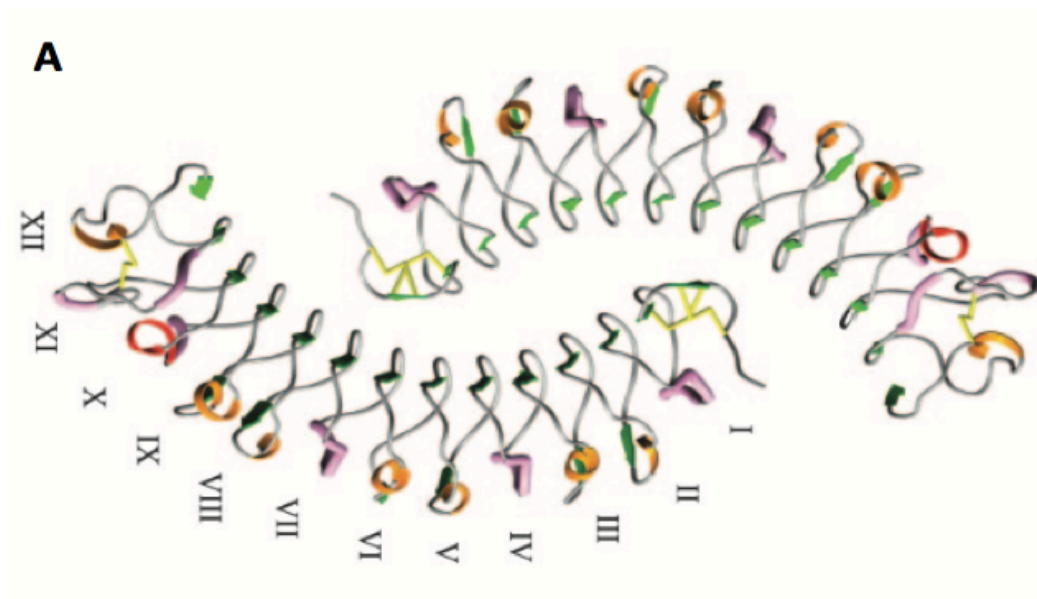


**Figure 3. Schematic diagram of decorin.** LRRs shown as orange boxes in the domain of the core protein, flanked by cysteine-rich clusters on both sides that can form disulfide bonds (red boxes). Decorin carries one dermatan sulfate or chondroitin sulfate glycosaminoglycan in the N-terminal region, marked by the yellow triangle. The green lines indicate potential N-linked glycosylation sites, Adapted from (Islam, 2014).

Based on the interaction of decorin core protein with other proteins in the ECM, several important functions have been attributed to decorin, including regulation of collagen fibrillogenesis. Decorin has a role in delaying fibril assembly and reducing fibril diameter (Chen & Birk, 2013; Kalamajski & Oldberg, 2010). Decorin has been shown to bind to different types of collagen, such as type I, II, III, VI and XIV (Bidanset, 1992; Douglas, 2006; Vogel, 1984).

The structural elements of decorin that bind to collagen type I have been studied. A previous study analyzed the decorin core protein, showing that neither the N-terminal half nor the central LRR repeats of decorin core protein can, by themselves, bind to

fibrillar collagen (Pogány & Vogel, 1992; Van Wart & Birkedal-Hansen, 1990). Another study reported that decorin binds to collagen type I primarily via LRR 4-5 (Svensson, Heinegard, & Oldberg, 1995), and a later report identified LRR 6 (Met-176 – Lys-201) to be important for the binding of decorin to collagen (Kresse, 1997). Recently, Kalamajeski et al demonstrated that LRR 5-6 of decorin, encompassing Arg-207 and Asp-210, is essential for collagen binding. Their data also showed that the Glu-181 residue, which was previously shown to be essential for collagen interaction, was not directly involved in the binding site, but its mutation might lead to mis-folding or structural changes in the collagen-binding site of the full-length decorin. These three amino acids, Arg-207, Asp-210, and Glu-181, were shown to be conserved in fifteen species, emphasizing their structural role and involvement in collagen binding (Kalamajski, Aspberg, & Oldberg, 2007). Moreover, in the same report by Kalamajeski, the binding region of decorin to collagen type I could be further localized to the specific sequence SYIRIADTNIT (SYI peptide) (Kalamajski et al., 2007) (Figure 4). In a solid-phase collagen binding assay, SYI peptide was reported to inhibit decorin-collagen interaction, whereas collagen-fibromodulin interaction was unaffected. Therefore, in this report, SYI peptide was documented to bind collagen and compete with the full length decorin for the same collagen binding site (Kalamajski et al., 2007).



**B**

	Sequence	Region
MKATLIFFLAQVSWA	1-16	signal peptide
GPFEQRGLDFDFMLEDEASGIIPYDPD	17-42	
NPLISMCPYRCQCHLRVVQCSDLGL	43-67	
DKVPWDFPPDPTLLDLQNNKI	68-88	LRR 1
TEIKEGAFKNLKDLHTLILVNNKI	89-112	LRR 2
SKISPEAFKPLVKLERLYLSKNQL	113-136	LRR 3
KELPEKMPRTLQELRVHENEI	137-157	LRR 4
TKLRKSDFNGLNNVLVIELGGNPL	158-181	LRR 5
KNSGIENGAFQGLKSLSYIRISDTNI	182-207	LRR 6
TAIPQGLPTSLETVHLDGNKI	208-228	LRR 7
TKVDAPSLKGLINLSKLGLSFNSI	229-252	LRR 8
TVMENGLANVPHLRELHLDNNKL	253-276	LRR 9
LRVPAGLAQHKYIQVVYLHNNNI	277-299	LRR 10
SAVGQNDFCRAGHPSRKASYSVSLYGNPV	300-329	LRR 11
RYWEIFPNTFRVCVYVRSAILGNYK	330-354	LRR 12

**Figure 4. The structure and amino acid sequence of decorin.** (A). A diagram of decorin LRR domain structure. The roman numbers correspond to the LRRs. Green arrows,  $\beta$ -strands; red ribbons,  $\alpha$ -helical turns; pink tubes, segments of polyproline II helix; orange ribbons, short segments of  $3_{10}$  helices and  $\beta$ -turns; yellow sticks, disulfide bonds (Scott et al, 2004). (B). Amino acid sequence of house mouse decorin with internal organization of LRRs. Yellow highlight, SYI peptide, Adapted from (Scott et al, 2004).

### **Collagen and Decorin Interaction**

The presence of decorin on the surface of the collagen fibrils was suggested to inhibit collagen fibril degradation by collagenases (Geng et al., 2006). It was suggested that the binding of decorin to the surface of collagen fibrils may form a barrier, possibly limiting the access of collagenase to its collagen cleaving site, which could lead to the protection of collagen by inhibiting its degradation (Geng et al., 2006). Since SYI peptide is derived from the collagen binding site of decorin, and its binding to collagen and competition with decorin at the same collagen binding site have been documented (Kalamajski et al., 2007), it is feasible that SYI peptide binding to collagen may also inhibit collagen degradation by collagenases. This inhibition of collagenase degradation could occur either by limiting the access of collagen degrading enzymes to their collagen cleavage site or by diverting these enzymes to degrade SYI peptide rather than the collagen molecule. Therefore, SYI peptide could modify the properties and biodegradability of collagen.

## **HYPOTHESIS**

Binding of SYI peptide to collagen may inhibit MMP1-mediated collagen type I degradation.

## **AIMS**

1. Investigate the effect of decorin-derived synthetic peptide SYIRIADTNIT in inhibiting MMP1-mediated collagen degradation in vitro.
2. Investigate the binding of decorin-derived synthetic peptide SYIRIADTNIT to collagen and determine its binding site(s) on collagen.
3. Investigate the effect of decorin-derived synthetic peptide SYIRIADTNIT on the activity of MMP1.

## **METHODS AND EXPERIMENTAL DESIGNS**

### **MMP1-Collagen Degradation Assay**

Pepsin-extracted acid-solubilized collagen type I (PureCol, Catalog #5005-100ML (formerly #5005-B), Advanced BioMatrix, San Diego, USA) was incubated in Tris buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, pH 7.5) at a concentration of 2 μM overnight at 4 °C to form collagen fibril solution. Four samples were prepared from the prepared collagen fibril solution.

MMP1 (R&D systems, 901-MP-010, Minneapolis, MN, USA) at a concentration of 541.5 nM was incubated in Tris buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, pH 7.5) containing 0.5 mM p-Aminophenylmercuric Acetate (APMA) for 30 min. at 37 °C for MMP1 activation. Immediately, the activated MMP1 was added at final concentrations of 38, 76, and 152 nM to the collagen samples, in a total volume of 80 μl per sample. The samples were incubated overnight at 37 °C. A sample of collagen only without MMP1 was included as a control.

For collagen cleavage analysis, SDS sample buffer was added to each sample. Then, 50 μl from each sample was loaded on SDS-PAGE NuPAGE 4-12% Bis-Tris gel (Invitrogen). To visualize proteins, including collagen and its degraded fragments, SDS-PAGE was stained with Coomassie Brilliant Blue (CBB).

### **The Effect of SYI Peptide on MMP1-Mediated Collagen Degradation**

Collagen type I fibril solution was formed by mixing 100  $\mu$ l collagen (3  $\mu$ g/ $\mu$ l in 0.01 M HCl) with Tris Buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, pH 7.5) overnight at 4 °C. Eight samples were prepared from the collagen fibril solution. The samples were incubated with various concentrations of SYI peptide or its scrambled peptide IYITDRNSTIA, 0.006, 0.02, 0.05, and 0.17 mM, overnight at 37 °C.

MMP1 was activated by incubation with 0.5 mM APMA in Tris buffer for 30 min. at 37 °C. Immediately, the activated MMP1 was added to the samples to a final MMP1 concentration of 76 nM. A control sample of collagen was incubated with MMP1 without previous incubation with any peptide. The other control of collagen was incubated alone without incubation with neither peptide nor MMP1. The samples were incubated overnight at 37 °C.

For collagen cleavage analysis, SDS sample buffer was added to each sample. Then, 50  $\mu$ l from each sample was loaded on SDS-PAGE NuPAGE 4-12% Bis-Tris gel. To visualize proteins, including collagen and its degraded fragments, SDS-PAGE was stained with CBB.

The non-degraded collagen in each sample, including alpha-1 chain, alpha-2 chain, and the multi-form collagen, was quantified using ImageJ software program as shown in Figure 6 B. The intensity of non-degraded collagen obtained from the control sample, in which collagen alone was loaded on SDS-PAGE was considered as the total collagen. The percentage of non-degraded collagen to the total amount of collagen was

calculated for each sample. The percentage of non-degraded collagen for the samples were compared to evaluate the extent of collagen degradation.

The same experimental procedure was performed using higher SYI peptide concentrations, 0.25, 0.5, 1, 2 mM.

The synthetic SYI peptide (SYIRIADTNIT) was obtained from Peptide 2.0, Inc. The scrambled peptide (IYITDRNSTIA) was designed using Peptide 2.0 software program and obtained from Peptide 2.0, Inc.

### **Biotin Affinity Chromatography to Identify the Collagen Sequence that Binds SYI Peptide Using MMP1 and Trypsin as Collagen Degrading Enzymes**

#### *Biotin Affinity Chromatography to Identify Non-Degraded Collagen that Binds Biotin-SYI Peptide (A Control Experiment)*

Sreptavidin sepharose packed columns were used to separate the non-degraded collagen that binds biotin-SYI peptide. The experimental column was equilibrated by adding 400  $\mu$ l binding buffer, TBS (50 mM Tris, 150 mM NaCl, pH 7.5) and centrifuged for 1 min. at 150 rcf. These steps were repeated three times. Immediately after the equilibration, 360  $\mu$ g biotin-SYI peptide dissolved in 30% dimethyl sulfoxide (DMSO) (200  $\mu$ l) was added to the column and incubated with slow end-over-end shaking for 30 min. at room temperature. Then, unbound biotin-SYI peptide was removed by centrifuging for 1 min. at 150 rcf. The column was washed three times with 30% DMSO, followed by washing three times with the binding buffer, TBS (pH 7.5). After washing,

200  $\mu$ l of the collagen fibril solution (2  $\mu$ M), prepared as described above, was added to the column and incubated with rotating overnight at 37 °C. The unbound collagen was removed by centrifugation for 1 min. at 150 rcf. The flow-through was collected. Then, the column was washed with 400  $\mu$ l of the washing buffer (TBS, 2 M urea, pH 7.5) for four times, and the flow-through was collected from each wash separately. After washing, the column was eluted with 200  $\mu$ l of the elution buffer (0.1 M glycine, 2 M urea, pH 2.9, according to the manufacturer's protocol) and centrifuged for 1 min. at 1,000 rcf. The elution was performed three times and the elution solution was collected separately each time. The same proportions of the unbound collagen flow-through, the washing flow-through, and the elution solutions were loaded on SDS-PAGE. To visualize proteins, including collagen, SDS-PAGE was stained with CBB. Two other columns were used as controls, prepared as follows:

- A column loaded with the collagen fibril solution only, without any previous peptide loading.
- A column loaded with biotin-scrambled peptide instead of biotin-SYI peptide and then with the prepared collagen fibril solution.

