# New Insights in the Degradation of Elastin and Collagens by Matrix Metalloproteinases

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*To Mom, Dad, and TRYMEE*

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**Abbreviations and symbols**

| | |
|---|---|
| AAA | Abdominal aortic aneurysm |
| cDNA | Complementary DNA |
| CID | Collision-induced dissociation |
| COPD | Chronic obstructive pulmonary disease |
| DIOS | Desorption ionization on porous silicon |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EBP | Elastin binding protein |
| EC | Enzyme category |
| ECD | Electron capture dissociation |
| ECM | Extracellular matrix |
| EDP | Elastin-derived peptide |
| ESI | Electrospray ionization |
| FT-ICR | Fourier transform-ion cyclotron resonance |
| HPLC | High performance liquid chromatography |
| LC | Liquid chromatography |
| LIT | Linear ion trap |
| LOX | Lysyl oxidase |
| LTBP | Latent transforming growth factor β-binding protein |
| MAGP | Microfibril associated glycoprotein |
| MALDI | Matrix-assisted laser desorption/ionization |
| mRNA | Messenger RNA |
| MMP | Matrix metalloproteinase |
| MS | Mass spectrometry |
| MS/MS | Tandem mass spectrometry ($MS^2$) |
| MT-MMP | Membrane-type MMP |
| NanoESI | Nanoelectrospray ionization |
| PTM | Post-translational modification |
| QIT | Quadrupole ion trap |
| qTOF | Hybrid quadrupole TOF mass analyzer |
| RNA | Ribonucleic acid |

| | |
|---|---|
| SALDI | Surface-assisted laser desorption/ionization |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| TFA | Trifluoroacetic acid |
| TGF-β | Transforming growth factor-β |
| TIMP | Tissue inhibitors of metalloproteinase |
| TOF | Time-of-flight |
| UV | Ultraviolet |

Amino acids, abbreviations, and exact residue masses (amino acid - $H_2O$)

| Amino acid | Three letter code | Single letter code | Monoisotopic mass |
|---|---|---|---|
| Alanine | Ala | A | 71.0371 |
| Arginine | Arg | R | 156.1011 |
| Asparagine | Asn | N | 114.0429 |
| Aspartic acid | Asp | D | 115.0269 |
| Cysteine | Cys | C | 103.0092 |
| Glutamine | Gln | Q | 128.0586 |
| Glutamic acid | Glu | E | 129.0426 |
| Glycine | Gly | G | 57.0215 |
| Histidine | His | H | 137.0589 |
| Hydroxylysine | HyK | k* | 144.0899 |
| Hydroxyproline | HyP | p* | 113.0477 |
| Isoleucine | Ile | I | 113.0841 |
| Leucine | Leu | L | 113.0841 |
| Lysine | Lys | K | 128.0950 |
| Methionine | Met | M | 131.0405 |
| Phenylalanine | Phe | F | 147.0684 |
| Proline | Pro | P | 97.0528 |
| Serine | Ser | S | 87.0320 |
| Threonine | Thr | T | 101.0477 |
| Tryptophan | Trp | W | 186.0793 |
| Tyrosine | Tyr | Y | 163.0633 |
| Valine | Val | V | 99.0684 |

## NOTATIONS

*m/z* Mass-to-charge ratio

pH Negative logarithm of the hydronium ion activity

* Hydroxylysine and hydroxyproline are represented in this work by small letters k and p, respectively.

# 1 INTRODUCTION

Elasticity is one of the requirements as organisms transform into complex multicellular systems. Elastin, the main component of elastic fibers, offers tissues with this exceptional function. It is secreted as a monomer but it rapidly matures into a complex polymer through a process of extensive cross-linking between tropoelastin units as well as through interactions with other elements of the elastic fiber system such as fibrillins, fibulins, and emilin. Mature elastin is the most stable protein in the extracellular matrix (ECM) although some proteases, mainly matrix metalloproteinases (MMPs), are now known to compromise its integrity over time. Degradation of elastin has enormous influence on the fundamental functions of the ECM, particularly in tissues where movement is critical such as in the aorta and lung.

MMPs are a family of multidomain proteases consisting of pro-, catalytic, and hemopexin-like domains. Some members have additional domains such as fibronectin-type II inserted in the catalytic domain. They are synthesized as prepro- and secreted as pro-MMPs and thus need activation to induce their catalytic activity. Their activation involves disruption of the propeptide-zinc linkage (also called cysteine switch) and this cleavage makes the catalytic zinc free for activity. Ever since their first discovery nearly half a century ago, MMPs have been shown to mediate all forms of ECM transformations. Their proteolytic activity is central to normal physiological processes such as tissue development, cell-to-cell interactions and remodeling. There is now enough evidence to support MMPs' active participation in pathological conditions such as cancer progression. Under normal conditions, very few MMPs are constitutively expressed in tissues; the notable examples are MMP-2 and MMP-7. However, when tissues are under stress, for instance, during exposure of the skin to UV radiation, multiple MMPs can be upregulated and this has consequent catabolic effect on the ECM.

Several MMPs have been identified to degrade elastin and the major ones are MMP-2, -7, -9 and -12. These enzymes are markedly upregulated under certain pathological conditions such as aneurysms, atherosclerosis, emphysema, and cancer. In fact, they are claimed to contribute to these disease processes by degrading elastin and other ECM components. Their extensive involvement in inflammatory processes made them targets for extensive

research and drug development (Verma and Hansch, 2007). However, success is far too slow for reasons including the lack of clear understanding on the functional role of each enzyme in the degradation process. For example, in patients with chronic obstructive pulmonary disease (COPD) a myriad of MMPs and serine proteases are known to be upregulated (Elkington and Friedland, 2006). Under this circumstance, it is difficult to distinguish the relative contribution of each elastase to rationally target the problem. This fact calls for a detailed understanding of the molecular basis of interactions between proteases and components of the ECM. Part of the objective of the present study, therefore, was to provide a comprehensive insight in the degradation of both soluble and insoluble forms of elastin under the influence of MMPs.

Moreover, MMPs act broadly on other components of the ECM such as collagens, which are the major components of tissue proteins. Structurally, collagens are made from three polypeptide chains, called α-chains, wound together around a central axis to form a stable triple helix. Each chain is composed of a regularly organized Gly-X-Y triplet, where every third position is occupied by Gly and the HyP at the Y position confers collagens with their stability. Several collagen types have been identified and collagen type I is the most abundant of all in the ECM. As a result of its abundance, collagen I is in constant contact with MMPs. From the MMP family, MMP-1, -2, -8, -13, -14, -18, and -22 have been reported to possess collagenolytic activity (Lauer-Fields *et al.*, 2002). However, limited information is available on the functional role of others; for instance, the role of MMP-12 has not been defined. MMP-12 is known to be upregulated in various inflammatory disease processes and given that collagen degradation is one of the marked features of many inflammatory conditions, it is of high biological significance to explore the role of MMP-12 in the degradation of collagens. The second part of this dissertation, thus, investigates the role of MMP-12 on the degradation of native collagens I and III.

The mature elastin and collagens are structurally complex compounds. Thus, the biochemical investigation effort on these complex biomolecules will have to face a challenging task of overcoming several analytical setbacks in order to generate relevant information. In this regard, mass spectrometry (MS) has presented recently an unmatched sensitivity and versatility advantages to the analytics of increasingly complex biological samples as compared to conventional analytical techniques. As a result of recent technological advances, MS can

be interfaced with several protein chemistry assays and provide powerful information on proteins sequences, structures, and post-translational modifications (PTMs). This study sought to take the advantages of this powerful technique.

Therefore, the aim of the present study is to develop analytical methods based on complementary MS and employ them to comprehensively investigate the degradation of tropoelastin, elastin and collagens under the influence of MMPs. It also intends to apply these methods to provide insight in to the complex structures of elastin and collagens. Furthermore, MS has been employed to explore the role of MMPs in generating matrikines both from elastin and tropoelastin.

# 2 THE EXTRACELLULAR MATRIX

The ECM is composed of families of macromolecules including collagens, elastin, glycoproteins, and proteoglycans arranged in a unique, tissue-specific, age-dependent, and three-dimensional ultrastructure (Har-el and Tanzer, 1993; Lin and Bissell, 1993; Rosenbloom *et al.*, 1993; Venstrom and Reichardt, 1993). In most instances matrix molecules exist as large sparingly soluble aggregates that can not easily be solubilzed or dissociated into component units. When dissociated, the biological properties and functional complexities of the individual constituent often differ from the intact form. The ECM, beyond its obvious structural function, serves as a "virtual information highway" between cells. It also, through the use of elastic fibers, provides tissue with flexibility and extensibility, which have been essential requirements in the evolution of multicellular organisms (Adams and Watt, 1993; Badylak, 2002; Geiger *et al.*, 2001; Har-el and Tanzer, 1993; Lin and Bissell, 1993; Rosenbloom *et al.*, 1993; Venstrom and Reichardt, 1993).

## 2.1 Elastic fibers

Elastic fibers are components of all vertebrate elastic tissues with the exception of lower vertebrates such as lamprey (Har-el and Tanzer, 1993; Kielty *et al.*, 2002; Lin and Bissell, 1993; Rosenbloom *et al.*, 1993; Sage and Gray, 1977; Venstrom and Reichardt, 1993). Their function in different tissues is a consequence of their composition and organization or architecture. For example, whereas they form concentric fenestrated lamellae in the medial layer of the aorta, they exist as delicate latticework throughout the lung with slight accumulation in specific areas of stress such as the opening of the alveoli (Shifren and Mecham, 2006). Mature elastic fibers are composed of an outer microfibrillar mantle and an inner core of amorphous cross-linked elastin (~ 90 % of the elastic fibers) (Rosenbloom *et al.*, 1993). Microfibrils are principal component of the elastic system with central importance in providing long range elastic recoil to connective tissues. They are also present in some flexible tissues that do not express elastin, for instance, in the ciliary zonules that hold the lens in dynamic suspension (Ashworth *et al.*, 2000) or in invertebrates with low pressure closed circulatory system such as in lobster (Kielty *et al.*, 2002; Qian and Glanville, 1997; Sherratt, 2009). Elastin and microfibrils evolved independently and elastin's

evolution is thought to have happened more recently (at some point after the divergence of the cyclostome and gnathostome lines) to reinforce the high pressure of closed circulatory system of higher vertebrates (Faury, 2001; Kielty *et al.*, 2002; Rosenbloom *et al.*, 1993). Isolated microfibrils are 10–12 nm wide beaded structures exhibiting an average axial unit repeat of 56 nm (Kielty and Shuttleworth, 1997; Lu *et al.*, 2006; Wess *et al.*, 1998). While fibrillins are the major component in microfibrillar proteins, other members include microfibril associated glycoproteins (MAGPs), latent transforming growth factor β-binding proteins (LTBPs), emilin, fibulins, and proteoglycans (Bressan *et al.*, 1993; Cain *et al.*, 2006; Henderson *et al.*, 1996; Sakai *et al.*, 1986a; Sakai *et al.*, 1986b; Sherratt, 2009; Wagenseil and Mecham, 2007)..

## 2.2 Elastin

### 2.2.1 Biophysical properties

Elastin used to be defined as the remaining part when a tissue is subjected to an aggressive treatment of extreme temperature and pH (Daamen *et al.*, 2007; Mecham, 2008). However, the functional form of the protein is a large and highly cross-linked polymer that organizes as sheets or fibers in the ECM. More than 10 different extraction methods have been reported, which basically involve either one or a combination of autoclaving, treatment with chaotropic agents, buffers, and reducing agents. The most common methods have been reviewed by Mecham (Mecham, 2008) and comparisons on the relative efficiency of some of these methods have been reported (Daamen *et al.*, 2001; Soskel and Sandburg, 1983). Pure elastin is devoid of collagen, carbohydrates, and has Gly level that represents about one-third of the total residue. The purity of elastin fiber is traditionally assessed by amino acid analysis. However, in most instances, the levels of Asp and Glu give the most accurate assessment of elastin purity (Mecham, 2008; Ritz-Timme *et al.*, 2003).

Elasticity is a property that originates from structural features of elastin. The backbone of elastin structure is highly mobile and the Ala-rich hydrophilic region exhibits predominantly α-helical structure, while the hydrophobic region exhibits both ordered conformation (conformations that are stabilized by internal hydrogen bonds like the β-strands and β-turns) and random structure (Fig. 2.1). The covalent cross-links impart restriction to the

molecule so that while stretching one chain is restricted from slipping past another. Moreover, elastin is rich in small amino acids such as Gly, which provides kinetic freedom, and Pro, a helix breaker, which may help to keep the chain free (Debelle and Alix, 1999; Debelle and Tamburro, 1999; Rauscher *et al.*, 2006; Tamburro *et al.*, 2006; Vrhovski and Weiss, 1998).