Both control columns were centrifuged, washed, and eluted in the same manner as the experimental column.

*Biotin Affinity Chromatography to Identify the Collagen Sequence that Binds Biotin-SYI Peptide Using MMP1 as a Collagen-Degrading Enzyme*

*Preparation of collagen fibril solution:* The collagen fibril solution was prepared as described above in the MMP1-mediated collagen degradation assay with 2  $\mu$ M collagen.

*Preparation of the MMP1-mediated collagen degradation solution:* MMP1 was activated by incubating with 0.5 mM APMA in Tris buffer for 30 min. at 37 °C. Immediately following activation, MMP1 was added to the collagen fibril solution and incubated overnight at 37 °C.

*Separation of the MMP1-degraded collagen fragment that binds biotin-SYI peptide:* Streptavidin sepharose packed columns were used to separate the collagen sequence that binds biotin-SYI peptide. The experimental column was equilibrated and loaded with biotin-SYI peptide in the same manner as described previously in the control experiment (page 21). Then, 200  $\mu$ l of the prepared MMP1-degraded collagen solution was added to the column and incubated with shaking overnight at 37 °C. The unbound collagen was removed by centrifuging for 1 min at 150 rcf. The flow-through was collected. Then, the column was washed with 400  $\mu$ l of the washing buffer (TBS, 2 M urea, pH 7.5) three times, and the flow-through was collected separately each time. After washing, the column was eluted with 200  $\mu$ l of the elution buffer (0.1 M glycine, 2 M urea, pH 2.9) and centrifuged for 1 min. at 1,000 rcf. The elution was performed three times and the elution solution was collected each time separately. The same proportions

of the unbound collagen flow-through, the washing flow-through, and the elution solutions were loaded on SDS-PAGE and stained with CBB.

Two more columns were used as controls, described as follows:

- A column loaded with the MMP1-degraded collagen solution only.
- A column loaded with biotin-scrambled peptide instead of biotin-SYI peptide and then with the prepared MMP1-degraded collagen solution.

Both control columns were centrifuged, washed, and eluted in the same manner as the experimental column.

*Biotin Affinity Chromatography to Identify the Collagen Sequence that Binds Biotin-SYI Peptide Using Trypsin as a Collagen Degrading Enzyme*

*Degradation of collagen with trypsin:* In order to optimize the collagen to trypsin ratio, non-fibrillized collagen solution, which likely contains monomer collagen molecule, was incubated with various concentrations of trypsin (Promega), 0.43, 0.86, and 1.72  $\mu\text{M}$ , in 50 mM Tris buffer. Non-fibrillized collagen was used as trypsin is not a physiological enzyme for collagen and does not require the presence of collagen fibril form to degrade it. The final concentration of collagen was 1  $\mu\text{M}$  per sample. The samples were incubated overnight at 37 °C. A sample of collagen only without trypsin was included as a control.

SDS sample buffer was added to each sample. Then, 50  $\mu\text{l}$  from each sample was loaded on SDS-PAGE. To visualize collagen and its degraded fragments, SDS-PAGE was stained with CBB.

*Preparation of trypsin-degraded collagen solution:* Trypsin-digested collagen solution was prepared for the affinity chromatography experiment by incubating non-fibrillized collagen with trypsin at a ratio of 20:1 in 50 mM Tris buffer overnight at 37 °C. After the incubation, trypsin activity was inhibited by adding 100 µM Tosyl-L-lysyl-chloromethane hydrochloride (TLCK) (Abcam) and incubating the solution for one hour at room temperature.

*Separation of the trypsin-degraded collagen fragment that binds biotin-SYI peptide:* The same experimental procedure was performed as described for the separation of the MMP1-degraded collagen fragment that binds biotin-SYI peptide except that the column was loaded with trypsin-degraded collagen solution instead of MMP1-degraded collagen solution.

Four columns were used as controls, described as follows:

- A column loaded with biotin-SYI peptide and then with the non-fibrillized collagen solution.
- A column loaded with the non-fibrillized collagen solution only.
- A column loaded with the trypsin-digested collagen solution only.
- A column loaded with biotin-scrambled peptide instead of biotin-SYI peptide and then with the prepared trypsin-digested collagen solution.
- A column loaded with biotin-SYI peptide and then with trypsin alone, without collagen.

All control columns were centrifuged, washed, and eluted in the same manner as the experimental column.

### **MMP1 Activity Assay**

The activity of MMP1 was measured using a MMP1 inhibitor screening kit (Abcam), which utilizes a thiopeptide as a chromogenic substrate. Hydrolysis of the thiopeptide by MMP1 produces a sulfhydryl group, which reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form 2-nitro-5-thiobenzoic acid, which can be detected by its absorbance at 420 nm. The assay was performed using a 96-well microplate. SYI peptide at concentrations of 0.023, 0.07, 0.23, and 0.7 mM, dissolved in urea, were incubated with 20  $\mu$ l MMP1 (153 mU/ $\mu$ l) in the assay reaction buffer. MMP1 inhibitor, N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH), was incubated with MMP1 as a positive control at a final concentration of 1.3  $\mu$ M. Another control was incubating MMP1 with neither SYI peptide nor MMP1 inhibitor. The volume was adjusted to 90  $\mu$ l per well. The urea concentration was 2 M in each sample. The microplate was incubated for 30 min. at 37 °C to allow peptide or inhibitor/MMP1 interaction. Then, the reaction was started by addition of the thiopeptide substrate (10  $\mu$ l). The final concentration of the thiopeptide substrate was 100  $\mu$ M per well. The optical densities (ODs) of the samples were recorded at 420 nm every 3 minutes for 30 minutes. A standard enzyme progress curve was prepared based on the OD and time according to the manufacturer's protocol.

### *Statistical analysis*

One-way ANOVA with Tukey post-hoc test was used to evaluate the statistical significance between the samples. P value with less than 0.05 was considered significant.

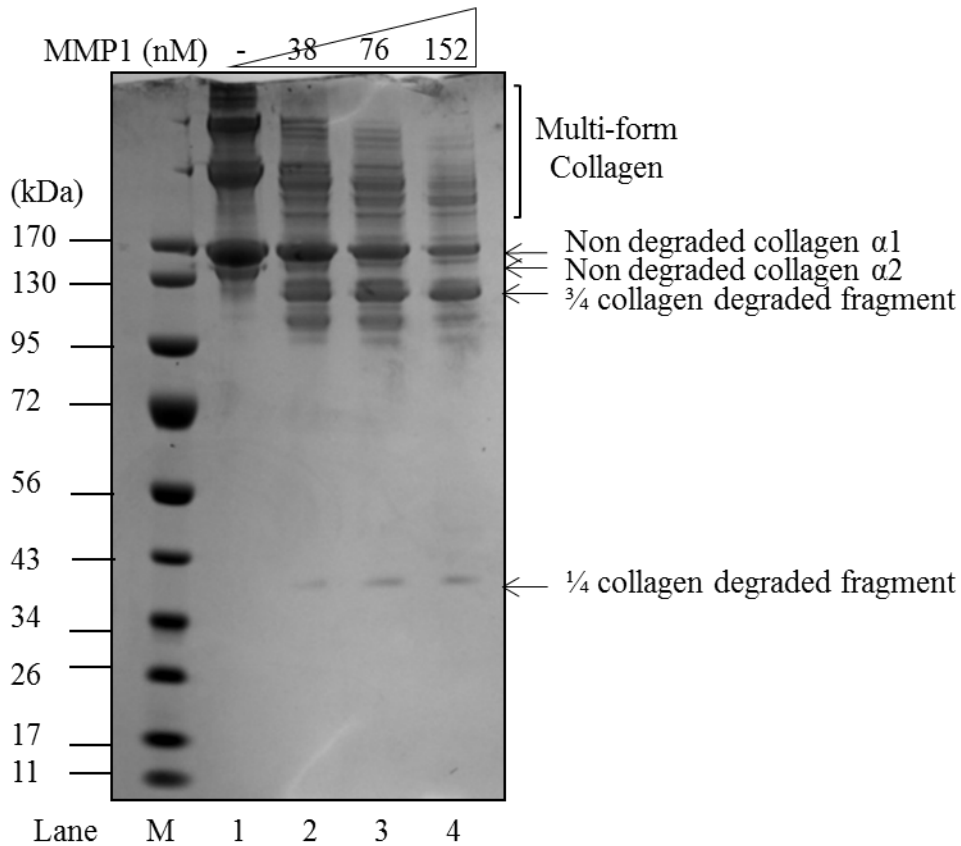
## RESULTS

### **The Effect of SYI Peptide on MMP1-Mediated Collagen Degradation**

Collagen type I was used in the current study because it is the major protein in bone and dentin. In mammals, collagenases include MMP1, MMP8, and MMP13. Previous report which investigated the effect of decorin on collagen degradation by collagenases mainly used MMP1 (Geng et al., 2006); therefore, MMP1 was used in the current study.

In order to optimize the collagen type I to MMP1 ratio that would be used in the subsequent experiments, 2  $\mu$ M collagen type I samples were incubated with various concentrations of MMP1 (38, 76, and 152 nM). Then, the samples were applied to SDS-PAGE. To visualize proteins, including collagen, SDS-PAGE was stained with CBB (Figure 5). Collagen alone, without MMP1, served as a control. Two positively stained bands were observed at around 150 kDa and 140 kDa, corresponding to collagen alpha-1 and alpha-2 chains, respectively. Positively stained bands migrating higher than 150 kDa were also observed, corresponding to  $\alpha$ -dimeric and  $\alpha$ -trimeric forms of collagen. When collagen type I samples were incubated with various concentrations of MMP1, positively stained bands were observed at around 127 kDa and 40 kDa (Figure 5, lanes 2-4), corresponding to the three quarters and one quarter collagen degraded fragments. As the MMP1 concentration was increased, the amount of non-degraded collagen appeared to decrease and that of the degraded collagen fragments appeared to increase. In the absence of MMP1 incubated with collagen, only positively stained bands corresponding to alpha-1, alpha-2, and multi-form collagen were observed (Figure 5, lane 1). Therefore, the

results indicate that MMP1 appears to degrade collagen type I in a dose-dependent manner. Incubation of collagen type I with 76 nM MMP1 (Figure 5, lane 3) was selected for subsequent experiments because the amount of MMP1 appeared to be sufficient for collagen degradation. The collagen to MMP1 molar ratio used in this sample was calculated to be 1 collagen to  $0.37 \times 10^{-3}$  MMP1.



**Figure 5. MMP1 collagen degradation assay.** Collagen fibril solution was incubated with various concentrations of APMA-activated MMP1. The CBB staining demonstrated that MMP1 appears to degrade collagen type I in a dose-dependent manner. The non-degraded collagen, including alpha-1, alpha-2, and multi-form (indicated by arrows and a bracket), appeared to be decreased and the degraded three quarters and one quarter collagen fragments (indicated by arrows) appeared to be increased as MMP1 concentration was increased. The control sample of collagen without MMP1 (lane 1) showed only non-degraded collagen. This experiment was performed three times ( $n = 3$ ) and a representative image was shown.

It has been reported that recombinant decorin core protein inhibited MMP1-mediated collagen type I degradation (Geng et al., 2006). SYI peptide is a synthetic peptide derived from decorin binding sites for collagen type I. It was demonstrated that

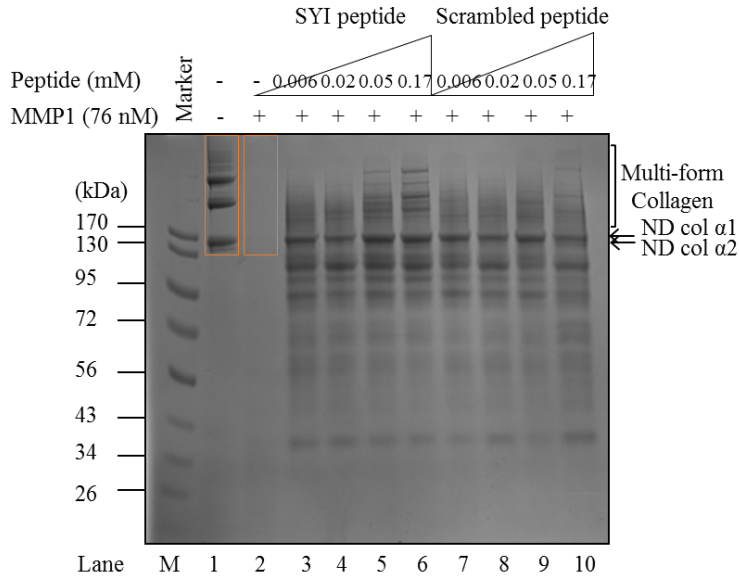
SYI peptide bound to collagen type I and competed with decorin core protein for the same collagen binding site (Kalamajski et al., 2007), the dose-dependent effect of SYI peptide on MMP1-mediated collagen type I degradation was investigated. Samples of 2  $\mu$ M collagen type I were incubated with various concentrations of SYI peptide or its scrambled peptide (0.006, 0.02, 0.05, and 0.17 mM). After incubation of collagen with each peptide overnight, the samples were further incubated with 76 nM of MMP1 to allow the degradation of collagen. The samples were then loaded on SDS-PAGE. To visualize proteins, including collagen and its degradation fragments, SDS-PAGE was stained with CBB (Figure 6 A). The non-degraded collagen in each sample, including alpha-1 chain, alpha-2 chain, and the multi-form collagen, was quantified using ImageJ software program as shown in Figure 6 B. The intensity of non-degraded collagen obtained from the control sample, in which collagen alone was loaded on SDS-PAGE (Figure 6 A, lane 1), was considered as the total collagen. The percentage of the non-degraded collagen to the total amount of collagen was calculated for each sample. The percentage of non-degraded collagen for the samples were compared to evaluate the extent of collagen degradation. Statistical analysis was performed using one-way ANOVA. The results showed that only non-degraded collagen bands were observed in the absence of MMP1 incubated with collagen (Figure 6 A, lane 1). There were no collagen bands detected when collagen was treated with MMP1 in the absence of SYI peptide (Figure 6 A, lane 2), suggesting that collagen was completely degraded by MMP1. Non-degraded collagen including alpha-1, alpha-2, and multi-form was detected when collagen was incubated with various concentrations of SYI peptide and further

treated with MMP1 (Figure 6 A, lane 3-6). As SYI peptide concentration was increased, the amount of non-degraded collagen appeared to be increased. Non-degraded collagen including alpha-1, alpha-2, and multi-form was also detected when collagen was incubated with various concentrations of the scrambled peptide and further treated with MMP1 (Figure 6 A, lane 7-10). Although the concentration of the scrambled peptide was increased, the amount of non-degraded collagen did not appear to be increased. The percentage of non-degraded collagen was compared between samples with SYI peptide (Figure 6 A, lanes 3-6) and that without any peptide (Figure 6 A, lane 2). There was no statistical difference between the percentage of non-degraded collagen in the presence of SYI peptide and that in the absence of any peptides (Figure 6 C). There was no statistical difference of the percentage of non-degraded collagen among samples at any SYI peptide concentration (Figure 6 C). Additionally, the percentage of non-degraded collagen was compared between samples with SYI peptide (Figure 6 A, lane 3-6) and those with the scrambled peptide (Figure 6 A, lane 7-10). The results showed that there was no statistical difference between the percentage of non-degraded collagen in the presence of SYI peptide and that in the presence of the scrambled peptide at any peptide concentration tested (Figure 6 C). In conclusion, the data demonstrated that there was no specific effect of SYI peptide on MMP1-mediated collagen degradation.

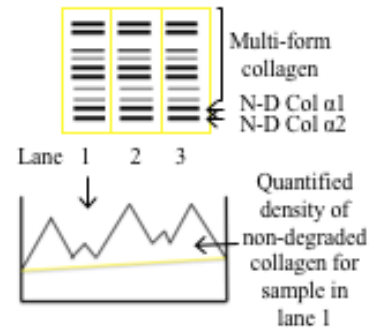
The scrambled peptide was made based on Peptide2.0 software program. The function of the scrambled peptide needs to be verified by a specific biological assay. The results showed that non-degraded collagen including alpha-1, alpha-2, and multi-form was detected when collagen was incubated with various concentrations of the scrambled

peptide and further treated with MMP1 (Figure 6 A, lanes 7-10). The percentage of non-degraded collagen was compared between samples with the scrambled peptide (Figure 6 A, lanes 7-10) and that without any peptides (Figure 6 A, lane 2). There was no statistical difference between the percentage of non-degraded collagen in the presence of the scrambled peptide and that in the absence of any peptides (Figure 6 C). Although there was no statistical difference, incubation of collagen with the scrambled peptide appeared to inhibit the collagen degradation to some extent as compared to the degradation of collagen in absence of any peptides (Figure 6 A, lane 2). The scrambled peptide did not seem to function as a control peptide for SYI peptide in this assay.

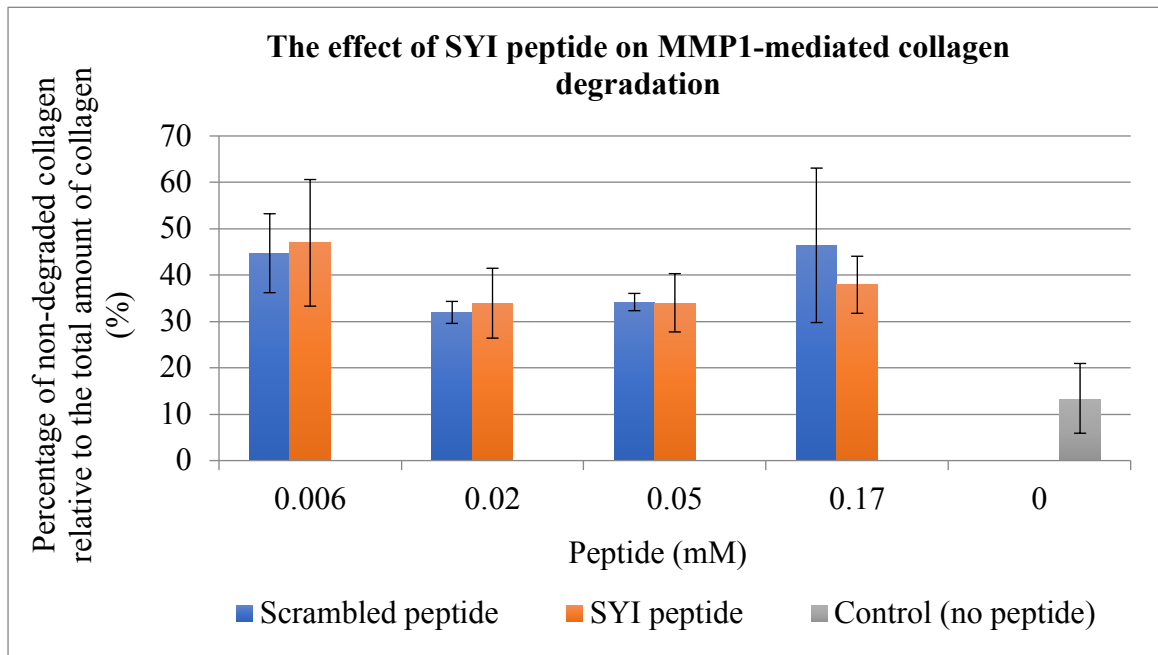
A



B



C



**Figure 6. The effect of the SYI peptide and its scrambled peptide at lower concentrations on MMP1-mediated collagen degradation.** (A). Samples of collagen fibril solution were incubated with various concentrations of SYI peptide and the scrambled peptide. APMA-activated MMP1 was then added to the samples. Samples

containing collagen only and collagen with MMP1 in the absence of any peptides were prepared as controls. The red box indicates non-degraded collagen that was quantified to compare the extent of collagen degradation. This experiment was performed three times ( $n = 3$ ), and a representative image was shown. (B). Schematic image demonstrating the method to quantify the intensity of non-degraded collagen, including alpha-1 (N-D col  $\alpha 1$ ), alpha-2 (N-D col  $\alpha 2$ ), and multi-forms of collagen, using Image J software. (C). A bar graph demonstrating the percentage of non-degraded collagen relative to the total amount of collagen for collagen samples incubated with various SYI peptide concentrations, incubated with the same concentrations of the scrambled peptide, and the control sample in which collagen was not incubated with any peptide. The values are shown as the mean  $\pm$  S.D. Statistical analysis was performed and there was no statistical difference between the percentage of non-degraded collagen in the presence of SYI peptide and that in the absence of any peptides ( $P$  value  $< 0.05$ ). There was also no statistical difference of the percentage of non-degraded collagen among samples at any SYI peptide concentration ( $P$  value  $< 0.05$ ). There was also no statistical difference between the percentage of non-degraded collagen in the presence of SYI peptide and that in the presence of the scrambled peptide at any peptide concentration tested ( $P$  value  $< 0.05$ ).

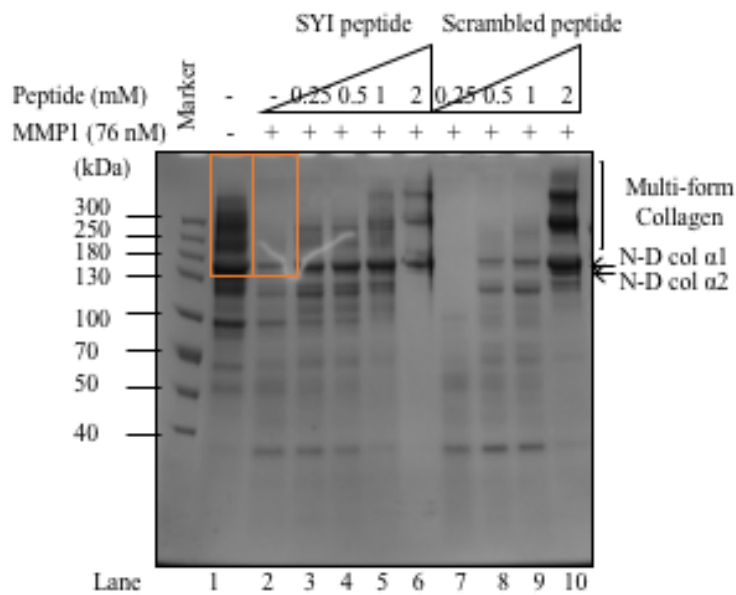
As the previous results showed no effect of SYI peptide on MMP1-mediated collagen degradation, the effect of higher concentrations of SYI peptide on MMP1-mediated collagen degradation was investigated. Samples of 2  $\mu$ M collagen type I were incubated with various concentrations of SYI peptide or its scrambled peptide (0.25, 0.5, 1, and 2 mM). After incubation of collagen with each peptide overnight, the samples were further incubated with 76 nM of MMP1 and visualized with CBB in the same manner as previously described (Figure 7 A). The non-degraded collagen in each sample, was quantified in the same manner as in Figure 6 B, and the percentage of the non-degraded collagen to the total amount of collagen was calculated and compared to evaluate the extent of collagen degradation. The results showed high level of non-degraded collagen bands in the absence of MMP1 incubated with collagen (Figure 7, lane

1). The level of non-degraded collagen bands was reduced when collagen was treated with MMP1 in the absence of SYI peptide (Figure 7 A, lane 2) as compared to collagen not treated with MMP1 (Figure 7, lane 1), suggesting that most collagen was degraded by MMP1. The level of non-degraded collagen bands appeared to be increased when collagen was incubated with various concentrations of SYI peptide and further treated with MMP1 (Figure 7 A, lane 3-6), suggesting that SYI peptide seemed to inhibit collagen degradation by MMP1. As SYI peptide concentration was increased, the extent of non-degraded collagen appeared to be increased. The percentage of non-degraded collagen was compared between the samples with SYI peptide (Figure 7 A, lanes 3-6) and that without any peptides (Figure 7 A, lane 2). There was no statistical difference between the percentage of non-degraded collagen in the presence of SYI peptide and that in the absence of any peptides (Figure 7 B). There was no statistical difference of the percentage of non-degraded collagen among samples at any SYI peptide concentration tested (Figure 7 B). In conclusion, the data demonstrated that there was no effect of higher concentrations of SYI peptide on MMP1-mediated collagen degradation.