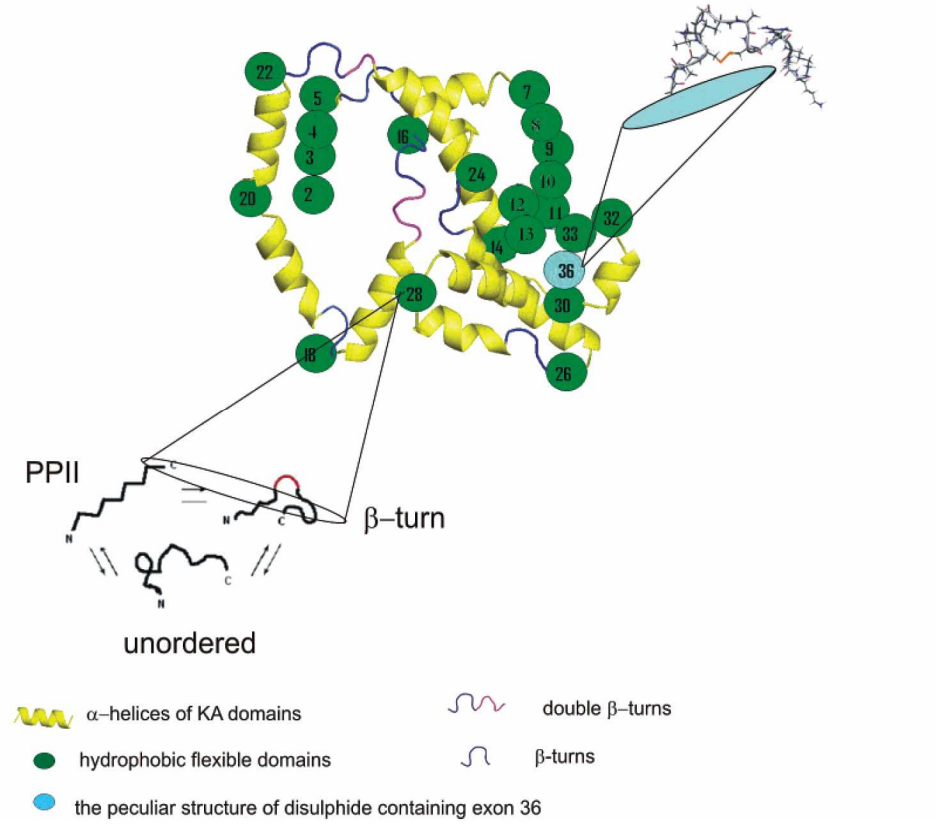



***Fig. 2.1:*** *Idealized model of tropoelastin structure (adopted from (Tamburro et al., 2006))*

Several mechanisms have been proposed to explain the physicochemical processes of elasticity (Mithieux and Weiss, 2005; Vrhovski and Weiss, 1998). One such theory considers elastin as a typical rubber consisting of a network of random chains of high entropy. While stress orders the chain and decreases the entropy of the system, a reduction in entropy provides the recoiling force when the stress is removed (Hoeve and Flory, 1974; Tatham and Shewry, 2002). The alternative model is based on the observation that elastin exhibits a regular structure of β-spiral comprising repetitive type-II β-turns. With stress the β-turns act as spacers between the turns of the spiral. This serves to suspend chain segments in a

kinetically free state. Stretching reduces the kinetic freedom and entropy of the system with consequent creation of a recoiling tendency (Tatham and Shewry, 2000; Tatham and Shewry, 2002; Urry, 1988; Urry *et al.*, 1986). Moreover, part of this restoring force may originate from the hydrophobic interaction between side chains and water. In this case, stretching of elastin exposes the hydrophobic side chain to an aqueous environment, decreasing the entropy of the surrounding water molecule. Hence, a restoring force will arise from the re-establishment of the hydrophobic interaction (Debelle and Alix, 1999; Gosline, 1978; Tatham and Shewry, 2000; Tatham and Shewry, 2002) .

### 2.2.2 Biochemistry

#### 2.2.2.1 The elastin gene

The human elastin gene has been mapped to chromosome 7q11.1-12.1 and it has an uncommon high intron-to-exon ratio of 20:1 (Rosenbloom *et al.*, 1993; Rosenbloom *et al.*, 1991). In most species the elastin gene is composed of 36 exons. Comparatively, the human elastin gene lacks sequences that correspond to exons 34 and 35 but contains the unusual hydrophilic sequence–encoding exon 26A (Bashir *et al.*, 1989; Cicila *et al.*, 1985; Tassabehji *et al.*, 1997; Vrhovski and Weiss, 1998) (domain composition is shown in Fig. 2.2). These two exons were lost sequentially during primate evolution and their loss is thought to have conferred a functional advantage to elastin (Bashir *et al.*, 1989; Indik *et al.*, 1987b; Mithieux and Weiss, 2005; Szabo *et al.*, 1999). Extensive alternative splicing of the primary elastin transcript has been confirmed by sequence analysis of mRNA and cDNA. It has further been described that the splitting of codons at the exon-intron borders occurs consistently throughout the molecule in a fashion that maintains the reading frame (Indik *et al.*, 1987b; Rosenbloom *et al.*, 1993; Vrhovski and Weiss, 1998). Alternative splicing can result in: (I) complete excision of an exon, for example exons 22 and 32 in human tropoelastin or (II) excision of portion of an exon as in the case of exon 26A (Indik *et al.*, 1987a; Vrhovski and Weiss, 1998).

From human tropoelastin, at least exons 22, 23, 24A, 24, 26A, 30, 32, and 33 are known to be alternatively spliced out (Indik *et al.*, 1987b; Parks and Deak, 1990; Pierce *et al.*, 1992a; Vrhovski and Weiss, 1998; Zhang *et al.*, 1999). Particularly, exon 22 is claimed to always

be spliced out, while in the case of exon 32, it is said to be subjected to frequent (~ 70 %) splicing. Moreover, exons 3, 5, 10, 11, and 20 are also suspected to be alternatively spliced out (personal communication with Dr. R.P. Mecham and Dr. Z. Urban, [also (Mecham, 2008; Tamburro, 2009)])

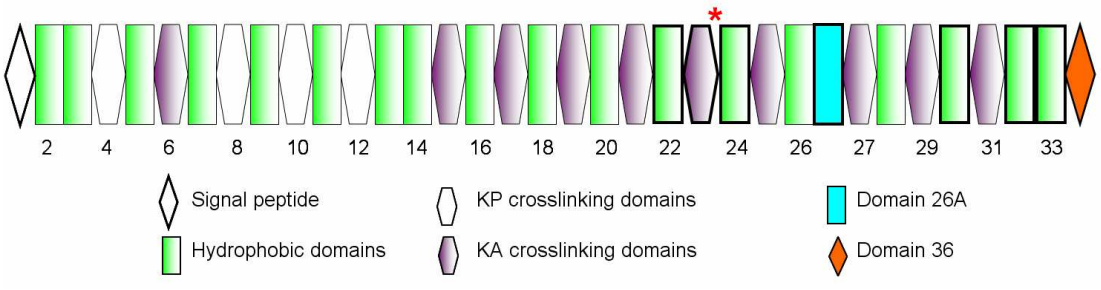



***Fig. 2.2:*** *Domain structure of human tropoelastin containing all possible exons. Exons subjected to alternative splicing are shown bordered in bold. The position of Exon 24A, which has been identified in elastin cDNA from fibroblast of human skin, is indicated by an asterisk (Fazio et al., 1988).*

#### 2.2.2.2 Tropoelastin

Tropoelastin is composed of sequences encoded by hydrophobic and cross-linking domains and with rare exception, these coding exons alternate (Indik *et al.*, 1987b; Rosenbloom *et al.*, 1991; Sandberg *et al.*, 1969; Tassabehji *et al.*, 1997). It was isolated first from copper-deficient pig and at present at least 11 human splice variants have been identified (Sandberg *et al.*, 1969; Vrhovski and Weiss, 1998). The functional role of these isoforms has not been yet fully determined. However, since alternative splicing may result in a scenario where either two cross-linking domains brought into apposition (e.g., deletion of exon 22) or the interval between cross-linked domains increased (e.g., deletion of exon 23), splicing can have influence on cross-linking with eventual consequence on the overall properties of the mature elastin (Rosenbloom *et al.*, 1993; Sato *et al.*, 2006; Yura *et al.*, 2006). Tropoelastin undergoes minimal PTMs and so far, only hydroxylation of some Pro residues and cross-linking have been reported (Brown-Augsburger *et al.*, 1995; Foster *et al.*, 1973; Getie *et al.*, 2005; Schmelzer *et al.*, 2005; Taddese *et al.*, 2008). Hydroxylation in elastin is catalyzed by prolyl hydroxylase (Uitto, 1979).

#### 2.2.2.3 Regulation of expression

Elastin synthesis starts early in development and is nearly repressed by maturity (Holzenberger *et al.*, 1993a; Holzenberger *et al.*, 1993b; Parks, 1997; Sephel *et al.*, 1987; Vrhovski and Weiss, 1998). It is synthesized and secreted from several cell types including smooth muscle cells, fibroblasts, endothelial cells, chondroblasts, and mesothelial cells (Starcher, 2000; Uitto *et al.*, 1991). Its expression is primarily controlled at the post-transcriptional level (Davidson, 2002; Parks, 1997). Compounds such as aprotinin (McGowan *et al.*, 1996; Parks, 1997), vitamin D, steroids, and phorbol ester have been shown to control tropoelastin's expression mainly by modifying the half-life of its mRNA (Hinek *et al.*, 1991; Parks *et al.*, 1992; Pierce *et al.*, 1992b). In contrast, studies of transcriptional regulation of elastin synthesis have identified a limited number of regulatory factors (Burnett *et al.*, 1982; Davidson, 2002; Pierce *et al.*, 2006). Given the fact that elastin is a stable biopolymer, post-transcriptional regulation of its expression is unexpected (Parks, 1997). Post-transcriptionally regulated proteins such as cytokines and oncogenes are expressed during physiologic transitions or for brief periods during developmental processes, where changes in the stability of their mRNAs rapidly regulate protein synthesis. In elastin, however, once the growth of tissue is complete new elastin synthesis is not required with the notable exception of the uterus (Urban and Boyd, 2000). Thus, it appears that unique and complex regulatory mechanism has evolved for the elastin gene (Parks, 1997; Vrhovski and Weiss, 1998).

#### 2.2.2.4 Secretion

Tropoelastin can be rapidly degraded by cysteine proteases if it is retained within cells (Davis and Mecham, 1996; Davis and Mecham, 1998; Grosso and Mecham, 1988). Secretion occurs at specific sites on the cell surface and this targeted secretion is thought to be mediated by an elastin receptor or chaperone complex (Davis and Mecham, 1996; Hinek and Rabinovitch, 1994; Hinek *et al.*, 1988). This chaperone complex consists of at least three protein subunits, where two of these subunits (61- and 55-kDa subunits) are cell membrane-associated proteins that immobilize the third, a 67-kDa peripheral subunit, also called elastin binding protein (EBP). EBP is an enzymatically inactive alternatively spliced variant of β-galactosidase and has two binding sites; one for tropoelastin and another for

lectin. EBP binds to hydrophobic domains in elastin with xGxxPG sequences but predominantly to the VGVAPG sequence (Grosso and Scott, 1993; Hinek *et al.*, 1993; Mecham *et al.*, 1989; Privitera *et al.*, 1998). On the outer side of the cell, the β-galactosugar-bearing moieties of the microfibrils bind to the lectin domain of EBP. This binding causes allosteric reduction in EBP's affinity to tropoelastin and dissociation of EBP from the integral proteins (Rodgers and Weiss, 2005). Thus, tropoelastin is separated and free for deposition, while the EBP is recycled by internalization (Privitera *et al.*, 1998).

#### 2.2.2.5 Elastic fiber assembly

Several *in vitro* reports have commended the hypothesis that tropoelastin is released and deposited on a pre-formed microfibrillar template in preparation for cross-linking (Cleary and Gibson, 1983; Fahrenba *et al.*, 1966; Kozel *et al.*, 2004). Microfibrils are expressed early in development and, thus, may play very important role in the maturation process of elastin. The high affinity cross-linking between tropoelastin and a sequence in the central region of fibrillin-1 is one strong example that shows the fundamental role of fibrillins (Rock *et al.*, 2004). Tropoelastin has also been shown to interact with fibrillins (Trask *et al.*, 2000), MAGPs (Brown-Augsburger *et al.*, 1996; Clarke and Weiss, 2004), proteoglycans (Reinboth *et al.*, 2002), and fibulins especially with fibulin-5 (Sasaki *et al.*, 1999; Wachi *et al.*, 2008). The C-terminal region of tropoelastin has been identified to mediate these protein-protein interactions and the β-sheet formation of the sequence encoded by exon 30, in particular, has been reported to initiate fiber formation (Kozel *et al.*, 2003). Fig. 2.3 gives an overall summary of known interactions between elastin and associated proteins (Wagenseil and Mecham, 2007).

However, even after characterizing several potential inter- and intra-protein interactions, the molecular basis on how microfibrils function as template for tropoelastin is still not fully understood. Several *in vivo* studies conducted to define the temporal hierarchy of interactions produced results, which led some even to question the need for microfibrils in the early phase of elastin synthesis (Kielty *et al.*, 2002). For example, all inactivation of genes encoding fibrillin-1 and -2, fibulins, MAGPs, or lysyl oxidases (LOXs) had little effect on elastic fibers formation (Chaudhry *et al.*, 2001; Kozel *et al.*, 2006; Pereira *et al.*, 1997). Furthermore, *in vitro* studies have shown tropoelastin's self-assembling capacity

without any or very minimal assistance from microfibrils. In this case, tropoelastin monomer under appropriate temperature and ionic strength has been confirmed to undergo a process of ordered self-aggregation (also known as coacervation) (Cox *et al.*, 1974; Kozel *et al.*, 2004; Volpin *et al.*, 1976). Coacervation is caused by multiple and specific interactions between hydrophobic domains with some degree of cooperativity with cross-linking domains (Kumashiro *et al.*, 2006; Vrhovski *et al.*, 1997).

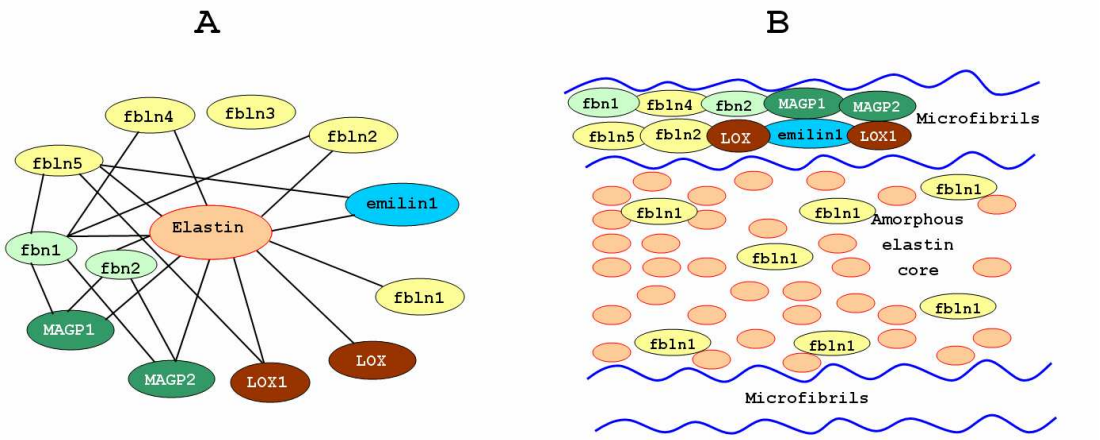



***Fig. 2.3:*** *Reported binding interactions (A) and spatial localization (B) of major components of elastic fiber proteins (elastin; fibrillin, fbn; fibulins, fblns; MAGPs; LOXs; and emilin)(modified after (Wagenseil and Mecham, 2007)).*

The self-assembling capacity of tropoelastin has also gained support from *in vivo* studies. Kozel *et al.*, for instance, described the sequence of events in elastic fibers formation with the help of a reporter protein (Kozel *et al.*, 2006). According to their observation, the first step in elastic fibers synthesis is the formation of small cell-surface associated tropoelastin globules. This early assembly process is known as microassembly and it may also involve fibulin-4 and/or -5 and LOXs. These small cross-linked aggregates remain on the cell-surface long enough until they become sufficiently large with the addition of new tropoelastin molecules. The elastin globules are then transferred to pre-existing microfibrils (primarily fibrilin-1 and/or -2 and probably MAGPs) and individual globules finally coalesce to form a larger elastin structure through a process of further cross-linking. This last step is called macroassembly. Therefore, the function of microfibrils in the entire process is not to initiate assembly, but rather to restrict either the extent or rate of tropoelastin aggregation.