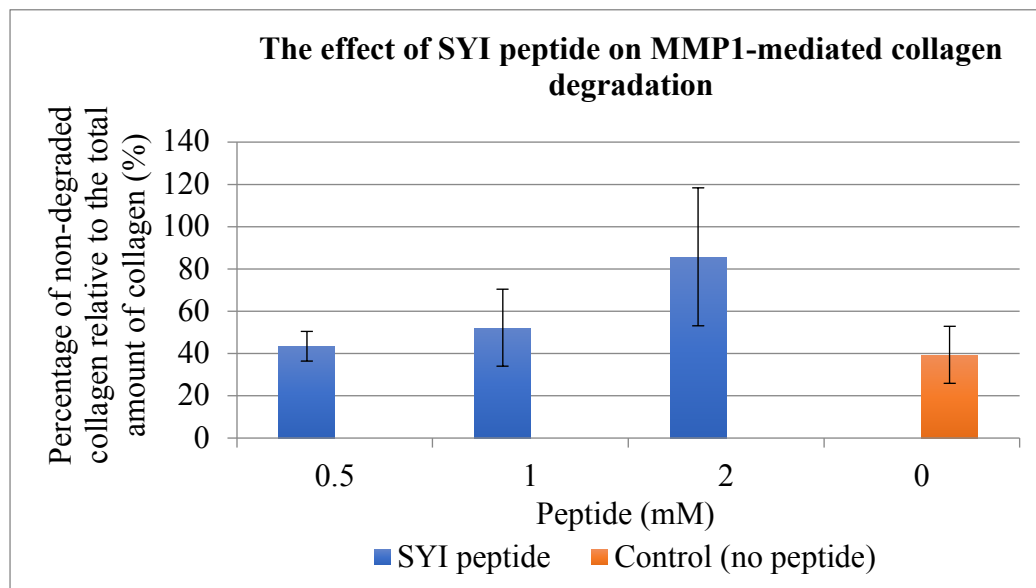
The results showed that non-degraded collagen was not detected when collagen was incubated with the least concentration of the scrambled peptide (0.25 mM) and further degraded by MMP1 (Figure 7 A, lane 7). The extent of non-degraded collagen appeared to be almost at a similar level when collagen was incubated with 0.5 or 1 mM of the scrambled peptide (Figure 7 A, lanes 8 and 9). When collagen was incubated with 2 mM of the scrambled peptide (Figure 7 A, lane 10), the extent of non-degraded collagen appeared to be greater than that when collagen was incubated without any peptides

(Figure 7 A, lane 2). Statistical analysis was not performed because the experiment with the scrambled peptide was done only one time.

A



B



**Figure 7. The effect of higher concentrations of SYI and its scrambled peptide on MMP1-mediated collagen degradation.** (A). Samples of collagen fibril solution were incubated with higher concentrations of SYI peptide and the scrambled peptide. APMA-activated MMP1 was then added to the samples. Samples containing collagen only and collagen with MMP1 in the absence of any peptides were prepared as controls. The red box indicates non-degraded collagen that was quantified to compare the extent of collagen degradation. N-D col  $\alpha$ 1, non-degraded collagen alpha-1; N-D col  $\alpha$ 2, non-degraded collagen alpha-2. This experiment was performed three times ( $n = 3$ ), and a representative image was shown. (B). A bar graph demonstrating the percentage of non-degraded collagen relative to the total amount of collagen for samples in which collagen was incubated with various SYI peptide concentrations and the control sample in which collagen was not incubated with any peptide. The values are shown as the mean  $\pm$  S.D. Statistical analysis was performed and there was no statistical difference between the percentage of non-degraded collagen in the presence of SYI peptide and that in the absence of any peptides ( $P$  value  $< 0.05$ ). There was also no statistical difference of the percentage of non-degraded collagen among samples with any SYI peptide concentration tested ( $P$  value  $< 0.05$ ). Statistical analysis was not performed because the experiment with the scrambled peptide was done only one time.

### **Biotin Affinity Chromatography to Identify Collagen Sequence that Binds SYI Peptide**

Our previous results (Figures 6 and 7) demonstrated no inhibitory effect of SYI peptide on MMP1-mediated collagen degradation. In this set of experiments the aim was to investigate SYI peptide binding to collagen.

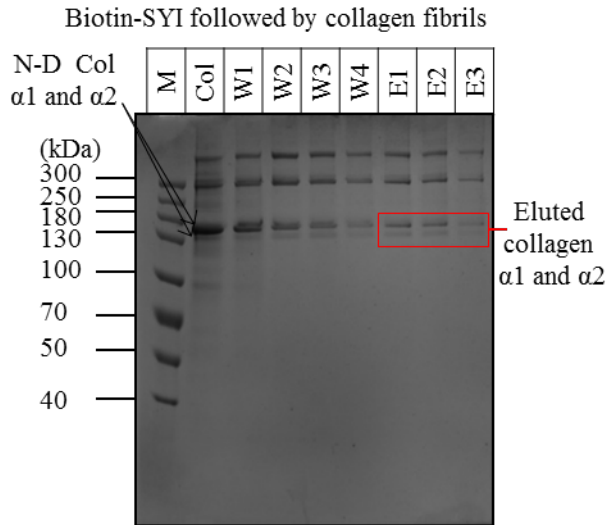
Biotin affinity chromatography was used to identify whether SYI peptide binds to collagen and to detect the collagen fragment that binds SYI peptide. Subsequently, the amino acid sequence of the collagen fragment was planned to be identified by mass spectrometry.

A control experiment was done initially to evaluate the binding of biotin-SYI peptide to non-degraded collagen fibril solution in streptavidin sepharose packed

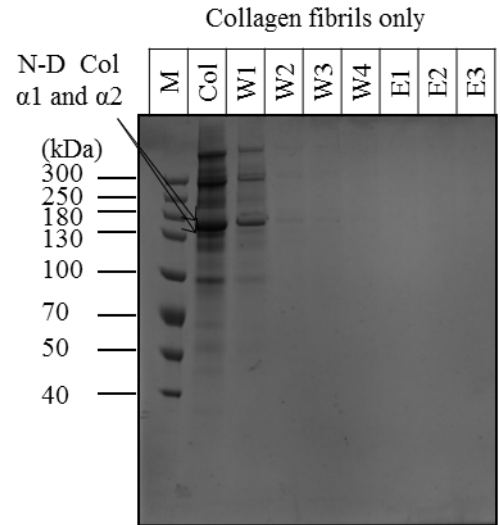
columns. First, the column was loaded with biotin-SYI peptide to allow the biotin and streptavidin binding. Then, non-degraded collagen fibril solution was loaded on the column to allow biotin-SYI peptide and collagen binding (Figure 8 A). Subsequently, the column was centrifuged and the collagen flow-through was collected (Figure 8 A, col), followed by washing the column four times and collecting the flow-through each time the column was washed (Figure 8 A, W1-W4). Finally, the column was eluted three times, collecting the elution each time (Figure 8 A, E1-E3). Two control columns were used in this experiment - a column loaded with collagen fibril solution alone (Figure 8 B) and a column loaded with biotin-scrambled peptide instead of biotin-SYI peptide (Figure 8 C). The same proportional amount of the collagen flow-through, the washing flow-through, and the elution were loaded on SDS-PAGE. To visualize proteins, including collagen, SDS-PAGE was stained with CBB. The results showed that non-degraded collagen alpha 1 and alpha 2 were detected when biotin-SYI peptide and non-degraded collagen fibrils solution were used (Figure 8 A, E1-E3), indicating that biotin-SYI peptide bind to collagen fibrils. There were no collagen bands observed in the elution of the control column loaded with non-degraded collagen only (Figure 8 B, E1-E3), thus confirming the specificity of biotin-SYI peptide and collagen binding observed. There were non-degraded collagen alpha 1 and alpha 2 bands observed in the elution of the control column loaded with biotin-scrambled peptide (Figure 8 C, E1-E3). However, the amount of collagen in the elution appeared to be less than the amount of collagen observed in the washing flow-through from this column (Figure 8 C, W1-W4). Therefore, the collagen bands observed in the elution of this column suggest lower peptide/collagen binding

compared to SYI peptide. These data indicate that non-degraded collagen fibrils bind to biotin-SYI peptide, and could be separated and visualized with a low degree of specificity using the streptavidin sepharose packed columns.

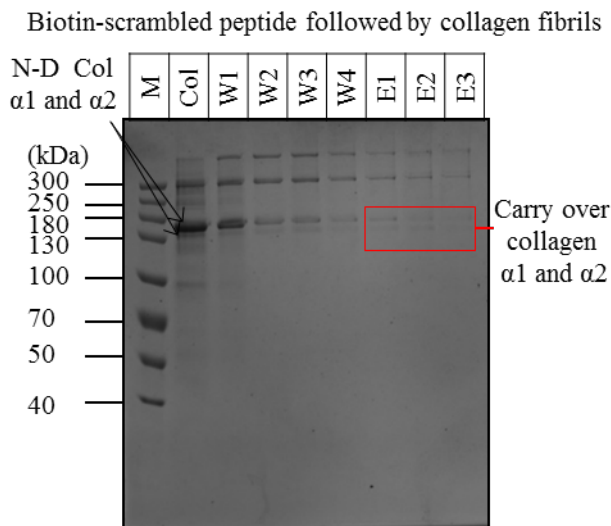
A



B



C

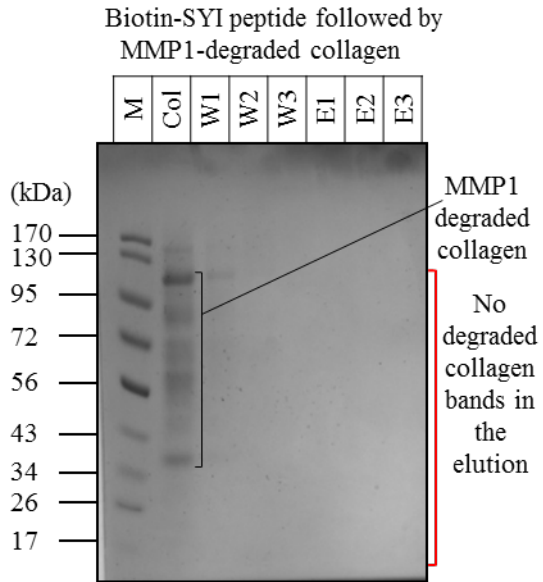


**Figure 8. Biotin affinity chromatography to identify non-degraded collagen fibril that binds to SYI peptide.** (A). A column was loaded with biotin-SYI peptide, then with collagen fibril solution. The column was centrifuged and non-bound collagen flow-through was collected. Then, the column was washed four times with the washing buffer, and eluted three times with 0.1 M glycine, 2 M urea, pH 2.9. The washing flow-through was collected from each washing time. The same proportion of the unbound collagen flow-through, each washing flow-through, and each elution was loaded on SDS-PAGE. SDS-PAGE was stained with CBB. M, marker; Col, flow-through of unbound collagen; W1, flow-through of the first washing; W2, flow-through of the second washing; W3,

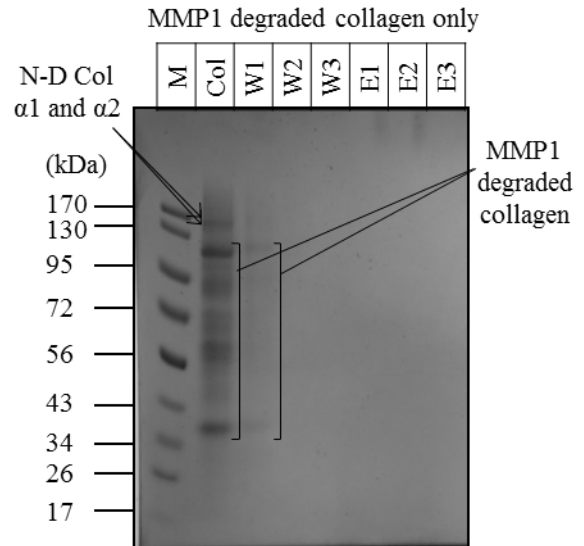
flow-through of the third washing; W4, flow-through of the fourth washing; E1, the first elution; E2, the second elution; E3, the third elution. (B). A control column loaded with collagen fibril solution only. (C). A control column loaded with biotin-scrambled peptide instead of biotin-SYI peptide and then with collagen fibril solution. All experiments in A, B, and C were performed three times ( $n = 3$ ), and representative images were shown.

In order to identify the collagen sequence that binds to SYI peptide, biotin affinity chromatography was first used to separate the MMP1-degraded collagen fragment that SYI peptide binds to. Biotin-SYI peptide was loaded on the column to allow streptavidin and biotin-SYI peptide binding. Then, MMP1-degraded collagen solution was incubated in the column to allow biotin-SYI peptide and collagen fragment binding. Subsequently, the column was centrifuged, washed, and eluted, and applied on SDS-PAGE in the same manner as described above (Figure 9 A). Two control columns were used in this experiment - a column loaded with MMP1-degraded collagen solution only (Figure 9 B) and a column loaded with biotin-scrambled peptide instead of biotin-SYI peptide (Figure 9 C). The results showed that no positively stained bands were observed when the column was loaded with biotin-SYI peptide and then with MMP1-degraded collagen solution (Figure 9 A, E1-E2). There were no positively stained bands observed in the elution of the two control columns, i.e. the column loaded with MMP1-degraded collagen solution only (Figure 9 B, E1-E3) and the column loaded with biotin-scrambled peptide (Figure 9 C, E1-E2). In conclusion, when MMP1 was used as a collagen-degrading enzyme, the collagen fragment that biotin-SYI peptide binds to was not detected using streptavidin sepharose packed columns.