A hypothetical model that tries to incorporate all known experimental results about the elastic fibers assembly process can be referred elsewhere (Wagenseil and Mecham, 2007).

Taken together, elastic fiber assembly has been proven to be a complex multistep process with several players; some have a direct while others have an indirect role, some have known while others have unknown functions. Alterations in the functions of these components have been shown to result in defects in fiber assembly, which can be expressed by elastic tissue phenotypes including in Marfan syndrome, supravalvular aortic stenosis (SVAS), and Williams-Beuren syndrome (Urban and Boyd, 2000). Some of these diseases are severe and at times life-threatening (Kielty, 2006). For this reason, the interest to understand the cellular and molecular basis of elastic fiber synthesis is still very high.

#### 2.2.2.6 Cross-linking in elastin

Cross-linking is a multistep process that begins with the alignment of monomers so that Lys residues come to proximity. Coacervation has been thought to facilitate the alignment process and thus help the oxidative deamination of certain Lys residues to form α-aminoadipic-δ-semialdehyde (allysine). Deamination is catalyzed by a family of enzymes known as LOXs (Uitto, 1979; Wise *et al.*, 2005). Of the LOX family members, only the mature enzymes LOX and LOX like-1 (LOXL-1) have been confirmed to play a role in elastin cross-linking (Borel *et al.*, 2001; Cenizo *et al.*, 2006; Smith-Mungo and Kagan, 1998; Wagenseil and Mecham, 2007). Once the reactive aldehyde derivative is formed, extensive cross-linking takes place in a rather complex and spontaneous fashion to form stable cross-links known as desmosine and isodesmosine through intermediate products of allysine aldol, lysinonorleucine, and merodesmosine (Eyre *et al.*, 1984; Narayanan *et al.*, 1978; Uitto, 1979; Vrhovski and Weiss, 1998).

Several investigators have tried to describe the nature and mechanism of cross-linking in mature elastin including the domains involved in the cross-linking (Baig *et al.*, 1980; Davis and Anwar, 1970; Foster *et al.*, 1974; Gerber and Anwar, 1975; Mecham and Foster, 1978; Vrhovski and Weiss, 1998). However, the complicated nature of elastin has made the progress in this regard slow and our knowledge with regard to the actual participation of domains in mature elastin is still not comprehensive. The only demonstration on specific in-

teractions that involve cross-linking of more than two chains describes the association of sequences encoded by exons 10, 19, and 25 joined together by one desmosine and two lysinonorleucine cross-links (Brown-Augsburger *et al.*, 1995). Recently though, with the help of sensitive analytical techniques such as MS and by employing novel approaches other investigators have provided some indirect but important insights into the cross-linking mechanism. For example, the central hydrophobic region of tropoelastin (domains 17–27) has been identified to be a "hot-spot" for potential cross-linking (Dyksterhuis *et al.*, 2007; Mithieux *et al.*, 2005; Wise *et al.*, 2005). Moreover, it is hoped that the application of modern techniques and the introduction of selective cross-linking agents combined with intelligent algorithms as tools to study proteins' structure can help to solve the puzzle of investigating cross-linking in elastin.

## 2.3 Collagens

Collagens are the most abundant proteins in the ECM and are involved in a broad range of functions. The name collagen is used to refer to a protein that forms a characteristic triple helix of three polypeptide chains. However, not all triple helix forming proteins are necessarily collagens by definition. Each polypeptide chain is mainly composed of Pro-rich Gly-X-Y repeating units where X and Y positions are frequently occupied by Pro and 4-hydroxyproline, respectively (Gelse *et al.*, 2003; Kadler *et al.*, 2007). Collagens also have non-triple helical domains at their N- and C-termini, termed as 'non-collagenous' (NC) domains and are numbered from the C-terminus (NC1, NC2, etc.) (Kadler *et al.*, 2007). The three α-chains are numerically numbered as α-1, -2, -3 and a collagen can have identical α-chains (homotrimer, e.g., collagen II) or can be different (heterotrimer, e.g., type I collagen, two identical α-chains and a third chain that differs). Each of these α-chains forms an extended left-handed helix with a pitch of 18 amino acid residues per turn. The three chains are wrapped one after another around a central axis in a right handed manner to form a triple helix. The chains are coiled around an axis in such a way to position all Gly residues in the center of the triple helix, while bulky side chains occupy the outer positions (Fraser *et al.*, 1979; Gelse *et al.*, 2003; Hofmann *et al.*, 1978).

Collagens have been known for long time now and to date at least 28 distinct types have been identified in vertebrates (numbered I- XXVIII) (Kadler *et al.*, 2007). There are also at

least 15 collagen-like proteins such as acetyl cholinesterase, adiponectin, ficolin, macrophage receptor and surfactant protein (Myllyharju and Kivirikko, 2001). Collagen type I exists widespread across the animal and plant kingdom. It is well characterized and is a model for other collagens in the family. Its triple helix has no imperfections, it assembles into fibrils, and it has a predominant structural role in tissues. Other collagens may lack several properties, for example, they have imperfections in their triple helix and thus are not fibrillar protein in their own right (Badylak, 2002; Kadler *et al.*, 2007). Collagens are a subject of numerous reviews with the emphasis on their synthesis, structural information, and their function (Bornstein and Sage, 1980; Canty and Kadler, 2005; Gelse *et al.*, 2003; Greenspan, 2005; Kadler *et al.*, 1996; van der Rest and Garrone, 1991).

## 2.4 Proteolytic processing in the ECM

Proteolysis in the ECM is one very important cause of changes in the cellular environment. Metalloproteinases are widely claimed to mediate almost all degradation processes in the ECM both under physiological and pathological conditions. Metzincins are very important members of metalloproteinases and they are recognized to play crucial role during ECM transformation. They are distinguished by a highly homologous motif containing three His residues that bind to zinc at the catalytic site and a conserved Met-turn that sits beneath the active site zinc. Their signature zinc-binding motif reads HExxHxxGBxHZ, where B is a bulky hydrophobic residue and Z is a family-specific amino acid (Sternlicht and Werb, 2001). MMPs are members of this superfamily (Mott and Werb, 2004) and most of them have a Ser residue at the Z position (Stöcker *et al.*, 1995). MMPs are the focus of the present work and are introduced further in the following sections.

### 2.4.1 Matrix metalloproteinases

Ever since the first discovery of a MMP (MMP-1) that dates back to 1962 (Gross and Lapiere, 1962; Woessner, 2002) and its purification almost a decade later (Bauer *et al.*, 1970; Brinckerhoff and Matrisian, 2002), the exploration of MMPs (matrixins) has been constantly expanding. At least 25 distinct vertebrate MMPs and 4 tissue inhibitors of metalloproteinases (TIMPs) are known until now. All of them are multidomain proteases synthesized with an approxim. 20 amino acid signal peptide and an approxim. 80 residues long

N-terminal propeptide followed by an approxim. 170 residue long catalytic domain (ctD). Some also have additional domains such as a hinge region, a hemopexin-like domain and repeats of fibronectin type II. On the basis of their domain organization, sequence similarity, and substrate specificity vertebrate MMPs can be divided into: **(1)** collagenases (MMP-1, -8, -13, and -18); **(2)** gelatinases (MMP-2 and -9); **(3)** stromelysins (MMP-3 and -10); **(4)** matrilysins (MMP-7 and -26); **(5)** membrane-type MMPs (MMP-14, -15, -16, -17, -24, and -25); and **(6)** those which cannot be classified in any of the above categories and this list includes MMP-12, -19, -20, -22, -23, and -28 (Visse and Nagase, 2003).

#### 2.4.1.1 Mechanism of catalytic action

The polypeptide chain folds of the ctD of MMPs are markedly similar and a given ctD is composed of 5 stranded β-pleated sheets, 3 α-helices, and connective loops. It also contains His-coordinated zinc and a water molecule. Glu adjacent to the first His (in the HExxHxxGxxH sequence) is essential for catalysis. The catalysis involves binding of a substrate into the catalytic site cleft through coordination of the carbonyl group of the peptide bond and the active site zinc. This arrangement displaces a water molecule from the zinc atom and allows the carboxyl group of the Glu to draw a proton from the displaced water molecule. This facilitates the nucleophilic attack of the water molecule on the carbonyl carbon of the peptide scissile bond (Chakraborti *et al.*, 2003; Pirard, 2007; Visse and Nagase, 2003).

#### 2.4.1.2 Regulation of activity

The activity of MMPs is tightly regulated at multiple steps including their synthesis, storage, activation, inhibition and clearance from the system. Their synthesis, in particular, is regulated primarily at the level of transcription by specific signals that are temporally limited and spatially confined (Curran and Murray, 2000; Mancini and Di Battista, 2006; Matrisian, 1994; Ra and Parks, 2007). Several *cis*-elements in the promoter regions of MMPs allow cell-specific expressions (Yan and Boyd, 2007; Ye, 2000). Although transcriptional regulation is the major control mechanism, some reports suggest that post-transcriptional mechanism is also involved, possibly by affecting the half-life of MMPs' mRNA. As an

example, transforming growth factor-β (TGF-β) increases MMP-2 and -9 levels in human gingival fibroblasts and prostate cancer cells (Yan and Boyd, 2007).

Once MMPs are synthesized, cells employ different mechanisms to confine proteolysis to specific locations within the ECM. For example, they use surface receptors to 'sniff out' the identity and location of specific substrates in the ECM. In this regard, integrin-ligand contacts provide cells with crucial signals to mediate the release of a particular enzyme to an identified location (Parks and Shapiro, 2001; Ra and Parks, 2007). Moreover, cells support targeted proteolytic activity within a pericellular space by storing MMPs anchored at specific locations on the cell membrane or specific parts of the ECM, thereby maintaining a locally high enzymes concentration. The binding of MMP-2 to the $\alpha_5\beta_3$ integrin (Brooks *et al.*, 1996), MMP-1 to the $\alpha_2\beta_1$ integrin (Dumin *et al.*, 2001), MMP-9 to CD44 (Yu and Stamenkovic, 2000), and MMP-7 to surface proteoglycans (Yu and Woessner, 2000) are good examples of compartmentalization effort to confine MMPs' activity.

#### 2.4.1.3 Inhibition

Inhibition is another mechanism of controlling the activity of MMPs. TIMPs are endogenous inhibitors of MMPs that bind in a 1:1 stoichiometry. They are secreted proteins, but may be found at the cell surface in association with membrane-bound proteins (Baker *et al.*, 2002). They are variably glycosylated and have N- and C-terminal domains of ~125 and 65 amino acids, respectively, with each containing 3 conserved disulfide bonds. The N-terminal domain folds as a separate unit and is capable of inhibiting MMPs although the C-terminal domain can also mediate some interactions (Nagase *et al.*, 2006; Wojtowicz-Praga *et al.*, 1997). The TIMP-MMP interaction is very similar to that of MMP-substrate interaction. For example, Fig. 2.4 illustrates the interaction between the ctD of membrane type-1 MMP (MT1-MMP) and TIMP-2, where the N-terminal four residues of the TIMP bind in the catalytic site cleft of the enzyme (Visse and Nagase, 2003). Other endogenous proteins that contain residues with some similarity to the N-terminal sequences of TIMPs can also act as MMP inhibitors (Baker *et al.*, 2002; Banyai and Patthy, 1999; Herman *et al.*, 2001). From these proteins, α2-macroglobulin (α2-M) has been identified to be the most prominent and clinically relevant inhibitor (Nagase *et al.*, 1994; Tchetverikov *et al.*, 2003). It is a large (750 kDa) protein produced mainly by the liver hepatocytes (Herman *et*

*al.*, 2001) and it is capable of entrapping and inactivating MMPs. However, as a consequence of its size, the inhibitory action of α2-M is mainly limited to the intravascular compartment (Baker *et al.*, 2002; Brew *et al.*, 2000; Nagase *et al.*, 1994; Wojtowicz-Praga *et al.*, 1997).

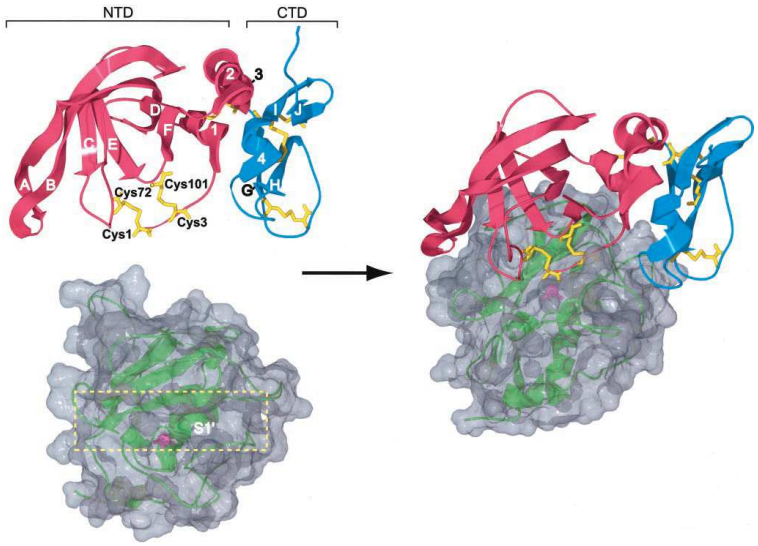



***Fig. 2.4:*** *Inhibition of MT1-MMP (bottom) by TIMP-2 (top). A complex is formed as a result of interaction between them. The location of the catalytic site cleft is indicated by a dashed rectangle while the active site zinc is visible as a pink sphere (adopted from (Visse and Nagase, 2003))*

## 2.5 MMP-mediated elastin degradation and its consequences

MMP-2, -7, -9, and -12 are the major elastinolytic proteases expressed in the ECM (Curci *et al.*, 1998). While MMP-2 and -7 are known to be constitutively expressed, the other two MMPs have to be stimulated by stress factors (Wielockx *et al.*, 2004). The upregulation of MMPs as a result of stresses usually causes damage to elastin and other components of the ECM (Chen, 2004; Murphy *et al.*, 1991). The degradation of elastin affects not only the structural integrity of the ECM but also produces fragments with capacities to regulate a variety of other cellular functions including disease processes (Nenan *et al.*, 2005; Shapiro, 1999). The subsequent sections provide a brief summary of common diseases, where MMP-mediated elastin degradation is claimed to be a common feature.