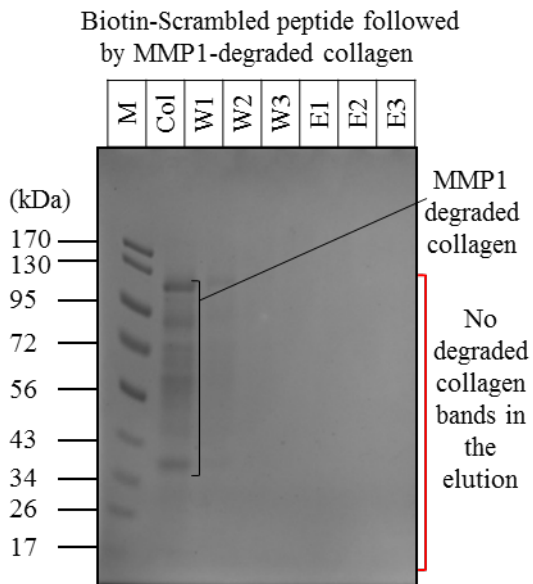
A



B



C

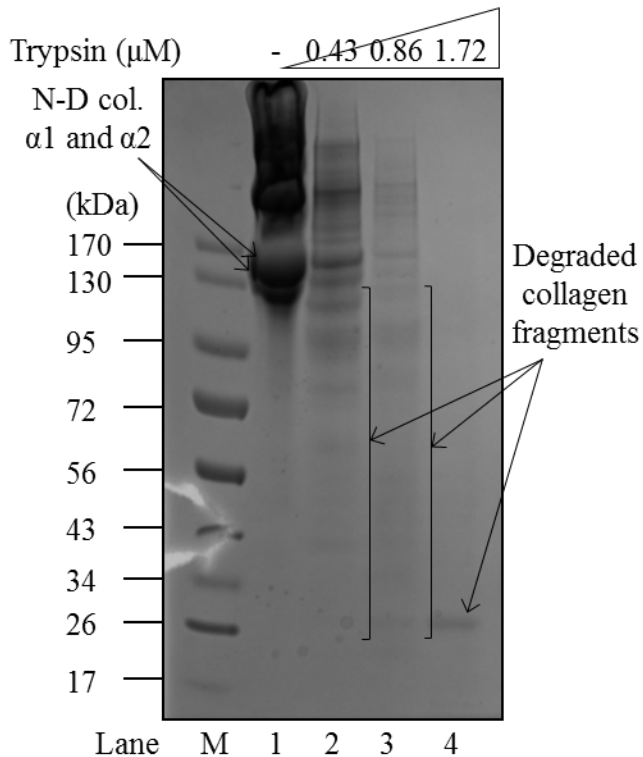


**Figure 9. Biotin affinity chromatography to identify the collagen fragment that binds to SYI peptide using MMP1 as a collagen-degrading enzyme. (A).** A column loaded with biotin-SYI peptide then with MMP1-degraded collagen solution. The column was centrifuged and non-bound collagen flow-through was collected. Then, the column was washed three times with the washing buffer, and eluted three times with 0.1 M glycine, 2 M urea, pH 2.9. The washing flow-through was collected from each washing

time. The same proportion of the unbound collagen flow-through, each washing flow-through, and each elution was loaded on SDS-PAGE. SDS-PAGE was stained with CBB. M, marker; Col, flow-through of unbound collagen fragment; W1, flow-through of the first washing; W2, flow-through of the second washing; W3, flow-through of the third washing; E1, the first elution; E2, the second elution; E3, the third elution. (B). A control column loaded with MMP1-degraded collagen solution only. (C). A control column loaded with biotin-scrambled peptide instead of biotin-SYI peptide and then with MMP1-degraded collagen solution. All experiments in A, B, and C were performed two times ( $n = 2$ ), and representative images were shown.

As we could not detect any MMP1-degraded collagen fragments that biotin-SYI peptide binds to, degrading collagen by another enzyme, producing different collagen fragments, may allow the binding of the degraded collagen fragments to SYI peptide and subsequent separation by biotin affinity chromatography. Trypsin was used as an alternative collagen-degrading enzyme. First, collagen to trypsin ratio was optimized by incubating samples of collagen type I with various concentrations of trypsin (0.43, 0.86, and 1.72  $\mu\text{M}$ ). These concentrations were selected based on previous studies in the literature (Dung et al, 1994). Collagen alone without trypsin served as a control. Then, the samples were loaded on SDS-PAGE. To visualize proteins, including collagen and its degraded fragments, SDS-PAGE was stained with CBB (Figure 10). Collagen alpha-1 chain, collagen alpha-2 chain, and multi-form collagen bands were detected when collagen was incubated with the two lowest trypsin concentrations and not when collagen was incubated with the highest trypsin concentration. When collagen type I samples were incubated with various concentrations of trypsin (Figure 10, lanes 2-4), positively stained bands migrated at lower molecular weights than non-degraded collagen alpha-2 band

were detected, indicating degradation of collagen by trypsin. As trypsin concentration was increased, the amount of non-degraded collagen appeared to be decreased and the amount of degraded collagen fragments appeared to be increased. In the control sample in which collagen was not incubated with trypsin, only collagen alpha-1 chain, collagen alpha-2 chain, and multi-form collagen bands were detected (Figure 10, lane 1). The data demonstrated that trypsin appeared to degrade collagen type I in a dose-dependent manner. When collagen was incubated with 0.86  $\mu\text{M}$  trypsin (Figure 10, lane 3), less non-degraded collagen bands were detected compared to when collagen was incubated with 0.43  $\mu\text{M}$  trypsin (Figure 10, lane 2) and more degraded collagen fragment bands were detected compared to when collagen was incubated with 1.72  $\mu\text{M}$  trypsin (Figure 10, lane 4). Therefore, a trypsin to collagen molar ratio of 1:2 (Figure 10, lane 3) was selected to be used for subsequent experiments. This ratio exhibited adequate collagen degradation and clearly visualized trypsin-degraded collagen fragments by CBB-stained SDS-PAGE.



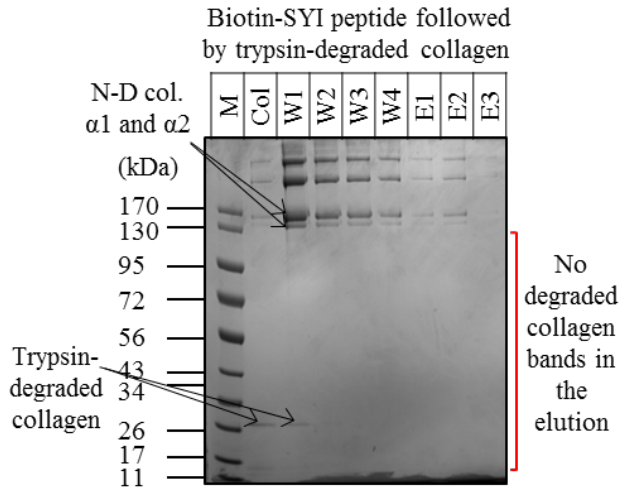
**Figure 10. Degradation of collagen with trypsin.** Collagen solution was incubated with different concentrations of trypsin. The data demonstrated that trypsin degraded collagen in a dose-dependent manner. The non-degraded collagen, including alpha-1 (N-D col.  $\alpha 1$ ), alpha-2 (N-D col.  $\alpha 2$ ), and multi-form, appeared to be decreased and the degraded collagen fragments (indicated by brackets) appeared to be increased as trypsin concentration was increased. The control sample of collagen without trypsin (lane 1) showed only non-degraded collagen. This experiment was performed once ( $n = 1$ ).

Subsequently, in order to identify trypsin-degraded collagen fragments that may bind to biotin-SYI peptide, biotin-SYI peptide was loaded on streptavidin sepharose packed column. Trypsin-degraded collagen solution was then incubated in the column to allow biotin-SYI peptide and collagen fragment binding. The column was centrifuged, washed, eluted, and applied on SDS-PAGE in the same manner as described above

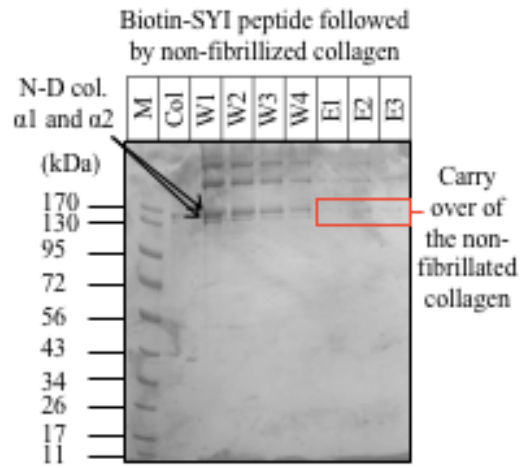
(Figure 11 A). Five controls were used in this experiment - a column loaded with biotin-SYI peptide and then with non-degraded non-fibrillized collagen solution only (Figure 11 B), a column loaded with non-degraded non-fibrillized collagen solution only (Figure 11 C), a column loaded with trypsin-degraded collagen solution only (Figure 11 D), a column loaded with biotin-scrambled peptide instead of biotin-SYI peptide and then with the prepared trypsin-degraded collagen solution (Figure 11 E), and a column loaded with biotin-SYI peptide and then with trypsin alone, without collagen (Figure 11 F). All the control columns were centrifuged, washed, and eluted in the same manner. The results showed that no positively stained degraded collagen fragments were observed in the elution of the column loaded with biotin-SYI peptide and then with trypsin-degraded collagen solution (Figure 11 A, E1-E3). Collagen alpha-1 and alpha-2 bands were detected in the control column loaded with biotin-SYI and then with non-degraded non-fibrillized collagen solution (Figure 11 B, E1-E2). However, the amount of collagen in the elution was less than that observed in the washing flow-through of this column (Figure 11 B, W1-W3), indicating that collagen monomer did not bind to biotin-SYI peptide. Collagen alpha-1 and alpha-2 bands were also detected in the control column loaded with non-degraded non-fibrillized collagen solution only (Figure 11 C, E1-E3); however, these bands do not indicate binding of non-degraded collagen for the same reason described above. There were no collagen bands observed in the elution of the other control columns, loaded with trypsin-degraded collagen solution only (Figure 11 D, E1-E3), loaded with biotin-scrambled peptide then with trypsin-degraded collagen solution (Figure 11 E, E1-E3), and loaded with biotin-SYI peptide and then with trypsin

alone (Figure 11 F, E1-E3), confirming the absence of any non-specific binding other than biotin-SYI peptide and collagen binding. In conclusion, collagen fragment that may bind to biotin-SYI peptide was not detected using streptavidin sepharose packed columns when trypsin was used as a collagen-degrading enzyme.