### 2.5.1 Vascular diseases

Aneurysmal diseases are the result of excessive breakdown of the ECM, primarily of elastin (Loftus and Thompson, 2002). Abdominal aortic aneurysm (AAA) is a progressive structural deterioration, gradual expansion, and eventual rupture of elastin-rich aorta. In aneurysmal aorta, the loss of elastin is a striking histological feature (only 8 % remain intact from 35 %) (Loftus and Thompson, 2002; Steinmetz *et al.*, 2003). A prominent infiltration of inflammatory cells and upregulation of a myriad of MMPs such as MMP-2, -8, -9, -12, -13, and MT1-MMP have been reported (Loftus and Thompson, 2002; Sakalihasan *et al.*, 1996; Thompson *et al.*, 1995; Thompson and Parks, 1996). MMP-9 and MMP-12 have been found overexpressed in macrophages associated with elastic fibers disruption in specimens of human AAA. In particular, MMP-12 has been observed to localize in the active "transition zone" of aneurysm, where remodeling and elastin degradation was still active. In fact, MMP-12 has been detected specifically bound to fragmented elastic fibers. This is in agreement with the *in vitro* results, which confirmed the superior binding affinity of MMP-12 to elastin compared to other elastases. Therefore, these and other similar findings imply that MMP-12 plays a central role in aneurysm formation in humans (Curci *et al.*, 1998; Shapiro, 1999; Thompson *et al.*, 1995).

Atherosclerosis is another important MMP-mediated vascular disease. It is a result of continuous inflammatory processes involving various cells, growth factors, and ECM degrading proteases. Studies have shown that disproportionate increase in the expression of MMPs plays a major role at different stages of atheroma formation (Knox *et al.*, 1997). During the initial stage, for example, MMP-9 along with other MMPs has been found to contribute to the degradation of endothelial cell basement membrane. As a consequence of this, the barrier function was altered and diapedesis of inflammatory cells was aggravated (Smeglin and Frishman, 2004). In addition, MMP-2 and MMP-9 have been reported to degrade the ECM and internal elastic lamina, clearing a path for the migration of smooth muscle cells from the media to intima (Bendeck *et al.*, 1994; Pauly *et al.*, 1994; Smeglin and Frishman, 2004). After plaques are formed increasing evidence suggests that several MMPs including MMP-2, -7, -9, -13, and -14 are involved in the degradation of the fibrous cap and plaque through their elastinolytic and collagenolytic activity (Galis and Khatri, 2002; Kong *et al.*, 2005; Loftus *et al.*, 2000).

### 2.5.2 Destructive pulmonary pathology

The primary structural fibrils of the lung are collagen and elastin (Starcher, 1986; Starcher, 2000). COPD is one of the major causes of mortality and morbidity and emphysema is a major source of COPD. Emphysema is characterized by relative excess of proteases and a relative paucity of anti-proteolytic defenses (Churg and Wright, 2005; Elkington and Friedland, 2006; Lagente *et al.*, 2005; Parks *et al.*, 2004). Historically, neutrophil elastase was considered to cause the major destruction, however, it is now known that MMPs are particularly involved in the process (Tetley, 2002). Clinically, the upregulation of MMP-1, -2, -8, -9, and -14 has been associated with the degradation of collagens (Elkington and Friedland, 2006; Turino, 2007), while the increase in quantity of MMP-12 has been claimed to be responsible for the degradation of elastin (Lagente *et al.*, 2009; Lagente *et al.*, 2005). In animal models, moreover, the involvement of MMP-12 and MMP-9 has been shown to play a role in pathological changes of cigarette smoke exposure (Churg and Wright, 2005; Hautamaki *et al.*, 1997; Lanone *et al.*, 2002). In particular, the upregulation of MMP-12 has been observed in all animals that developed COPD (Churg and Wright, 2005; Lagente *et al.*, 2005). Although MMP-7 is a potent elastase and is known to be upregulated in COPD, not many studies have been dedicated to investigate the contribution of MMP-7 in this disease process (Elkington and Friedland, 2006). In any case, taking animal and clinical data together, it becomes apparent that elastin-degrading MMPs play central role in the pathology of COPD and emphysema.

### 2.5.3 Tumor progression

The cellular and non-cellular compartments of the tumor microenvironment communicate mainly through the cooperation of multiple proteases, which create a milieu permissive of tumor progression, invasion and dissemination (Noel *et al.*, 2008; Overall and Kleifeld, 2006). MMPs have been confirmed to play a significant role in cancer progression (Egeblad and Werb, 2002; Fingleton, 2006; Ii *et al.*, 2006). Several *in vitro* and *in vivo* experiments showed a modulation of tumor growth and metastasis either through the use of inhibitors or deliberate deletion of MMP genes (Fingleton, 2006; Noel *et al.*, 2008). However, their involvement has proven to be complex and not all MMPs contribute to the pro-

gression of tumor. In fact, some play anti-tumorigenic role through processing of their substrates (Egeblad and Werb, 2002; Fingleton, 2006; Golubkov and Strongin, 2007; Lynch and Matrisian, 2002; Noel *et al.*, 2008; Yoon *et al.*, 2003). Elastin is also involved in several ways; on the one hand, the strong binding of tumor cells (melanoma) to insoluble elastin correlates with the metastatic potential of cancer cells (Lapis and Timar, 2002; Timar *et al.*, 1991). On the other hand, elastases including some MMPs generate bioactive products (also called matrikines) from elastin that cause multiple responses such as chemotaxis to tumor cells and induction of other MMPs that play a complex role in the carcinogenesis process (Lapis and Timar, 2002). Specific evidence on the role of elastinolytic MMPs during tumor progression include MMP-2 and MMP-9 (Lambert *et al.*, 2003; Masson *et al.*, 2005; Noel *et al.*, 2008), MMP-7 (Adachi *et al.*, 1999; Ii *et al.*, 2006; Yamamoto *et al.*, 2001), and MMP-12 (Heppner *et al.*, 1996; Kerkelä *et al.*, 2000). Taken together, through complex mechanisms including the generation of bioactive peptides, elastinolytic MMPs can control cell behavior with relevant consequences on cancer progression.

### 2.5.4 Generation of cryptic bioactive fragments

The degradation of ECM components releases fragments capable of initiating multiple signaling pathways (Maquart *et al.*, 2004). These cryptic bioactive fragments are small ligands from the entire parent molecule and are capable of interacting with cellular receptors such as EBP, integrin avβ3, and galectin-3 to induce biological activities (Duca *et al.*, 2004; Kohidai *et al.*, 2004; Maquart *et al.*, 2005; Maquart *et al.*, 2004; Pocza *et al.*, 2008). Elastin has been identified as one major source of matrikines in the ECM. For example, unidentified components of κ-elastin were found to unfold bioactivities such as induction of monocytes, chemotaxis to white blood cells, stimulation of fibroblasts, and also enhance angiogenesis (Maquart *et al.*, 2004; Robinet *et al.*, 2005). The rarely expressed domain 26A has also been reported to exert a relaxation activity on the vascular tone (Ostuni *et al.*, 2002). Several other ligands from human elastin including GLGVGAGVP, GFGVGAGVP, PGAIPG, and VGVAPG have also been confirmed to induce multiple bioactivities (Adair-Kirk and Senior, 2008; Duca *et al.*, 2004; Pocza *et al.*, 2008). Studies that aimed to associate structures of matrikines to biological activities have revealed that generally fragments that contain the xGxxPG consensus motif and form a type VIII β-

conformation can correctly bind to receptors to stimulate bioactivities (Duca *et al.*, 2004; Floquet *et al.*, 2004). Thus, it can be concluded that proteolytic modification in the ECM will not only lead to structural failure, as was once thought, but also activates cryptic molecules, which otherwise cannot be active before modification.

## 2.6 Analytical challenges of investigating elastin

Elastin remains one of the insufficiently characterized components of the ECM. The challenge of investigating elastin begins with its tissue distribution. Some tissues are rich in elastin, e.g., the aorta, and it is hence comparatively easier to isolate elastin, but others are poor in their elastin content. Furthermore, elastin exists covalently bound to several elastic fiber components and isolation of pure elastin requires harsh treatment to remove other components (Mecham, 2008). To date, isolation of free and intact elastin, particularly from diseased tissues, is a tremendously challenging task. Even after isolation, the mature elastin is not amenable for conventional wet-biochemical techniques. It can only be dissolved after hydrolysis. In effect, very important techniques such as X-ray crystallography and solution NMR can not be applied on intact elastin (Keeley *et al.*, 2002). Nonetheless, because of its central role in tissue physiology and pathologies the interest to understand the biochemistry and biomechanical properties of elastin remains high. Several methods, for example, circular dichroism (Debelle and Alix, 1995; Foster *et al.*, 1976), imaging techniques (Kozel *et al.*, 2006), and other biochemical methods (Brown-Augsburger *et al.*, 1996; Brown-Augsburger *et al.*, 1995) have been employed to get insights into the physical and chemical properties of this complex molecule. Each of these approaches has contributed to the present knowledge on elastin and its role in the ECM. However, the application of other novel techniques can obviously add another dimension to the research endeavor and generate helpful results. In this regard, MS has recently become one of the most successful analytical methods to investigate complex compounds. Continuous technical and conceptual advances in areas of MS, most notably on ionization techniques, analyzers, and data processing have given MS-based analysis an edge over other conventional biochemical techniques (Aebersold and Mann, 2003; Feng *et al.*, 2008). Therefore, the present study aspires to develop methods based on MS to investigate the biochemical basis of elastin and collagens degradation.

### 2.6.1 Application of MS in protein and peptide analytics

MS can be used for accurate mass determination or to study complex structures of proteins and peptides (Figeys *et al.*, 2001; Free *et al.*, 2009; Gingras *et al.*, 2005). In the latter case, the mass-to-charge ratio *(m/z)* of an isolated peptide/protein is initially determined followed by structural investigation after controlled fragmentation. Such experiments are called tandem MS (also known as MS/MS or $MS^2$). The subsequent sections briefly highlight the most important components of MS, i.e., ionization techniques and mass analyzers, followed by strategies of peptide sequencing from tandem MS. Since a typical workflow of MS experiments usually starts with separation of complex analytes, it is dealt with briefly first.

#### 2.6.1.1 The need for separation prior to MS analysis

On the account of their insoluble nature elastin and collagens cannot be analyzed directly with MS and a hydrolytic step is always necessary. However, the resultant hydrolytic products are complex mixtures of peptides, which are often challenging for analysis. For example, released peptides contain various modifications including cross-links. The hydrophobic nature of the peptides also affects the analysis since such peptides are less ionizable. Therefore, separation prior to MS analysis enhances the quality of data that can be generated by MS significantly. In this regard, gel-based separation techniques such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) can be used. However, gel separation may not necessarily perform well with the hydrophobic peptides of elastin (Barroso *et al.*, 2006). In addition, gel-based separation techniques have limited application for low abundant and low mass peptides. Thus, chromatographic separation has been the method of choice for sample treatment. The principle of liquid chromatography (LC) involves separation of the analyte by passing a sample solution through a column containing succession of stationary phases. The subtle structural differences between molecules are sources of separation. Both conventional and miniaturized LC methods can be implemented but the latter has the added advantage of concentrating the analyte and hence provides better sensitivity. Nanoflow LC, in particular, has been proven to enhance sensitivity significantly (Cutillas, 2005).

#### 2.6.1.2 Ionization techniques

Ionization techniques are critical in converting molecules in solution into ions in the gas phase. The most common soft ionization techniques applied in protein analysis are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). ESI creates ions from solution by spraying an electrically generated fine mist of droplets at atmospheric pressure. The formed ions are then transmitted into the ion optics of the MS for further processing. The physicochemical processes involved in the generation of ions are sufficiently described elsewhere (Bruins, 1998; Iavarone and Williams, 2003). In the case of MALDI, an analyte is allowed to dissolve and co-crystallize with an excess of a matrix on a metal target (plate). Most matrices are organic compounds of low molecular mass with a capacity to absorb laser energy of a certain wavelength. The sample plate with crystallized analyte is brought into a vacuum chamber of the MS and laser pulses are applied to cause a rapid desorption of the matrix into the gas phase and liberation of ionized analyte (Glückmann *et al.*, 2001; Yates, 2004; Zenobi and Knochenmuss, 1998). Advances in the refinement of ESI and MALDI have been made. The most notable improvements in the case of ESI have come from a reduction in the flow rate of the liquid, thereby improving the efficiency of ionization with an added advantage of concentrating the analyte as it elutes off the column. NanoESI is widely applied either on static mode or online by coupling LC to MS. MALDI has also led to the generation of matrix free ionization techniques such as surface-assisted laser desorption/ionization (SALDI) and desorption ionization on porous silicon (DIOS) (Pan *et al.*, 2007; Shen *et al.*, 2001).

#### 2.6.1.3 Analyzers

**A. TOF analyzers**

In time-of-flight (TOF) analyzers the *m/z* of an analyte ion is deduced from its flight time through a tube of defined length that is under vacuum. All the ions receive the same kinetic energy from a source and since they have different *m/z* values they will have different corresponding velocities as they traverse through the field-free region. Thus, they are resolved into groups or packets according to their *m/z* values (Domon and Aebersold, 2006; Guilhaus, 1995; Mamyrin, 2001). Steady technological advances in TOF analyzers have im-

proved the ions resolution significantly (Mamyrin, 2001). As an example, poor resolution arising from lack of discreteness of an initial ion packet in conventional linear TOF analyzers has been improved by extending the field free region and more importantly by introducing a unique technique called time-lag focusing. In the latter case, a time delay between the end of the ionization pulse and the start of the extraction pulse has been shown to improve resolution (Guilhaus, 1995; Mamyrin, 2001; Vestal, 2009). Another development utilizes an ion mirror to improve the resolution. An ion mirror works according to the principle that ions of the same *m/z* values but of slightly different kinetic energies enter into an electric field that opposes the electric field in the acceleration region. Ions with relatively higher kinetic energy travel deeper in the ion mirror than those with second higher energy. When ions leave the mirror, they leave with the same distribution of kinetic energies and velocities as when they entered but with different path lengths. The differences in path lengths correct time differences between isomasses; in effect, ions of the same *m/z* values will reach the detector at the same time (Marshall and Hendrickson, 2008). This operating principle is applicable in reflectron TOF analyzers. Further resolution enhancement has been achieved by orthogonal acceleration of ions. In this case, an ion beam travels in a direction perpendicular to the axis of the flight path and by doing so; the dispersion in kinetic energy can be minimized. TOF analyzers can be used to perform MS and MS/MS experiments especially when they are implemented as qTOF (a hybrid) or as TOF/TOF. Moreover, they can be operated with both ESI and MALDI (Domon and Aebersold, 2006; Guilhaus, 1995; Vestal, 2009).