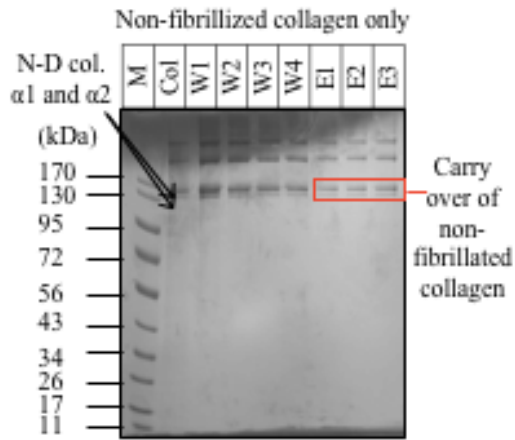
A



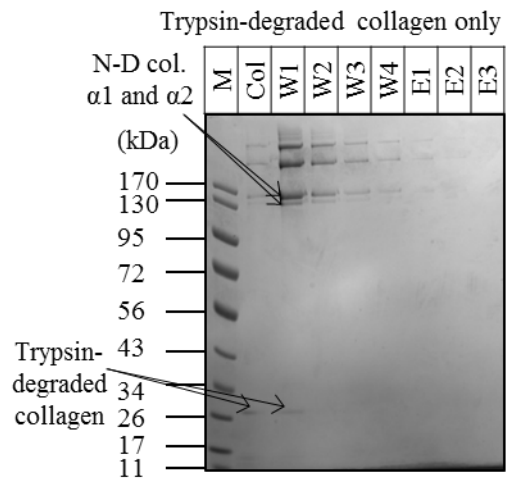
B



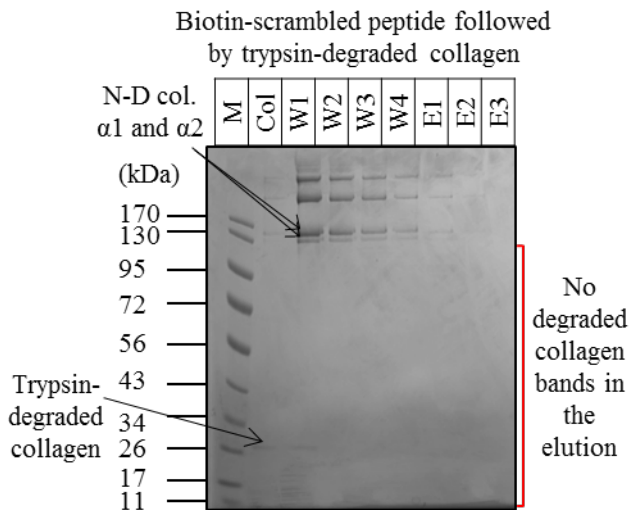
C



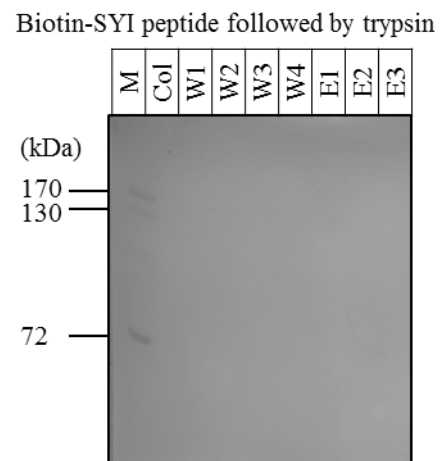
D



E



F



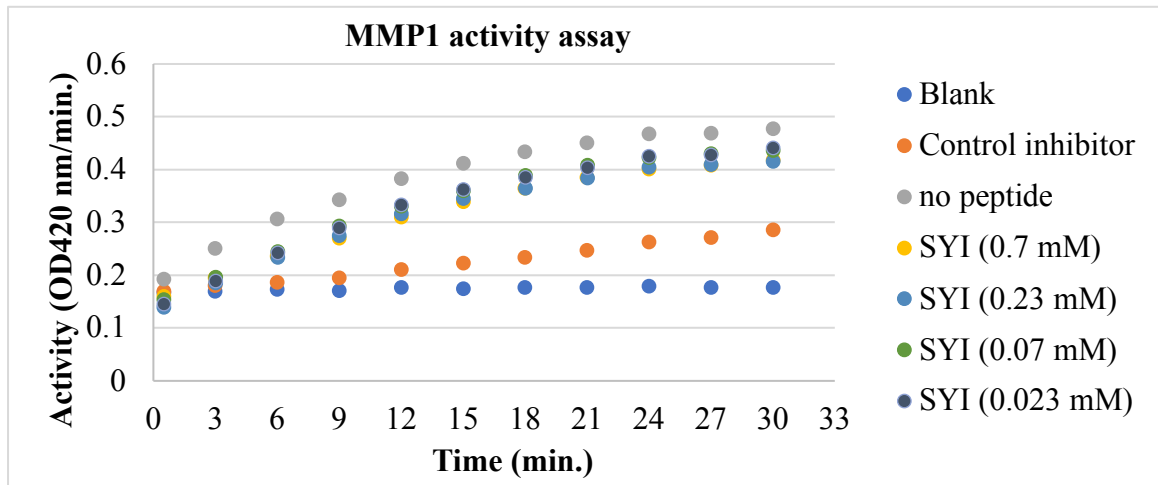
**Figure 11. Biotin affinity chromatography to identify collagen fragment that binds to SYI peptide using trypsin as a collagen-degrading enzyme.** (A). A column loaded with biotin-SYI peptide then with trypsin-degraded collagen solution. The column was centrifuged and unbound collagen flow-through was collected. Then, the column was washed four times with the washing buffer, and eluted three times with 0.1 M glycine, 2 M urea, pH 2.9. The washing flow-through was collected from each washing time. The same proportion of the unbound collagen flow-through, each washing flow-through, and each elution was loaded on SDS-PAGE. SDS-PAGE was stained with CBB. M, marker; Col, flow-through of unbound collagen fragment; W1, flow-through of the first washing; W2, flow-through of the second washing; W3, flow-through of the third washing; W4, flow-through of the fourth washing; E1, the first elution; E2, the second elution; E3, the third elution. (B). A control column loaded with biotin-SYI peptide and then with non-degraded non-fibrillized collagen solution. (C). A control column loaded with non-degraded non-fibrillized collagen solution only. (D). A control column loaded with trypsin-degraded collagen solution only. (E). A control column loaded with biotin-scrambled peptide instead of biotin-SYI peptide and then with prepared trypsin-degraded collagen solution. (F). A control column loaded with biotin-SYI peptide and then with trypsin alone. All experiments in A, B, C, D, E, and F were performed only once (n = 1).

### **MMP1 Activity Assay**

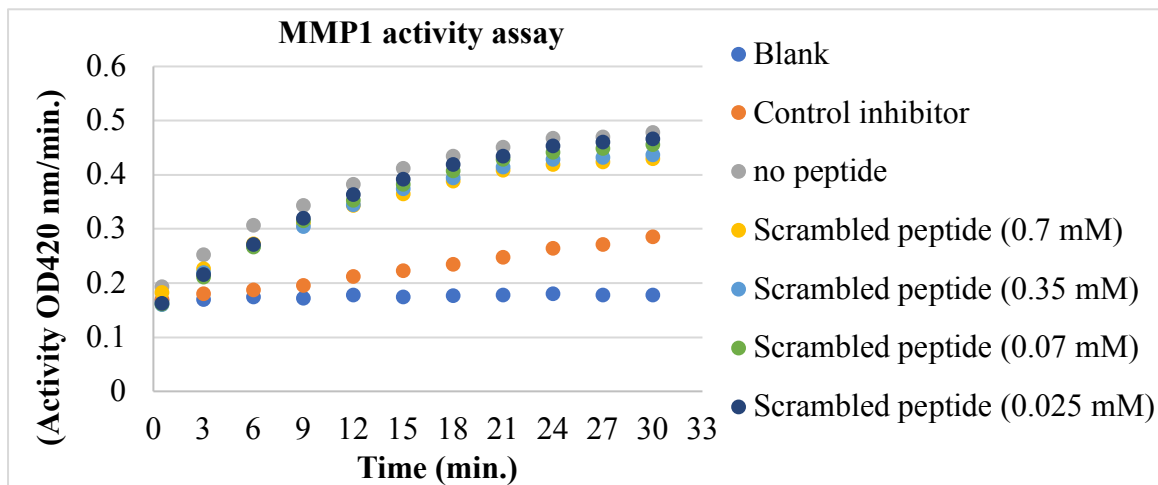
Initially, we thought that SYI peptide inhibited MMP1-mediated collagen degradation. However, the statistical analysis of our previous data showed no effect of SYI peptide on MMP1-mediated collagen degradation (Figure 6; Figure 7). The purpose of this experiment was to investigate whether SYI peptide inhibited MMP1-mediated collagen degradation as a competitive substrate of MMP1. MMP1 activity in the presence of SYI peptide or its scrambled peptide was assessed using a MMP1 activity assay kit. A chromogenic thiopeptide substrate was employed as a synthetic substrate for MMP1. The optical density (OD) of degrading the thiopeptide substrate by MMP1 was measured every three minutes. The enzymatic reaction by MMP1 alone served as a positive control, while the enzymatic reaction in the presence of the known MMP1 inhibitor, N-Isobutyl-

N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH), served as another control. The dose of SYI peptide used in the collagen degradation assay (Figure 6; Figure 7) was tested in this MMP1 activity assay. After obtaining the OD values, the initial rate of the reaction was calculated using the curve of absorbance versus time, expressed as OD/min. (Figure 12) (Table 2). The rate of the MMP1 enzymatic reaction was high in the control where there was no peptide (indicated by the gray dot curve, Figure 12 A and Figure 12 B), while that of the MMP1 enzymatic reaction in the presence of NNGH was markedly suppressed (indicated by the orange dot curve, Figure 12 A and Figure 12 B). On the contrary, the rate of the MMP1 enzymatic reaction was essentially unaffected by SYI peptide (Figure 12 A) and by the scrambled peptide (Figure 12 B) at the concentrations tested. Statistical analysis was performed using one-way ANOVA and the results showed that there was no statistical difference between MMP1 activities without SYI peptide and with SYI peptide at any of the concentrations tested (Figure 12 C). There was also no statistical difference between MMP1 activities with SYI peptide and with the scrambled peptide at any of the concentrations tested (Figure 12 C). Therefore, the results clearly demonstrated that SYI peptide did not affect MMP1 activity and was not a competitive substrate for MMP1.

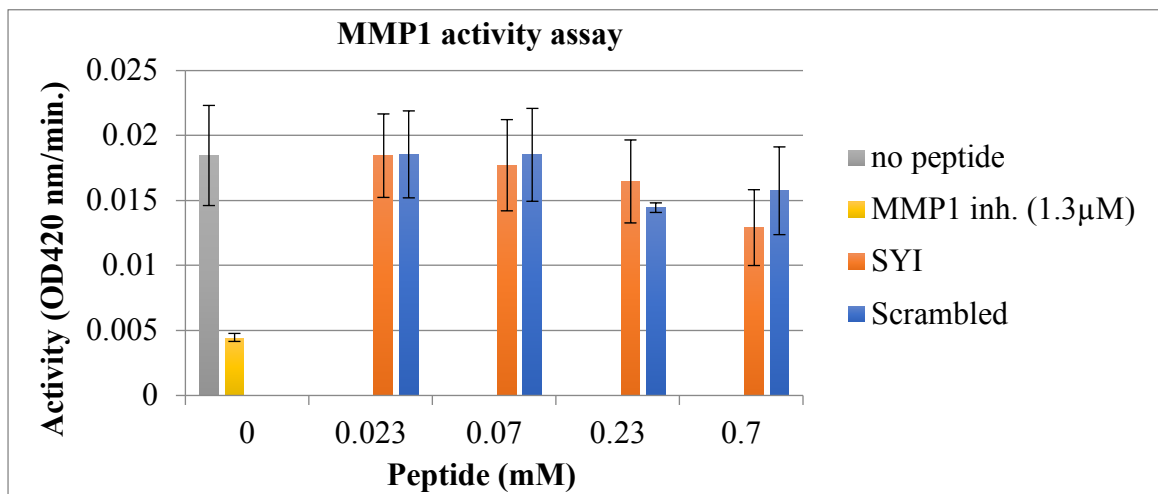
A



B



C



**Figure 12. MMP1 activity assay.** (A-B). A chromogenic thiopeptide was degraded by MMP1 in the presence of various SYI peptide concentrations (A) or scrambled peptide (B). The optical density (OD) of the samples was recorded at 420 nm every 3 min. This experiment was performed three times ( $n = 3$ ). Data shown was for one representative assay. (C). Quantitative analysis of the MMP1 activity assay. The activity of MMP1 was measured as the initial rate of the reaction, represented by the slope of the best-fit line of the plotted points for the straight region of the reaction curve. The values are shown as mean  $\pm$  S.D. based. Statistical analysis was performed and there was no statistical difference between MMP1 activities without SYI peptide and with SYI peptide at any concentrations tested ( $P$  value  $< 0.05$ ). There was also no statistical difference between MMP1 activities with SYI peptide and with the scrambled peptide at any concentrations tested ( $P$  value  $< 0.05$ ).

		Average MMP1 activity (OD 420 nm/min.)
Control inhibitor (NNGH)		$4.46 \times 10^{-3} \pm 0.31 \times 10^{-3}$
Control, no peptide		$18.46 \times 10^{-3} \pm 3.85 \times 10^{-3}$
Scrambled peptide (mM)	0.7	$15.75 \times 10^{-3} \pm 3.37 \times 10^{-3}$
	0.23	$14.45 \times 10^{-3} \pm 0.37 \times 10^{-3}$
	0.07	$18.51 \times 10^{-3} \pm 3.57 \times 10^{-3}$
	0.023	$18.55 \times 10^{-3} \pm 3.35 \times 10^{-3}$
SYI peptide (mM)	0.7	$12.91 \times 10^{-3} \pm 2.91 \times 10^{-3}$
	0.23	$16.46 \times 10^{-3} \pm 3.19 \times 10^{-3}$
	0.07	$17.71 \times 10^{-3} \pm 3.51 \times 10^{-3}$
	0.023	$18.45 \times 10^{-3} \pm 3.21 \times 10^{-3}$

**Table 2. Average MMP1 Activity.** The values are shown as the mean +/- S.D. based on three experiments (n = 3).

## DISCUSSION

### **The Effect of SYI Peptide on MMP1-Mediated Collagen Degradation**

The peptide SYIRIADTNIT (SYI peptide) is derived from decorin. It is located at LRR 6 and encompasses Arg-207 and Asp-210, which are important for collagen binding (Kalamajski et al., 2007). It has been previously reported that SYI peptide inhibited decorin-collagen interaction, which showed the capacity of SYI peptide to bind collagen type I (Kalamajski et al., 2007). It also has been reported that decorin inhibited MMP1-mediated collagen degradation in vitro (Geng et al., 2006). Consequently, we speculated that SYI peptide may inhibit MMP1-mediated collagen degradation. However, our data showed that there was no statistical difference between the extent of collagen degradation by MMP1 in the presence and absence of SYI peptide; hence we concluded there was no effect of SYI peptide on MMP1-mediated collagen degradation (Figures 6 and 7). There might be some pitfalls in the current study regarding the data. First, MMP1 was not reproducibly active in each experiment. The control experiment was successful to show degradation of collagen to three quarters and one quarter collagen fragments (Figure 5); however, the subsequent experiments, which investigated the effect of SYI peptide, were not reproducible, showing complete collagen degradation without any positively stained CBB collagen degraded-fragments in the control sample where collagen was incubated with MMP1 without the presence of any peptide (Figure 6, lane 2), while some collagen degraded bands were detected in the same control of other experiments (Figure 7, lane 2). The pattern of collagen degradation was also different between the experiments, which

made it difficult to identify a consistent read out. Second, the percentage of non-degraded collagen relative to the total amount of collagen was used as the quantitative read out for the extent of collagen degradation. However, this may not be the appropriate read out to show the inhibitory effect of SYI peptide on MMP1-mediated collagen degradation.

There are a couple of alternative read outs that may serve as a better evaluation criterion that were not considered in the present study. These read outs could be the degraded collagen three quarters and/or one quarter fragments. The amount of degraded collagen fragments produced in the control experiment (Figure 5) appeared to serve as a more reliable readout compared to the remaining non-degraded collagen used in the current study. However, the degraded collagen fragments could not be used as a read out in the current study because of the non-reproducibility in MMP1 activity between the experiments.

Although the effect of SYI peptide on MMP1-mediated collagen degradation needs to be further studied, the reason the results suggest that there is no effect of SYI peptide on MMP1-mediated degradation may be explained as follows. Decorin core protein is a large molecule consisting of 354 amino acids. It is approximately 40 kDa in size. It has been reported that decorin inhibited MMP1-mediated collagen type I degradation by binding to collagen, possibly at or near the MMP1 cleavage site, thus masking the cleavage site and limiting access of MMP1 to its collagen cleavage site (Geng et al, 2006). It was reported that collagenases catalyze the cleavage of collagen types I peptide bond between Gly775 and Ile776 (Lauer-Fields et al, 2002). It has been reported also that a major binding site of decorin core protein corresponded to alpha-1

cymongen bromide 6 collagen peptide (CB6) (Keene et al, 2001). CB6 collagen peptide was reported to consist of 192 amino acids beginning from around the amino acid corresponding to the site 819 (Henkel et al, 2007). Based on these reports, the MMP1 cleavage site appeared to be not far from the decorin binding site on collagen. On the other hand, SYI peptide consists of eleven amino acids and is 1,266.4 Da in size. Although SYI peptide was derived from the active collagen-binding site of decorin, it is a small peptide compared to decorin. This suggests that even though decorin and SYI peptide bind to the same region in collagen, the region of decorin which is not responsible for collagen binding may mask MMP1 cleavage site while SYI peptide may not. Several structural studies showed the binding and processing mechanisms of MMP1 against collagen type I (Arnold et al., 2011; Manka et al., 2012). However, it is still not clear whether the binding region of MMP1 within collagen type I overlaps with that of decorin. Further studies are still needed to conclude how decorin inhibits MMP1-mediated collagen degradation.

Additionally, a crystal structure of a decorin-collagen complex suggests that decorin has multiple binding sites to collagens, showing the complexity of the interaction (Orgel, Eid, Antipova, Bella, & Scott, 2009). The existence of multiple low-affinity binding sites of decorin to collagen has also been suggested in other previous studies (Kalamajski et al., 2007; Svensson et al., 1995). Therefore, if decorin interferes with MMP1-mediated collagen degradation and SYI peptide does not, that might be due to a possible existence of multiple binding sites of decorin to collagen. This may be another

reason why our data did not show any effect of SYI peptide on MMP1-mediated collagen degradation.

### **Biotin Affinity Chromatography to Identify the Collagen Fragment that Binds to SYI Peptide**

Identifying the collagen sequence that binds to SYI peptide will support our previous results that showed no inhibitory effect of SYI peptide on MMP1-mediated collagen degradation. Biotin affinity chromatography was used to separate the collagen fragment that binds SYI peptide. The amino acid sequence of the separated collagen fragment that bound SYI peptide was planned to be identified by mass spectrometry. Initially, MMP1 was used as a collagen-degrading enzyme. There were no degraded collagen fragments identified when biotin-SYI peptide was used (Figure 9 A). However, non-degraded collagen fibrils were found as a biotin-SYI peptide-binding protein (Figure 8 A). These results indicated that MMP1 could cleave collagen-SYI peptide binding site, thus preventing their binding. Another explanation might be as follows. If the amount of collagen loaded on the column was insufficient to detect the collagen fragment that bound biotin-SYI peptide, degraded collagen fragment might indeed be present, but might not be found because the staining level of the collagen fragment was below the detection limit for CBB staining. Another possibility is that the collagen fragment that bound to biotin-SYI peptide was too small to be detected by SDS-PAGE and it migrated out or to the bottom of SDS-PAGE.

Secondly, trypsin was used as a collagen-degrading enzyme to digest collagen differently from MMP1, aiming to separate the degraded collagen fragment that may bind to biotin-SYI peptide using biotin affinity chromatography. The results showed no trypsin-degraded collagen fragment that bound to biotin-SYI peptide was separated (Figure 11 A). Non-degraded, non-fibrillized collagen was also not separated (Figure 11 B). These results may suggest that the amount of collagen loaded on the column was not sufficient to show the separated non-degraded collagen in the control column. However, if we assume that the amount of collagen loaded on the column was sufficient, the data may also indicate that SYI peptide might not bind to non-fibrillized collagen, and collagen fibril form may be crucial for SYI peptide-collagen binding. Moreover, in the current study, non-fibrillized collagen was degraded by trypsin to generate the collagen fragments solution that was loaded on the streptavidin sepharose packed column, aiming to separate the degraded collagen fragment that may bind to biotin-SYI peptide. Since the data suggest that collagen fibril form may be crucial for SYI peptide-collagen binding, degrading fibril collagen by trypsin instead on non-fibrillized collagen used in the current study may be considered in future studies to separate the degraded collagen fragment that may bind biotin-SYI peptide. To detect the collagen fragment that binds SYI peptide, the method described by Keene (2001) to identify the cymongen bromide (CB)-digested collagen fragment that binds to decorin core protein might be useful. It has been reported that a major binding site of decorin core protein corresponded to alpha-1 CB6 collagen peptide. A photoaffinity labeling and cross-linking approach was reported to identify alpha-1 CB6 collagen peptide as a decorin binding site. Decorin core protein was

radiolabeled with  $^{125}\text{I}$  and derivatized with sulfosuccinimidyl-2-[*p*-azidosalicylamido]ethyl-1,3-dithiopropionate (SASD). Derivatized decorin was then allowed to bind to collagen fibrils. Decorin and collagen was further covalently cross-linked by ultraviolet light; then, the covalent linkage was severed by reduction with  $\beta$ -mercaptoethanol, resulting in tagging of the decorin-binding site on collagen. The labelled collagen was cleaved with CB and subjected to SDS-PAGE, autoradiography, and CBB staining. Similar methods may lead to successful identification of the collagen fragment that binds SYI peptide, and decorin core protein could be used as a positive control in this experimental plan.

Alternatively, modifying the experimental procedure used in the current study may lead to successful separation of the collagen fragment that binds SYI peptide. For example, more sensitive staining techniques may be used, including silver staining, which has higher sensitivity than CBB staining and is able to detect less than 1 ng of protein. Silver staining is compatible with mass spectrometry analysis. Fluorescent stains could also be used, such as Sypro Ruby. The sensitivity of Sypro Ruby staining is as good as or better than silver staining techniques. It interacts non-covalently with proteins. Thus, mass spectrometry data can be obtained immediately after staining without modification steps.

Moreover, if the separated collagen fragment that binds SYI peptide is too small to be detected by SDS-PAGE and CBB staining, additional purification methods to further purify the elution from biotin affinity column prior to mass spectrometry analysis may be used. Reversed-phase HPLC is a widely used protein and peptide separation

technique with high resolving capability. It utilizes the attachment of peptides and proteins to a highly hydrophobic particle surface. Protein or peptide is retained by adsorption to that surface until desorbed and eluted by specific concentration of organic solvent. The use of modified silica (C18) in the separation surface is particularly useful for the separation of small peptides and is usually the surface of choice for the separation of peptides resulting from protease digestion of proteins.

### **The Effect of SYI Peptide on MMP1 Activity**

It was thought that SYI peptide inhibited collagen degradation by MMP1. However, the statistical analysis of the collagen degradation assays showed no effect of SYI peptide on MMP1-mediated collagen degradation. The effect of SYI peptide on MMP1 activity using a different substrate than collagen was studied to investigate whether SYI peptide inhibited MMP1-mediated collagen degradation as a competitive substrate or inhibitor of MMP1 or by sterically blocking the access of MMP1 to its collagen cleavage site. Our data showed that MMP1 activity was not affected by the presence of SYI peptide (Figure 12). These results are supported by two previous reports. The first report studied whether recombinant decorin could be acting as a competitive substrate for MMP1 and thereby inhibiting its activity towards collagen fibrils (Geng et al., 2006). The second report studied the susceptibility of human recombinant decorin to MMP1 (Imai, Hiramatsu, Fukushima, Pierschbacher, & Okada, 1997). Both reports concluded that decorin was not digested by MMP1. Their results indicate that decorin is

not a competitive substrate for MMP1. However, decorin still could be an inhibitor for MMP1.

Recombinant human MMP1, used in Figure 12, was purchased from Abcam (ab139443). The recombinant MMP1 protein was produced in HEK293 cells; it consists of amino acids Met1 to Asn469 and is fused with a polyhistidine tag at the C-terminus. In contrast, recombinant human MMP1 used in the collagen degradation assay (Figures 6 and 7) was purchased from R&D systems. This MMP1 protein was produced in a mouse myeloma cell line; it is a pro-form consisting of amino acids Phe20 to Asn469 with no tag. Although MMP1 purchased from R&D systems has been investigated in the MMP1 activity assay, the recombinant MMP1 was not activated under the condition tested. To be consistent with the previous data from the collagen degradation assay (Figures 6 and 7), the effect of SYI peptide on the activity of MMP1 from R&D systems needs to be further studied.

## BIBLIOGRAPHY

- Ameye, L., & Young, M. F. (2002). Mice deficient in small leucine-rich proteoglycans: novel in vivo models for osteoporosis, osteoarthritis, Ehlers-Danlos syndrome, muscular dystrophy, and corneal diseases. *Glycobiology*, *12*(9), 107R–16R. <https://doi.org/10.1093/glycob/cwf065>
- Arnold, L. H., Butt, L. E., Prior, S. H., Read, C. M., Fields, G. B., & Pickford, A. R. (2011). The Interface between Catalytic and Hemopexin Domains in Matrix Metalloproteinase-1 Conceals a Collagen Binding. *Journal of Biological Chemistry*, *286*(52), 45073–45082. <https://doi.org/10.1074/jbc.M111.285213>
- Bertini, I., Calderone, V., Fragai, M., Luchinat, C., Mangani, S., & Terni, B. (2004). Crystal Structure of the Catalytic Domain of Human Matrix Metalloproteinase 10. *Journal of Molecular Biology*, *336*(3), 707–716. <https://doi.org/10.1016/j.jmb.2003.12.033>
- Birkedal-Hansen, H., & Taylor, R. E. (1982). Detergent-activation of latent collagenase and resolution of its component molecules. *Biochemical and Biophysical Research Communications*, *107*(4), 1173–1178. [https://doi.org/10.1016/S0006-291X\(82\)80120-4](https://doi.org/10.1016/S0006-291X(82)80120-4)
- Bode, W., Gomis-Rüth, F. X., & Stöckler, W. (1993). Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the “metzincins.” *FEBS Letters*, *331*(1–2), 134–140. [https://doi.org/10.1016/0014-5793\(93\)80312-1](https://doi.org/10.1016/0014-5793(93)80312-1)
- Chen, S., & Birk, D. E. (2013). The regulatory roles of small leucine-rich proteoglycans in extracellular matrix assembly. *FEBS Journal*, *280*(10), 2120–2137. <https://doi.org/10.1111/febs.12136>
- Dung, S. Z., Li, Y., Dunipace, A. J., Stookey, G. K. (1994). Degradation of insoluble bovine collagen and human dentine collagen pretreated in vitro with lactic acid, pH 4.0 and 5.5. *Archives of Oral Biology*, *39*(10), 901-905.
- Esko, J. D., Kimata, K., & Lindahl, U. (2009). Proteoglycans and Sulfated Glycosaminoglycans. In *Essentials of Glycobiology*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press (pp. 229–248). <https://doi.org/10.0-87969-559-5>
- Galazka, G., Windsor, L. J., Birkedal-Hansen, H., & Engler, J. A. (1996). APMA (4-aminophenylmercuric acetate) activation of stromelysin-1 involves protein interactions in addition to those with cysteine-75 in the propeptide. *Biochemistry*,