### B. Quadrupole ion traps

Quadrupole uses an oscillating electric potential to focus ions toward the center of the traps. The quadrupole ion trap (QIT) functions both as an ion store in which case ions can be confined for a period of time and as a MS. In the later case, the *m/z* of the confined ion species can be measured by tipping the electric field of the ion trap in a particular direction so that ions tumble out of the potential well and leave the ion trap in order of ascending *m/z* values (Jonscher and Yates, 1997; March, 1997; Stafford, 2002). Conventional three dimensional analyzers (3D-QIT) employ a 3D quadrupole electric field to store ions in concentric 3D orbitals. Ions of the lowest stored *m/z* values occupy the outer layer and ions of successively higher *m/z* values occupying orbitals of progressively shorter radii. Older 3D-

QIT analyzers have typically limited trapping capacity and low resolution. However, a series of technological improvements produced modern 3D-QIT instruments with extended *m/z* upper limits up to 6000 and reduced lower mass limit especially while conducting MS/MS experiments (March, 1997). In the case of linear quadrupole ion trap *m/z* analyzers (LIT) ions are confined radially by a two-dimensional 2D radio frequency field, and axially by stopping potentials applied to the end electrodes. In comparison to 3D-QIT, LIT has higher injection efficiencies and ion storage capacities. Thus, their function is not limited to simply storing ions, rather, they can be implemented with multiple analyzers (e.g., Q-Q-LIT) to offer multiple functionalities with enhanced sensitivity (Domon and Aebersold, 2006; Douglas *et al.*, 2005).

#### 2.6.1.4 Sequencing strategies using tandem MS

Different approaches have been devised to sequence proteins using MS. Peptide mass fingerprinting was among the first to gain widespread popularity. In this method, an isolated protein is enzymatically digested with a specific protease (e.g., with trypsin) and the resulting peptide masses are measured typically with MALDI-TOF MS. The measured peptide masses are then searched against a database of proteins that have been digested *in silico* (Gevaert *et al.*, 2001; Thiede *et al.*, 2005). Another common strategy, called shotgun or bottom-up approach, involves pre-separation and/or enrichment of an enzymatically digested protein or protein mixture followed by interrogation of selected ions by MS/MS. The third method is called top-down approach and it involves direct analysis of the intact protein. In this case, since proteins are difficult to ionize identification requires MS capable of high resolution (lower ppm range) with the ability to fragment large analyte, for example, FT-ICR MS (Coon *et al.*, 2005; Ge *et al.*, 2002; Resing and Ahn, 2005).

The latter two sequencing approaches involve fragmentation of an ion of interest and record the *m/z* values of the resulting fragments. Fragmentation is usually performed by collision with an inert gas (CID). In this case, energy is applied to a precursor ion in the collision cell of the MS and depending on the quantity of the energy applied fragmentation will occur. Lower energies (typically 10–50 eV) tend to produce fragments generated by cleavages at peptide bonds, while higher energies (~ 1 kV) can produce other ions including those produced by internal fragmentations (Hernandez *et al.*, 2006). Results of fragmenta-

tion experiments are recorded as continuous signals and they need to be processed to get discrete values. Various signal processing algorithms can be used to convert the raw data into tandem mass spectra, wherein each fragment ion is represented by its *m/z* and the corresponding intensity value. Once tandem mass spectra are obtained, interpretation to get the original peptide or protein sequence is the next step. This step has also its own formidable challenges arising from incompleteness of fragmentation, complicated and less-understood fragmentation patterns and poor quality fragment spectra. However, it is often possible to influence the interpretation process positively by generating quality data through careful selection of methods including those involved during sample preparation and MS analysis. Generally, two approaches can be used to deduce sequences from tandem mass spectra as described in the following two sections.

### A. Sequence database searching

The governing principle in this case is for every unique peptide the complete spectrum of fragment ions is also unique. Depending on the mode of fragmentation different types of ions can be formed; the most common ions with soft ion activation methods are the y- and b-fragments. Searching a protein sequence database using MS/MS begins with the generation of candidate peptides by *in silico* digestion of theoretical proteins from the database. Those peptides with *m/z* values that match to the precursor ion mass are selected for further comparison and scoring. Filtering criteria such as enzyme specificity, species, and accurate mass can be used to reduce the number of candidates and computing time. Once candidates are selected, the experimental tandem mass spectra are then compared to the theoretical fragment spectra generated for each candidate peptide (Hernandez *et al.*, 2006). Various scoring methods are applied to judge the validity of each match; the most common ones are MASCOT (Perkins *et al.*, 1999) and SEQUEST (Eng *et al.*, 1994). Some scoring algorithms use both *m/z* value and intensity component of a spectrum, while others use only *m/z* component. However, predictable pattern of intensities of fragment ions, which is dependent on the collision energy and specific composition of the peptide, can help to confirm the identity of the matched peptide (Shadforth *et al.*, 2005).

## B. *De novo* sequencing

*De novo* sequencing seeks to use MS/MS spectrum of peptides as the sole reference for deducing their sequences. Therefore, this requires high quality spectra and complete enough set of fragment ions. *De novo* sequencing has particular advantages, for instance, when a reference database is not available or sequencing of artificial peptides is required, or when there are extensive PTMs. Several algorithms have been designed to automate *de novo* sequencing and the list may include PEAKS, MassSeq, Sherenga, and AuDeNs. The approaches these algorithms use to deduce sequences can be grouped into two broad categories. The first group works by building all possible permutations of sequences that can match the *m/z* value of the experimental precursor ion. Then, the theoretical fragmentation patterns created from these "pseudo-sequences" are compared against the measured tandem mass spectra and peptides with higher scores are presented as candidates for the original peptide sequence. However, the challenge of this approach is the degree of complexity will dramatically increase with the increase in the precursor mass value. In this regard, other additional information such as immonium ions, prior information on the amino acid composition, and accurate *m/z* measurements of the precursor are helpful in limiting the number of theoretical sequences. The software PEAKS, in particular, uses a robust algorithm to limit the number of pseudo-sequences to 10,000. Alternatively, other software tools depend on step-by-step building of a peptide sequence from the experimental tandem mass spectra until the sequenced peptide mass equals the measured precursor mass. This approach is referred to as peak succession approach and it is evidently much more sensitive to the quality of the MS/MS data submitted. The working principles of these algorithms are reviewed elsewhere (Hernandez *et al.*, 2006; Pevtsov *et al.*, 2006; Reinders *et al.*, 2004; Shadforth *et al.*, 2005; Xu and Ma, 2006).

# 3 RESULTS AND DISCUSSION

Elastin and collagens are the major components of connective tissues and they are subjects of damage when tissues are exposed to stress factors. For example, as compared to the area of skin protected from UV light (Fig. 3.1 A) elastin has been observed damaged when the skin was exposed to UV radiation (Fig. 3.1 B) (Pasquali-Ronchetti and Baccarani-Contri, 1997). Damage of important ECM components has been frequently associated with diseases such as atherosclerosis, emphysema, and cancer. At times, these diseases are severe, if not life-threatening. Therefore, it is of high interest to understand the biochemical basis of damages in tissues and the present study is designed to provide a comprehensive insight into the degradation of elastin, tropoelastin, and selected collagens of human skin under the influence of MMPs.

As described in the previous sections, molecular investigations on elastin and collagens will face hurdles arising from their structural complexities and the present study addresses these problems by developing MS-based analytical strategies. The methods basically involve a combination of separation techniques and MS to characterize degradation processes and the resulting products. The same methods were also employed to gain insight into the complex molecular structure of elastin and collagens. Experiments were performed either online, i.e., LC coupled to MS or offline, i.e., by fractionating complex samples into lesser complex components using LC followed by MS experiments. For exhaustive characterization of degradation products, a combination of different acquisition methods and MS analyzers were employed. MS instruments were typically equipped with an ion trap, orbitrap, qTOF, or TOF/TOF analyzer and ionization techniques were based on ESI, nanoESI, and MALDI. Applying a combination of these techniques has been proven to provide complementary information (Stapels and Barofsky, 2004). Peptides were sequenced by *de novo* sequencing and/or comparing experimentally acquired tandem mass spectra with *in silico* generated fragment spectra from sequence databases. Computational analyses were assisted by programs based on multiple algorithms including MASCOT, PEAKS, SPIDER, and PHENYX. When necessary, fragment spectra were analyzed manually to confirm the identity of sequences. This was particularly necessary to verify PTMs, in which case several candidates with modifications at different sites could be suggested. Furthermore, molecular modeling was performed using crystal structures of enzymes and modeled peptides

from the natural substrate with the help of software tools. Modeling was conducted to explain some of the observed specificity differences.

The study utilized human recombinant tropoelastin expressed in *E. coli*, mature insoluble elastin isolated from human skin using a method described elsewhere (Starcher and Galione, 1976), and bovine neck ligament elastin. An example of an electron microscopic view of elastin fibers isolated from human skin using a slightly modified procedure after Daamen *et al.* is shown in Fig. 3.2 (Daamen *et al.*, 2005). Synthesized peptides from isolated domain of human elastin were also employed for specific cleavage studies. Moreover, collagen isolated from human skin was employed to explore the binding and collagenolytic behavior of one of the three MMPs. The results of the degradation of elastin are presented in the first part of this cumulative thesis, while results regarding collagenolytic activity of MMPs are summarized afterwards.

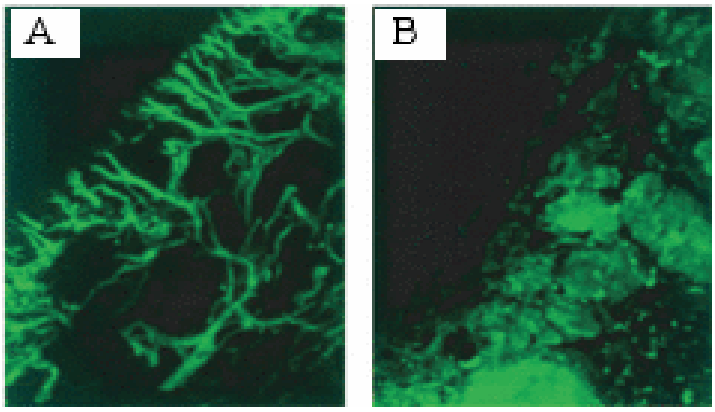



***Fig. 3.1:*** *Immunofluorescence staining of elastic fibers in sun-protected (A) and sun-damaged human skin (B) as visualized by confocal laser scanning microscopy (adopted from (Mahoney et al., 2009)).*

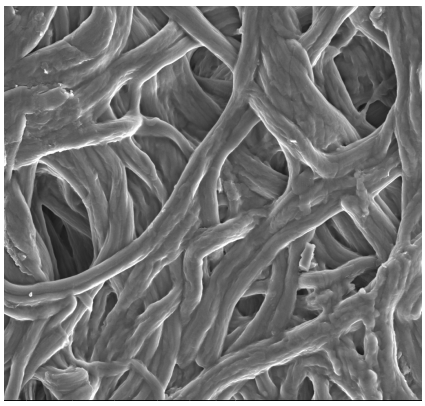


***Fig 3.2:*** *A transmission electron microscopic view of elastin (10 µm) isolated from human skin.*

## 3.1 Characterizing the degradation profile of human recombinant tropoelastin by MMPs

Human tropoelastin lacking domains encoded by exons 22, 24A, and 26A (Isoform 2, SHELΔ26A) prepared as described previously (Martin *et al.*, 1995) was used as substrate. The activated forms of MMP-7 and -12 were utilized as acquired from the manufacturer, while MMP-9 was activated as described in the manufacturer's guide. The profile of degradation was investigated by exposing tropoelastin to different concentrations of MMPs (e.g., enzyme-to-substrate ratios of 1:500, 1:5000, and 1:1x10000) in a buffer systems consisting of 50 mM Tris, 200 mM NaCl, and 10 mM $CaCl_2$ for MMP-7 and -12; 50 mM Tris, 100 mM NaCl, and 5 mM $CaCl_2$ for MMP-9 at pH 7.5. The degradation profiles were monitored with the help of MALDI-MS by taking aliquots from the respective digestion and control samples at different time intervals. The samples were mixed with the matrix solution (10 mg $mL^{-1}$ sinapinic acid in 0.1 % aqueous TFA/acetonitrile, 1:1, V/V) at a digest-to-matrix solution ratio of 1:5 (V/V). 0.5 µL of the mixture was spotted onto a stainless steel sample plate and measurements were performed after drying. To characterize the profile of degradation the appearance of released products as well as the disappearance of tropoelastin were monitored. The tests revealed that the three MMPs cleaved tropoelastin very fast and products could be detected even after the first minute of incubation. Interestingly, even at low enzyme concentrations (enzyme-to-substrate ratio 1:1x10000) perceptible degradation, especially by MMP-12, was detected very early (Fig. 1, appendix 6.1).