- 35(34), 11221–11227. <https://doi.org/10.1021/bi960618e>
- Geng, Y., McQuillan, D., & Roughley, P. J. (2006). SLRP interaction can protect collagen fibrils from cleavage by collagenases. *Matrix Biology*, 25(8), 484–491. <https://doi.org/10.1016/j.matbio.2006.08.259>
- Hanessian, S., Moitessier, N., Gauchet, C., & Viau, M. (2001). N-aryl sulfonyl homocysteine hydroxamate inhibitors of matrix metalloproteinases: Further probing of the S1, S1', and S2' pockets. *Journal of Medicinal Chemistry*, 44(19), 3066–3073. <https://doi.org/10.1021/jm010097f>
- Hasty, K. a, Jeffrey, J. J., Hibbs, M. S., & Welgus, H. G. (1987). The collagen substrate specificity of human neutrophil collagenase. *The Journal of Biological Chemistry*, 262(21), 10048–10052. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/3038863>
- Hedbom, E., & Heinegard, D. (1989). Interaction of a 59-kDa connective tissue matrix protein with collagen I and II. *Journal of Biological Chemistry*, 264(12), 6898–6905.
- Henkel, W., Dreisewerd, K. (2007). Cyanogen bromide peptides of the fibrillar collagens I, III, and V and their mass spectrometric characterization: detection of linear peptides, peptide glycosylation, and cross-linking peptides involved in formation of homo- and heterotypic fibrils. *Journal of Proteome Research*, 6(11):4269-89.
- Hildebrand, A., Romarís, M., Rasmussen, L. M., Heinegård, D., Twardzik, D. R., Border, W. A., & Ruoslahti, E. (1994). Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta. *The Biochemical Journal*, 534(Pt 2), 527–534. <https://doi.org/10.1042/bj3020527>
- Imai, K., Hiramatsu, A., Fukushima, D., Pierschbacher, M. D., & Okada, Y. (1997). Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. *The Biochemical Journal*, 322 ( Pt 3, 809–814. <https://doi.org/10.1042/bj3220809>
- Iozzo, R. V., & Schaefer, L. (2015). Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. *Matrix Biology*. 42, 11-55. <https://doi.org/10.1016/j.matbio.2015.02.003>
- Islam, M. (2014). *Biochemical analysis of collagen binding by invertebrate SPARC and engineered monomeric decorin*. Retrieved from ProQuest Theses and Dissertations Global. (Thesis/dissertation number 10078558).
- Jeng, A. Y., Chou, M., & Parker, D. T. (1998). Sulfonamide-based hydroxamic acids as

- potent inhibitors of mouse macrophage metalloelastase. *Bioorganic & Medicinal Chemistry Letters*, 8, 897–902.
- Kaku, M., & Yamauchi, M. (2014). Mechano-regulation of collagen biosynthesis in periodontal ligament. *Journal of Prosthodontic Research*, 58(4), 193–207. <https://doi.org/10.1016/j.jpor.2014.08.003>
- Kalamajski, S., Aspberg, A., & Oldberg, Å. (2007). The decorin sequence SYRIADTNIT binds collagen type I. *Journal of Biological Chemistry*, 282(22), 16062–16067. <https://doi.org/10.1074/jbc.M700073200>
- Kalamajski, S., & Oldberg, A. (2010). The role of small leucine-rich proteoglycans in collagen fibrillogenesis. *Matrix Biology*, 29(4), 248–253. <https://doi.org/10.1016/j.matbio.2010.01.001>
- Kjeuin, L., & Lindahl, U. (1991). Protoglycans : structures and interactions. *Annual Review of Biochemistry*, 60, 443-75.
- Last, J. A., & Reiser, K. M. (1984). Collagen biosynthesis. *Environmental Health Perspectives*, 55(9), 169–177.
- Lauer-Fields, J. L., Juska, D., Fields, G. B. (2002). Matrix metalloproteinases and collagen catabolism. *Biopolymers*, 66(1):19-32.
- Manka, S. W., Carafoli, F., Visse, R., Bihan, D., Raynal, N., & Farndale, R. W. (2012). Structural insights into triple-helical collagen cleavage by matrix metalloproteinase. *Proceeding of the National Academy of Sciences of the United States of America*, 109(31), 12461–12466. <https://doi.org/10.1073/pnas.1204991109>
- McEwan, P. A., Scott, P. G., Bishop, P. N., & Bella, J. (2006). Structural correlations in the family of small leucine-rich repeat proteins and proteoglycans. *Journal of Structural Biology*, 155(2), 294–305. <https://doi.org/10.1016/j.jsb.2006.01.016>
- Merline, R., Schaefer, R. M., & Schaefer, L. (2009). The matricellular functions of small leucine-rich proteoglycans (SLRPs). *Journal of Cell Communication and Signaling*, 3(3–4), 323–335. <https://doi.org/10.1007/s12079-009-0066-2>
- Mochida, Y., Kaku, M., Yoshida, K., Katafuchi, M., Atsawasuwan, P., & Yamauchi, M. (2011). Podocan-like protein: A novel small leucine-rich repeat matrix protein in bone. *Biochemical and Biophysical Research Communications*, 410(2), 333–338. <https://doi.org/10.1016/j.bbrc.2011.05.150>
- Myllyharju, J. (2008). Prolyl 4-hydroxylases, key enzymes in the synthesis of collagens and regulation of the response to hypoxia, and their roles as treatment targets. *Annals of Medicine*, 40(6), 402–417. <https://doi.org/10.1080/07853890801986594>

- Nagase, H. (2001). Substrate specificity of MMPs. *Matrix Metalloproteinase Inhibitors in Cancer Therapy*, (14), 39–66. [https://doi.org/10.1007/978-1-59259-011-7\\_2](https://doi.org/10.1007/978-1-59259-011-7_2)
- Nagase, H., Enghild, J. J., Suzuki, K., & Salvesen, G. (1990). Stepwise activation mechanisms of the precursor of the matrix metalloproteinase 3 (stromelysin) by proteinases and (4-aminophenyl)mercuric acetate. *Biochemistry*, 29(24), 5783–5789. <https://doi.org/10.1021/bi00476a020>
- Nagase, H., Visse, R., & Murphy, G. (2006). Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular Research*, 69(3), 562–573. <https://doi.org/10.1016/j.cardiores.2005.12.002>
- Nagase, H., & Woessner, J. (1999). Matrix Metalloproteinases. *Journal of Biological Chemistry*, 274(31), 491–494. <https://doi.org/10.1007/s13398-014-0173-7.2>
- Nakajima, M., Kizawa, H., Saitoh, M., Kou, I., Miyazono, K., & Ikegawa, S. (2007). Mechanisms for asporin function and regulation in articular cartilage. *Journal of Biological Chemistry*, 282(44), 32185–32192. <https://doi.org/10.1074/jbc.M700522200>
- Okada, Y., Gonoji, Y., Nakanishi, I., Nagase, H., & Hayakawa, T. (1990). Immunohistochemical demonstration of collagenase and tissue inhibitor of metalloproteinases (TIMP) in synovial lining cells of rheumatoid synovium. *Virchows Archiv B Cell Pathology Including Molecular Pathology*, 59(1), 305–312. <https://doi.org/10.1007/BF02899418>
- Orgel, J. P. R. O., Eid, A., Antipova, O., Bella, J., & Scott, J. E. (2009). Decorin core protein (decoron) shape complements collagen fibril surface structure and mediates its binding. *PLoS ONE*, 4(9). <https://doi.org/10.1371/journal.pone.0007028>
- Paladini, R. D., Wei, G., Kundu, A., Zhao, Q., Bookbinder, L. H., Keller, G. A., ... Frost, G. I. (2013). Mutations in the catalytic domain of human matrix metalloproteinase-1 (MMP-1) that allow for regulated activity through the use of Ca<sup>2+</sup>. *Journal of Biological Chemistry*, 288(9), 6629–6639. <https://doi.org/10.1074/jbc.M112.364729>
- Pogány, G., & Vogel, K. G. (1992). The interaction of decorin core protein fragments with type I collagen. *Biochemical and Biophysical Research Communications*, 189(1), 165–172. [https://doi.org/10.1016/0006-291X\(92\)91539-3](https://doi.org/10.1016/0006-291X(92)91539-3)
- Rada, J. A., Cornuet, P. K., & Hassell, J. R. (1993). Regulation of corneal collagen fibrillogenesis in vitro by corneal proteoglycan (lumican and decorin) core proteins. *Experimental Eye Research*, 56(6), 635–648. <https://doi.org/10.1006/exer.1993.1081>
- Remacle, A. (2003). Membrane type I-matrix metalloproteinase (MT1-MMP) is internalised by two different pathways and is recycled to the cell surface. *Journal of*

- Cell Science*, 116(19), 3905–3916. <https://doi.org/10.1242/jcs.00710>
- Schaefer, L., & Schaefer, R. M. (2010). Proteoglycans: From structural compounds to signaling molecules. *Cell and Tissue Research*, 339(1), 237–246. <https://doi.org/10.1007/s00441-009-0821-y>
- Scott, J. E. (1988). Proteoglycan-fibrillar collagen interactions. *Biochemical Journal*, 252(2), 313–323.
- Stricklin, G. P., Jeffrey, J. J., Roswit, W. T., & Eisen, A. Z. (1983). Human Skin Fibroblast Procollagenase: Mechanisms of Activation by Organomercurials and Trypsin. *Biochemistry*, 22(1), 61–68. <https://doi.org/10.1021/bi00270a009>
- Svensson, L., Heinegard, D., & Oldberg, A. (1995). Decorin-binding sites for collagen type I are mainly located in leucine-rich repeats 4-5. *Journal of Biological Chemistry*, 270(35), 20712–20716. <https://doi.org/10.1074/jbc.270.35.20712>
- Svensson, L., Oldberg, Å., & Heinegård, D. (2001). Collagen binding proteins. *Osteoarthritis and Cartilage*, 9(SUPPL. A). <https://doi.org/10.1053/joca.2001.0440>
- Sylvester, M. L., & Ratner, B. D. (2017). Collagen affinity coating for surface binding of decorin and other biomolecules : Surface characterization. *Biointerphases*, 12(2): 02C419.
- Tillgren, V., Ho, J. C. S., Önnarfjord, P., & Kalamajski, S. (2015). The novel small leucine-rich protein chondroadherin-like (CHADL) is expressed in cartilage and modulates chondrocyte differentiation. *Journal of Biological Chemistry*, 290(2), 918–925. <https://doi.org/10.1074/jbc.M114.593541>
- Van Doren, S. R. (2015). Matrix metalloproteinase interactions with collagen and elastin. *Matrix Biology*, 44-46, 224-231. <https://doi.org/10.1016/j.matbio.2015.01.005>
- Van Wart, H. E., & Birkedal-Hansen, H. (1990). The cysteine switch: A principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family (collagenase/gelatinase/stromelysin/zinc enzyme). *Biochemistry*, 87(July), 5578–5582. <https://doi.org/10.1073/pnas.87.14.5578>
- Vogel, K. G., Paulsson, M., & Heinegård, D. (1984). Specific inhibition of type I and type II collagen fibrillogenesis by the small proteoglycan of tendon. *The Biochemical Journal*, 223(3), 587–597. Retrieved from <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1144341&tool=pmcentrez&rendertype=abstract>
- Weber, I. T., Harrison, R. W., & Iozzo, R. V. (1996). Model Structure of Decorin and

Implication for Collagen Fibrillogenesis. *Journal of Biological Chemistry*, 271(18), 31767–31770. <https://doi.org/10.1074/jbc.271.50.31767>

Welgus, H. G., Jeffrey, J. J., & Eisen, A. Z. (1981a). The Collagen Substrate Specificity of Human Collagenase\* Skin Fibroblast. *The Journal of Biological Chemistry*, 256(18), 9511–9515.

Welgus, H. G., Jeffrey, J. J., & Eisen, A. Z. (1981b). The Collagen Substrate Specificity of Human Skin Fibroblast Collagenase. *Journal of Biological Chemistry*, 256(18), 9511–9515.

Yamauchi, M., & Sricholpech, M. (2012). Lysine post-translational modifications of collagen. *Essays in Biochemistry*, 52, 113–133. <https://doi.org/10.1042/bse0520113>

## CURRICULUM VITAE