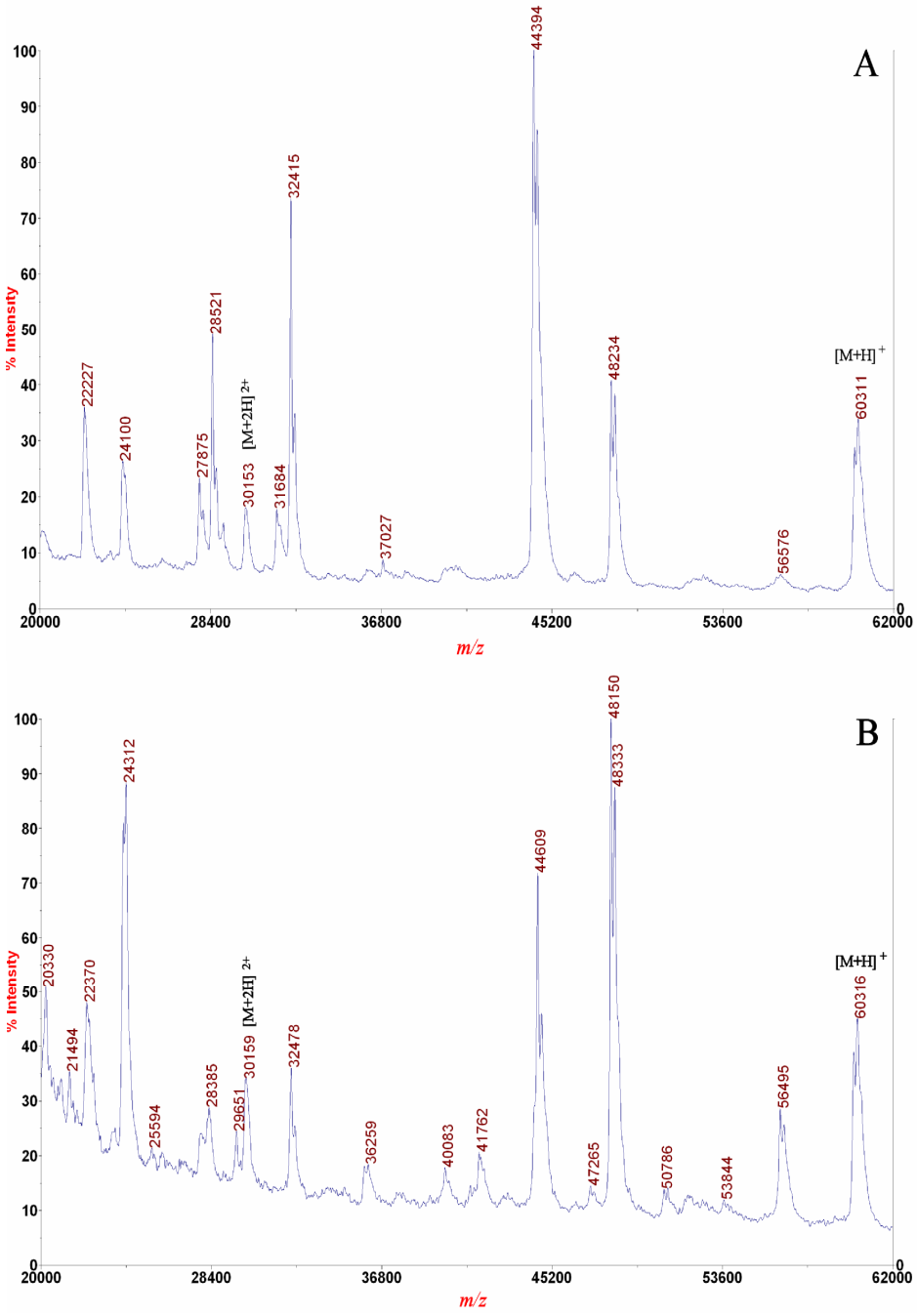
As the degradation continued it was possible to recognize that MMPs firstly generated higher mass polypeptides with characteristic patterns across the incubation period. These compounds include those with 32 kDa, 43 kDa, 44 kDa, 48 kDa, 53 kDa, and 56 kDa. These polypeptides were degraded to smaller products upon further incubation. During the early phase of degradation, MMP-7 and -12 typically produced polypeptides with masses of 32 kDa, 44 kDa, and 48 kDa. The compound with 44 kDa, in particular, was the most abundant peptide initially, while the compound with 32 kDa was the second most abundant. However, their abundance changed rapidly and the compound with 32 kDa became the most intense peak. In contrast, the 48 kDa polypeptide was produced rapidly and it was the most intense peak when tropoelastin was degraded by MMP-9. The pattern of forma-

tion of these polypeptides was reproducible over multiple concentrations of enzymes and separate experiments. Therefore, this may indicate that the most sensitive bonds that readily respond to MMP-9 reside at different locations in tropoelastin sequence as compared to sensitive bonds to MMP-7 and -12. Representative spectra that compare the formation of intermediate polypeptides by the three MMPs are shown in Fig. 3.3.

Further comparison of the three MMPs revealed that MMP-12 degraded tropoelastin faster than the other two enzymes. For example, intact tropoelastin could not be detected after 30 min incubation with MMP-12 (enzyme-to-substrate ratio 1:500, appendix 6.1), while it took at least 2 h with MMP-7 and even longer (3 h) with MMP-9 at the same enzyme-to-substrate ratio. With regard to the intermediate polypeptides, it took at least 2 h for MMP-12 to degrade them to smaller masses. In contrast, the intermediate polypeptides could be detected even after 4 h incubation in the presence of MMP-7 and MMP-9. In the control study, where tropoelastin was incubated in the absence of MMPs, tropoelastin remained intact during the course of incubation (Fig. 2, appendix 6.1). This ensured the cleavages in tropoelastin were induced exclusively by the MMPs.

Thus, this study confirmed that tropoelastin is very sensitive to the proteolytic activity of MMPs, especially to MMP-12. The degradation has been shown to proceed through the generation of intermediate higher mass polypeptides. Similar pattern of processing of tropoelastin was also reported both *in vitro* and *in vivo*. Serum proteases such as kallikrein and plasmin have been claimed to cause such processing in tissues (Romero *et al.*, 1986). The cleavages, particularly in domains 6 and 26, have been identified to form higher mass polypeptides with similar patterns (Jensen *et al.*, 2003; Jensen *et al.*, 2000; Kozel *et al.*, 2003; Mecham and Foster, 1977). The degradation of tropoelastin through intermediate polypeptides has also been reported to be mediated by metal-dependent enzymes (Hayashi *et al.*, 1995). In this study, the exact cleavage sites that led to the formation of the aforementioned intermediate products were not mapped. However, it was recognized that some of the peptides reported to exist in serum have similar masses with those polypeptides produced by MMPs. It should be underscored that the extreme sensitivity of bonds in tropoelastin to MMPs has several consequences. For example, the ability of tropoelastin to get involved in a process of new elastic fibers formation or repair of damaged elastin is de-

pendent on its intactness. Truncated tropoelastin has been proven to fail to undergo productive elastic fibers formation (Sato *et al.*, 2007).



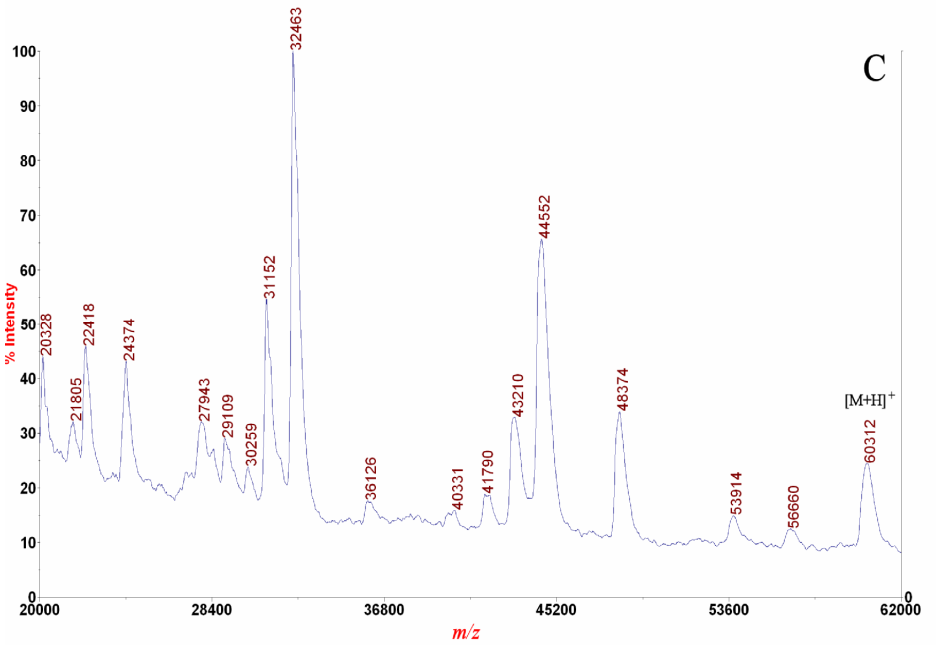



***Fig. 3.3:*** *Positive ion MALDI mass spectra of polypeptides generated when tropoelastin (shown by $[M + H]^+$ in the chromatogram) was incubated for 30 min in the presence of MMP-7 (A, enzyme-to-substrate ratio 1:500); MMP-9 (B, 1:500); and MMP-12 (C, 1:5000).*

## 3.2 The degradation of human recombinant tropoelastin by MMPs: mapping cleavage sites and analyzing specificities (Appendix 6.2, Heinz *et al.*, 2010)

To further understand the cleavage processes and map susceptible areas, tropoelastin was digested in the presence of MMP-7, -9, and -12 under the buffer conditions described in appendix 6.2. The digests were chromatographed using nanoHPLC and subsequently sequenced with the help of nanoESI-qTOF MS and MALDI-TOF/TOF MS. The sequence coverage of each digest is shown in Fig. 1 (appendix 6.2). Using these peptides, the susceptible areas in tropoelastin were mapped and cleavage specificities were characterized. As can be seen in Fig. 1, the three MMPs degraded tropoelastin extensively. MMP-12 produced at least 132 peptides, while 84 and 74 peptides were generated by MMP-7 and MMP-9, respectively. All three MMPs cleaved bonds throughout tropoelastin with frequent cleavages occurring mainly at the C- and N-terminus of the protein. In contrast, the central hydrophobic region of the tropoelastin sequence, particularly the region encoded by domains 17–25, was observed to notably resist proteolytic attacks. This region has been reported to contain residues responsible for inter- and intra-molecular contacts (cross-links)

during coacervation (Toonkool *et al.*, 2001; Wise *et al.*, 2005). It was, therefore, speculated that the incubation condition might support folding in the region and hide several bonds from MMPs attack. MMP-12 cleaved at least at 89 sites while MMP-7 and -9 cleaved at least at 58 and 63 sites, respectively. From the total cleavages, 24 were common sites for the three enzymes while 29 were shared by MMP-7 and MMP-9. Similarly, MMP-7 and MMP-12 shared 35 common cleavage sites, while MMP-9 and -12 exhibited the maximum common cleavage sites of 46. As also pointed out earlier, MMP-12 is the most active enzyme against tropoelastin and it was identified to cut at several sites, except in the domains encoded by exons 8, 9, and 11. In contrast, MMP-7 and -9 did cleave multiple linkages in several domains but no cleavage could be detected in domains encoded by exons 8-11, 19, 20, and 36.

MMPs involve primarily their ctDs to facilitate cleavage of substrates. The interaction between the zinc atom of the ctD and a residue constituting the scissile bond of the substrate has been identified to play the most important role in enzyme catalysis (Pirard, 2007). Other residue-subsite contacts that take place at pockets either to the right or to the left of zinc have also been recognized to support cleavage processes. Subsites differ in the manner they bind to substrates; some bind strongly while others exhibit weak interactions. The difference in the binding affinities can be tapped to design unique inhibitors that are selective in their interactions with a given MMP. However, the remarkable similarity between the 3D structure of MMPs and the resultant resemblance in substrate specificity always offer a formidable challenge to attain the objective of designing selective inhibitors. Fig. 3.4 depicts a ribbon structure of MMP-7, -9, and -12 aligned to demonstrate the degree of similarity these enzymes have.

A summary of specific residue-subsite interactions that show preferences of the respective subsites in the ctDs of the three MMPs is given in Tables 1 and 2 (appendix 6.2). As can be observed, the three MMPs cleaved typically N-terminal to hydrophobic and/or aliphatic residues such as Leu, Ala, Val, Gly, Ile, Tyr, and Phe with noticeable differences in their affinity towards individual residues. When residue preferences were determined on the basis of the number of cleavages each MMP initiated, the preference of the $S_1$` in MMP-7 followed the order Leu (48 %) >> Val/Gly (each 12 %) > Pro (10 %) > Tyr (5 %). The residue specificity of MMP-9 followed the order Leu (22 %) > Ala (19 %) > Gly (14 %) >

Lys (11 %) > Val (9 %), while MMP-12 showed a specificity according to the order Ala (26 %) > Leu (20 %) > Lys (12 %) > Val/Tyr (each 9 %) > Gly (7 %). It is evident that the three enzymes showed certain degree of preferences for amino acids at $S_1$`. To exemplify this, MMP-7 showed stronger preference for hydrophobic residues particularly for Leu (73 %, 29 out of a total of 40 Leu residues in tropoelastin) as compared to MMP-9 (35 %) and MMP-12 (48 %) at $S_1$`. It is also interesting to note that Ala, a relatively small aliphatic and hydrophobic residue, was moderately preferred at $S_1$` of MMP-9 and particularly preferred in MMP-12. However, very few cleavages (2 out of 157 possible x-Ala bonds) could be identified with Ala at $P_1$` for MMP-7. Moreover, all three MMPs appeared to cleave N-terminal to charged residues such as Lys with notable differences in their affinities. In particular, around 31 % of all x-Lys linkages in tropoelastin could be cleaved by MMP-12, while in the case of MMP-7 and MMP-9 the proportions were 3 % and 20 %, respectively. At other subsites, the three MMPs behaved more or less similarly and a detailed account has been provided in appendix 6.2.

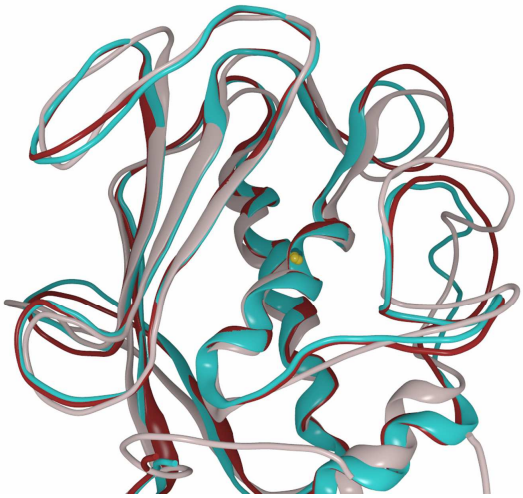


***Fig. 3.4:*** *Alignment of the ribbon structures of MMP-7 (gray), MMP-9 (red), and MMP-12 (cyan).*

Some of the observed residue-subsite interactions are interesting. Thus, to better explain these interactions the crystal structures of the three MMPs in complex with model peptides were graphically analyzed. As also described previously (Bode, 2003) and confirmed in this work (Fig. 3, appendix 6.2), the size and shape of individual subsites influenced residue-subsite interactions. For example, as evidenced by Fig. 3A, $Tyr^{214}$ of the $S_1$` loop in the

case of MMP-7 was identified to extend into the opening of the loop, while MMP-9 and -12 have the smaller Leu residue at the same position, giving the loop in MMP-9 and -12 an extended appearance. In addition, whereas the entrance of the $S_1$` loop in MMP-9 was restricted by the bulkier but flexible $Tyr^{393}$, the same position was occupied by the smaller $Thr^{210}$ residue in MMP-12. Therefore, bulky and aromatic residues such as Ser, Lys, and Arg were restricted from binding in the shallower $S_1$` pocket of MMP-7 but accommodated well in the deeper subsite of MMP-12. Furthermore, considering the hydrophobic nature of $S_1$` charged residues are not expected to be favored. However, the degree of hydrophobicity differs between the three MMPs. For example, the polar residue $Thr^{214}$ of MMP-12 is mutated to an aliphatic residue $Val^{398}$ in MMP-9 and $Ala^{215}$ in MMP-7. $Glu^{219}$ of MMP-7 and MMP-12 is also mutated to $Gln^{402}$ in MMP-9. Fig. 3C also confirmed that the bound model substrate with Lys at $P_1$` rests in the vicinity, where it is more hydrophobic in MMP-7 than MMP-12 and MMP-9. Therefore, the more hydrophilic and wider pocket in MMP-12 could accommodate Lys better than MMP-7 and -9. The other interesting residue-subsite interaction is that Pro was a preferred residue at $P_3$ (Tables 1 and 2, appendix 6.2). Modeling of the crystal structure of MMP-12 in complex with the natural substrate LPYGYGP (residues 226–233, tropoelastin isoform 2) revealed that Pro at $P_3$ appeared to force the backbone of the peptide into a conformation that favors the occupation of individual subsites (Fig. 4A, appendix 6.2). Furthermore, the positioning of Pro at $P_3$ allowed bulky residues such as Tyr at $P_2$ position to interact with $S_2$ (Figs. 4B and C, appendix 6.2).

Overall, as discussed in section 3.1 tropoelastin is degraded by MMPs rapidly and it is now known that most cleavages take place mainly close to the two terminal regions of the tropoelastin sequence. The rapid cleavages in the highly conserved C-terminal region, in particular, are interesting. The C-terminal domain of tropoelastin has been proven to be responsible for tropoelastin's binding with microfibrillar proteins and possibly cross-linking during elastin biosynthesis (Broekelmann *et al.*, 2008; Sato *et al.*, 2007). Products of terminally truncated tropoelastin sequences have been shown to cause errors in elastin biosynthesis. Examples of similar cases include SVAS and lamb ductus arteriosis diseases (Hinek and Rabinovitch, 1993; Tassabehji *et al.*, 1997; Wu and Weiss, 1999). Thus, the present study identified the susceptibility of tropoelastin to MMPs and further mapped susceptible bonds. The clear indication of these results is that tropoelastin will be rapidly processed when it is expressed in the presence of MMPs. Moreover, as previous results

showed, processed tropoelastin cannot effectively participate in elastin synthesis. Therefore, this underlines not only the direct degradative roles of MMPs on tropoelastin but it also shows the potential influence MMPs have on the ECM functions by undermining new synthesis and repair of damaged elastin. The information gained by mapping susceptible bonds in tropoelastin can also be applied in the designing of biomaterials based on tropoelastin. Efforts have already been made to develop resistant tropoelastin derivatives using recombinant technologies or chemical methods by modifying susceptible regions of tropoelastin without affecting functional properties (Ng *et al.*, 2008; Weiss, 2007). Furthermore, the cleavage site specificity study has provided several important results that can be used to understand tropoelastin-MMP interactions.

## 3.3 Investigation of the degradation of insoluble human skin elastin by MMPs

### A. Macrophage elastase-mediated degradation (Appendix 6.3, Taddese *et al.*, 2008)

MMP-12-mediated degradation of elastin is central to diseases like emphysema (Lagente *et al.*, 2005), atherosclerosis (Liang *et al.*, 2006), and certain cancerous diseases (Kerkelä *et al.*, 2000). However, it is not always easy to conclusively assign the role of MMP-12 in disease processes. The fact that other elastin-degrading MMPs are co-expressed makes the unequivocal determination of the primary etiological factor difficult. In addition, identification of other MMPs than MMP-12 as major players for the same disease but different disease models complicates the matter even further. In COPD, for example, while all animal studies have confirmed MMP-12 to be the most prominent player, some human studies suggested MMP-9 to play the major role (Elkington and Friedland, 2006). Therefore, under these circumstances a detailed investigation of the direct role of each MMP on the ECM components may help to characterize their role in disease processes. To assist this purpose, the present study investigated MMP-12-mediated degradation of elastin.

Elastin isolated from human skin was exposed to the activity of MMP-12 as described in appendix 6.3. The resultant peptides were characterized using HPLC-ESI-ion trap MS or offline by nanoESI-qTOF MS after HPLC fractionation. Since the biopolymer elastin releases a complex mixture of peptides upon degradation, it was necessary to develop an optimum chromatographic separation method that could also serve to characterize less

abundant products. Furthermore, fractionation and subsequent offline experimentation using nanoESI-qTOF MS gave the chance to conduct MS/MS measurements over longer time so that fragment spectra could be averaged to enhance quality. This was particularly necessary for the identification of less abundant species. The sequences of peptides were determined from their tandem mass spectra by database matching, *de novo* sequencing and a combination of *de novo* sequencing and database searching. Table 1 in appendix 6.3 summarizes the peptides generated by MMP-12 from insoluble elastin. A total of 41 peptides ranging from 4 to 41 amino acids were identified. Moreover, with the help of these peptides the cleavage sites were mapped and are shown in Fig. 2.

Additional measurements were also performed on the MMP-12 digest of elastin using nanoHPLC coupled to nanoESI-qTOF MS and offline on MALDI-TOF/TOF MS after nanoHPLC separation. Fig. 3.5, for instance, provides an overview of the peptides generated by MMP-12 when measured using nanoHPLC-nanoESI-QTOF MS (procedure is described in appendix 6.4). These additional experiments helped to characterize more degradation products than those reported in appendix 6.3. It was particularly possible to identify cleavages from domains in the central region of the elastin sequence. As described in appendix 6.3, almost no degradation product could be sequenced using ESI or nanoESI alone from this region. The identification of new degradation products can be ascribed to the complementary nature of the acquisition strategies (ESI and MALDI), which have been reported to preferentially ionize peptides of certain character. For example, MALDI has been identified to exhibit a slight bias towards peptides with basic or aromatic residues, while ESI tends to favor the identification of hydrophobic peptides (Stapels and Barofsky, 2004). Taken as a whole, using the combined strategies, it was possible to identify 96 peptides with lengths between 4 and 51 amino acids. In a similar study that aimed to characterize the MMP-12 degradation products of elastin, Barroso and coworkers could sequence much fewer peptides using an LC-MS approach (11 peptides with lengths ranging from 4 to11 residues) (Barroso *et al.*, 2006). Evidently, methods developed in this study achieved significantly better characterization of the cleavage products. It was, for example, possible to sequence low abundant peptides with masses up to 5 kDa. Thus, this underlines the advantages of employing multiple approaches to achieve better sequence coverage of a complex mixture like an elastin digest. The overall MMP-12-initiated cleavage sites are sum-

marized and discussed in section C in comparison with the MMP-7-mediated cleavage sites and specificities.

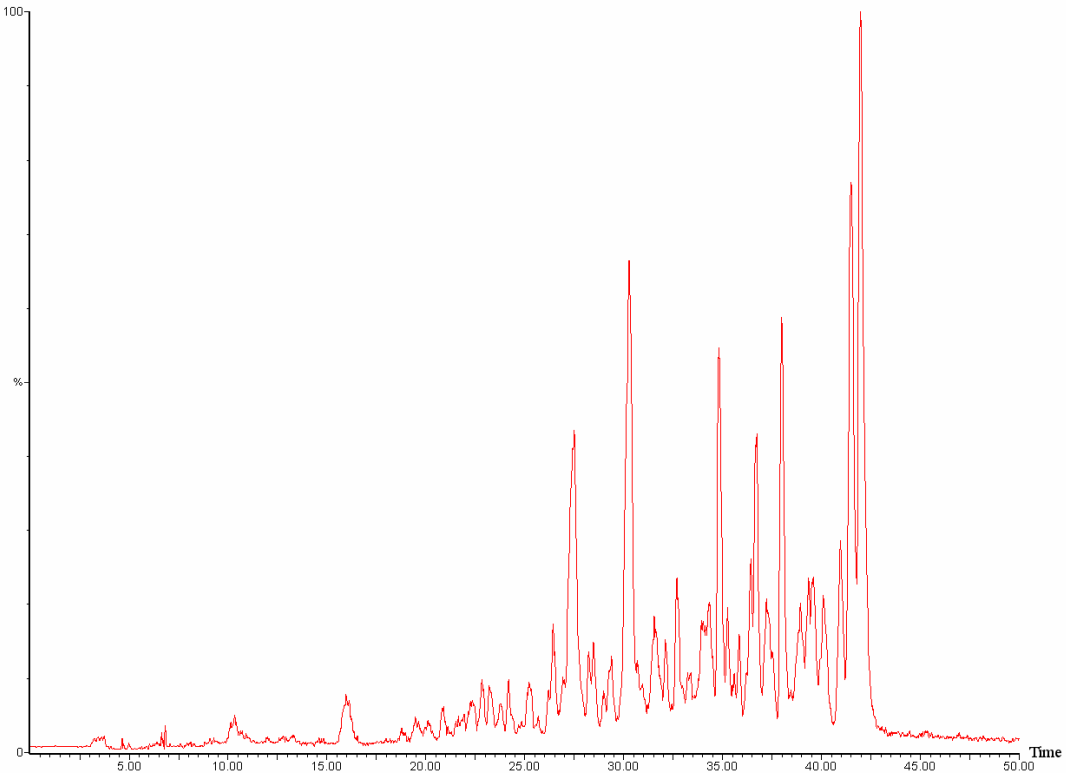



***Fig 3.5:*** *Base peak chromatogram of a MMP-12 digest of insoluble human skin elastin (enzyme-to-substrate ratio 1:100). The measurement was carried out using nanoHPLC-nanoESI-qTOF. It shows the elution profile of peptides released into solution using the method described in appendix 6.4.*

## B. Matrilysin-mediated degradation (Appendix 6.4, Taddese *et al.*)

Matrilysin is the smallest member of the MMP family and has been shown to be constitutively expressed in tissues (Ii *et al.*, 2006). It has important functions in the innate immunity of organs such as the lung and intestine (Burke, 2004; Wielockx *et al.*, 2004). However, its overexpression has been shown to lead to remodeling of the ECM. Of all the remodeling processes that MMP-7 has been claimed to be involved in, the most prominent and widely investigated one is its involvement in cancer (Ii *et al.*, 2006; Wielockx *et al.*, 2004). MMP-7 has been associated with almost all cancerous diseases of many organs of the body, for example, ovarian cancer (Wang *et al.*, 2005), esophageal adenocarcinoma

(Salmela *et al.*, 2001), and rectal cancer (Luo *et al.*, 2005). Its expression at almost every phase of cancer progression has been reported (Wielockx *et al.*, 2004). MMP-7 has also been associated with progressive inflammatory conditions such as atherosclerosis (Halpert *et al.*, 1996), elastotic material formation in the skin (Saarialho-Kere *et al.*, 1999), and other destructive pulmonary diseases (Elkington and Friedland, 2006). Although many diseases have been associated with the aberrant expression of MMP-7, its direct role has not been fully understood. Nevertheless, it is widely known that MMP-7 can degrade various components of the ECM, including elastin and its particular role in conferring macrophages with the most potent elastinolytic function has been demonstrated (Filippov *et al.*, 2003). The present study describes the degradative role of MMP-7 on mature elastin.

Human skin elastin was digested in the presence of MMP-7 (as described in appendix 6.4). The resultant degradation products were separated by nanoHPLC and analyzed either online with nanoESI-qTOF MS or offline with MALDI-TOF/TOF MS. Additionally, peptides were characterized offline using static nanoESI on qTOF MS after fractionation by HPLC. Fig. 1 in appendix 6.4 shows the elution profile of peptides generated by MMP-7 when analyzed by nanoHPLC coupled to nanoESI-qTOF MS. Peptides released by MMP-7 are summarized in Table 1 and with the help of these peptides it was possible to identify protease susceptible regions in mature elastin (Fig. 4).

Unlike MMP-7 and MMP-12, when elastin was incubated in the presence of activated MMP-9 no elastin-derived peptides could be detected even after 24 h incubation. This observation was unexpected considering the fact that the same enzyme could extensively degrade tropoelastin at the same buffer and pH conditions. Similar observations have been reported under slightly modified buffer conditions (12.5 mM Tris, 1.5 mM $CaCl_2$, 33 mM NaCl, 0.005 % Brij at pH 7.5) (Barroso *et al.*, 2006). Thus, given the fact that the same enzyme could effectively degrade tropoelastin, the result may lead to a speculation that MMP-9 binds less to elastin than tropoelastin. However, this has to be proven with further investigations.

## C. Comparison of matrilysin and macrophage elastase-mediated degradations

The overall sequence coverage and cleavage sites mapped using MMP-7 and MMP-12 digests of elastin are summarized in Fig 3.6. The sequence coverage is shown on the basis of the amino acid sequence of human tropoelastin isoform 9 (SwissProt accession number P15502-9). It can be observed that at least 81 susceptible linkages were mapped for MMP-12, while MMP-7 cut at least at 56 linkages in elastin. As a result of these cleavages, at least 95 peptides were released by MMP-12, while MMP-7 generated at least 52 peptides with lengths ranging from 5 to 87 amino acids. The comparison of the chromatograms of MMP-7 and -12 digests of elastin also provides a visual overview on the degree of complexity of the degradation products. Evidently, the chromatogram from the MMP-12 digest (Fig. 3.5) is more complex and appears to contain more detectable peptides than the MMP-7 digest (Fig. 1, appendix 6.4). This has also been reflected on the number of peptides identified from the two digests (52 vs. 95). This observation is consistent with results of a previous report, which has shown that MMP-12 binds to elastin more efficiently than MMP-7 (Curci *et al.*, 1998).

Furthermore, when cleavage sites recognized by MMP-7 are compared against those recognized by MMP-12 (Fig 3.6), it can be observed that approx. 40 % (38 out of 99) of the total cleavages are common sites for both MMPs. However, if the comparison is performed on the basis of the identities of peptides produced by the two MMPs, it becomes evident that MMP-12 produced several unique peptides (65 out of a total of 95) that could not be identified in MMP-7 digest. The difference in the sequence identities between these two digests is interesting and seems to present an opportunity to discern potential marker peptide or a set of peptides that may help to follow the role of MMPs in degradative disease processes. In this regard, a peptide mass fingerprint of the MMP-12 digest of elastin has been suggested as a predictor of disease processes such as in COPD (Broberg *et al.*, 2006). However, mass fingerprinting of complex biological samples requires the determination of masses with a high degree of accuracy, which is not suitable for routine purposes. Moreover, the reliability of mass fingerprinting may be limited by the fact that complex biological samples may contain several compounds with closely similar or identical masses. Thus, known peptides sequences generated by specific MMPs may be a better choice to address the setbacks of relying only on masses.


***Fig. 3.6*** *Sequence coverage from MMP-7 and MMP-12 digests of insoluble human skin elastin. Cleavage sites are highlighted by shaded triangles. The assignment is based on the sequence of human tropoelastin isoform 9 (SwissProt accession number P15502-9). Hydroxylated Pro residues are shown as "**p**".*

It also becomes clear from Fig 3.6 that both MMP-7 and -12 frequently cleaved linkages close either to the C- or N-terminus of the tropoelastin sequence. A similar pattern of preference was also noted in tropoelastin. Thus, at this point it can be concluded that elastinolytic MMPs prefer bonds in the region close either to C- or N-terminus, while bonds from residues encoded by domains close to the central regions of the elastin sequence are markedly resistant to the degradative activities of MMPs. Amino acids encoded by the exons 5–7, 26, and 30–33 were found to be particularly susceptible to MMP-7 attack, while none or few cleavages could be detected in linkages encoded by other exons. In contrast, MMP-12 cleaved in domains slightly more diverse than MMP-7. For instance, in addition to those mentioned for MMP-7, MMP-12 cleaved few linkages in the central hydrophobic region including the sequence encoded by exon 24. It is necessary to point out, however, that several cleavages mapped in tropoelastin could not be detected in elastin (compare Fig. 3.6 and Fig. 1 in appendix 6.2). Identification of a limited number of cleavages in elastin might be related to the extensive involvement of several domains in cross-linking. Cross-linking restricts the availability of stretches of residues to make productive interactions with enzymes. In this regard, the observation of the present study is in agreement with a recent result reported by Bertini and coworkers (Bertini *et al.*, 2009). They showed that at least 8–11 amino acid residues (4–7 residues N-terminal and 2–4 residues C-terminal to the amino acids containing the scissile bond) are required for MMP-12 to cleave a linkage in elastin. Fig. 3.7 shows, for example, an interaction between the ctD of MMP-12 and KPVPGGLAG of elastin (residues 64–72), where -Gly$^{69}$-Leu$^{70}$- is the scissile bond.

Moreover, to get insight on the specificities of the ctDs of MMP-7 and MMP-12, particularly of the $S_4$–$S_4$` subsites, residue preferences were comparatively analyzed and a summary is provided in Table 3.1. Similar to other MMPs in the family, both MMP-7 and -12 cleaved preferentially -Gly-Leu- bonds in elastin and as a possible consequence of its abundance, Gly is accepted relatively equally in $P_3$–$P_1$, $P_2$` and $P_4$` positions (Table 3.1). The $S_1$` subsite in both MMPs revealed a strong preference for Leu, which was followed by Gly and Val for MMP-7 and Val and Gly for MMP-12. MMP-7 also exhibited moderate affinity for Pro at the $S_1$` pocket and this contrasts a previous report, which showed that placing Pro at $S_1$` made cleavage of substrates undetectable (Netzel-Arnett *et al.*, 1993). However, the contributions of residues at other subsites have to be accounted in order to

make direct comparison of the influence of residues at $S_1$`. Whereas Pro at $P_3$ was accepted in both MMPs, it was further recognized that hydroxylation of Pro at $P_3$ did not affect the binding of Pro to $S_3$. HyP could still be accepted particularly by MMP-12. A detailed discussion on the residue preferences of each subsite in comparison with reported specificities is provided in the appendices.

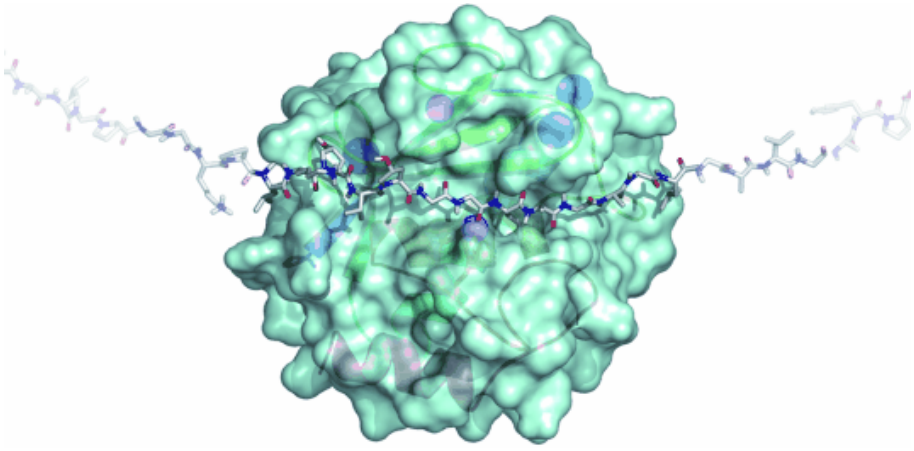


***Fig. 3.7:*** *A complex between the ctD of MMP-12 and an elastin fragment KPVPG**G**$^{69}$-**L**$^{70}$AG (residue 62-74). The ctD interacts with a stretch of residues which should be long enough to make a productive interaction (adopted from (Bertini et al., 2009)).*

***Table 3.1:*** *Residue preferences at $S_4$-$S_4$` subsites of the ctDs of MMP-7 and MMP-12 upon cleavage of insoluble human skin elastin.*

| | **$P_4$ / %** | | **$P_3$ / %** | | **$P_2$ / %** | | **$P_1$ / %** | | **$P_1$' / %** | | **$P_2$' / %** | | **$P_3$' / %** | | **$P_4$' / %** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **MMP-** | **7** | **12** | **7** | **12** | **7** | **12** | **7** | **12** | **7** | **12** | **7** | **12** | **7** | **12** | **7** | **12** |
| Gly | 28 | 27 | 37 | 32 | 40 | 39 | 47 | 51 | 18 | 12 | 50 | 47 | 23 | 28 | 37 | 28 |
| Ala | 13 | 15 | 15 | 17 | 25 | 24 | 12 | 20 | 5 | 11 | 15 | 20 | 28 | 29 | 13 | 23 |
| Val | 15 | 17 | 10 | 11 | 3 | 8 | 13 | 7 | 12 | 19 | 7 | 15 | 17 | 15 | 5 | 12 |
| Leu | 7 | 4 | 8 | 5 | 8 | 7 | 3 | 3 | 33 | 35 | 8 | 4 | 8 | 5 | 8 | 5 |
| Ile | 2 | 1 | 0 | 3 | 0 | 1 | 0 | 1 | 3 | 3 | 2 | 1 | 5 | 1 | 3 | 5 |
| Phe | 8 | 7 | 5 | 3 | 7 | 7 | 3 | 3 | 3 | 7 | 5 | 3 | 5 | 3 | 5 | 4 |
| Tyr | 2 | 0 | 0 | 0 | 2 | 3 | 2 | 0 | 3 | 7 | 2 | 1 | 2 | 3 | 2 | 0 |
| Pro | 12 | 13 | 13 | 15 | 5 | 5 | 7 | 4 | 15 | 8 | 2 | 0 | 7 | 11 | 15 | 11 |
| HyP | 5 | 7 | 3 | 8 | 3 | 4 | 5 | 7 | 2 | 1 | 2 | 0 | 0 | 3 | 0 | 4 |

Taken together, this study tried to characterize the degradation of elastin in terms of products released, susceptible degradation sites, and residue specificities of enzymes involved. The results provided insight as to how and where MMPs bind and degrade elastin. As also suggested, one potential application of the results of this investigation is the possibility of describing disease-associated products released into biofluids. In this regard the potential of developing marker peptide has been suggested. The results of residue specificity studies may also be used in the designing of inhibitors or better model substrates. Models, in particular, can be used in high-throughput screenings in the place of actual substrates.

### 3.4 Generation of matrikines from tropoelastin and elastin (Appendices 6.1, Taddese *et al.*, 2009 and 6.2, Heinz *et al.*, 2010)

As discussed in the preceding sections, elastin and tropoelastin are rich sources of bioactive peptides. Several proteases have been described to produce matrikines from elastin; the notable examples are leukocyte elastase (Lombard *et al.*, 2006) and proteinase 3 (Lombard *et al.*, 2005). However, limited or no information is available on the identity of MMP-generated matrikines from elastin and tropoelastin. Thus, the potential of the three MMPs to liberate peptides with bioactive and potentially bioactive sequences was analyzed. Tables 3 and 4 in appendix 6.2 summarize the sequences of peptides with bioactive motifs released from tropoelastin by the proteolytic actions of MMPs. All tested MMPs released several peptides from tropoelastin containing one or a combination of the GLGVGAGVP, PGAIPG, VPGVG, and VGVPG motifs with MMP-12 producing the highest number of potential matrikines. It is worth to note that some of the identified fragments contain bioactive motifs multiple times in their sequences. For example, the peptide 103–207 (*m/z* 9371) released by MMP-12 possesses 9 reported bioactive sequences or the peptide 121–207 (*m/z* 7860) released by MMP-7 and MMP-12 contains 8 bioactive sequences (Tables 3 and 4 in appendix 6.2). The generation of the entire sequence of domain 24 from tropoelastin by MMP-9 is also another notable example of the potential of MMPs in releasing multiple matrikines. Domain 24 contains several repeats of the VGVAPG sequence, a peptide that has been reported to be involved in various biological activities (Maquart *et al.*, 2005). The possible biological significance of having a repeated bioactive motifs in one peptide sequence has been proposed to favor overall bioactivity (Duca *et al.*, 2004).

Furthermore, the hexapetide VGVAPG has been reported to be the principal ligand of elastic receptor (Brassart *et al.,* 2001; Senior *et al.,* 1984). When released locally, it has been shown to induce series of signals that lead to myriads of activities. For instance, through its interaction with the galectin-3 receptor, it leads to biological functions that amplify melanoma invasion (Pocza *et al.,* 2008). Thus, it was of particular interest to explore the potential of the three MMPs in releasing VGVAPG from elastin. To study this, domain 24-encoded residues were synthesized as two fragments (PEPA and PEPB, appendix 6.1) and their susceptibilities to MMPs was thoroughly examined. As shown in Fig. 5 and Table 1 (appendix 6.1) several small-sized sequences were produced when PEPA and PEPB were exposed to the activity of MMP-12. It was observed that MMP-12 cleaved at several linkages in the synthesized peptides, provided that the active subsites ($S_4$ - $S_4'$) of the enzyme were occupied. The linkages -$Gly^{482}$-$Val^{483}$-, -$Gly^{500}$-$Val^{501}$-, and -$Val^{483}$-$Gly^{484}$- (on the basis of tropoelastin isoform 2) were identified to be susceptible to MMP-12 attack. Cleavages of these bonds resulted in abundant peptides as shown in the MS chromatogram (Fig 6, appendix 6.1). However, it is necessary to note that a considerable proportion of PEPA and PEPB was found to be undegraded after 10 h of incubation in the presence of MMP-12.

When PEPA and PEPB were exposed to the activities of MMP-7 and MMP-9 no cleavage could be detected. This shows that these two enzymes can hardly cleave in the region encoded by exon 24 even when the sequence is present in its isolated form. Consistent with these observations, MMP-12 cleaved at least at three linkages in domain 24 in intact tropoelastin (-$Val^{483}$-$Gly^{484}$-, -$Ala^{504}$-$Pro^{505}$-, and -$Gly^{523}$-$Val^{524}$-, Fig. 4 in appendix 6.1). These cleavages were identified in a 10 h digest of tropoelastin, while only one of the three cleavages could be detected in a 4 h digest (Fig. 4 in appendix 6.2). In insoluble elastin, four cleavage sites could be identified for MMP-12 (-$Gly^{507}$-$Leu^{508}$-, -$Gly^{531}$-$Leu^{532}$-, -$Ala^{545}$-$Pro^{546}$-, and -$Gly^{558}$-$Val^{559}$-, Fig. 3.7*)*, whereas no cleavages in domain 24 could be identified when elastin was incubated for 24 h in the presence of MMP-7 and MMP-9. It is necessary to recall from the preceding discussion that the linkages encoded by domains in the central region of the tropoelastin sequence are more resistant to proteolytic attack and that the domain 24-encoded sequence is one of the most hydrophobic sequences in tropoelastin.

Overall, the three MMPs produced several fragments with potential bioactivity as evidenced by the presence of motifs with reported activity. It appears that MMP-12 has a higher potential of producing matrikines. MMP-12 also showed a higher propensity of generating VGVAPG fragments from domain 24. These findings perhaps help to explain the biological activities observed in biological fluids containing the three MMPs. For example, monocytes recruitment has been observed in bronchoalveolar lavage fluid of MMP-12-expressing mice but not in MMP-12 deficient mice when model animals were exposed to cigarette smoke (Houghton *et al.*, 2006). EDPs were detected only in the lavage fluid from MMP-12 expressing mice but not from MMP-12 deficient mice. In another report, instillation of MMP-12 to the lung has been reported to recruit chemokines, macrophages or neutrophils to the lung (Greenlee *et al.*, 2007). However, additional experiments are needed to conclusively establish the *in vivo* significance of the potential matrikines identified by this study.

## 3.5 On the primary structure of elastin

### A. Identification of proline hydroxylation sites (Appendices 6.3, Taddese *et al.*, 2008 and 6.4, Taddese *et al.*)

MS provides an exceptional advantage to identify post-translationally modified sequences. Thus, in an attempt to characterize the Pro hydroxylation pattern in elastin, digests of MMPs were screened for the presence of peptides containing HyP residues. Table 3.2 provides a list of HyP-containing peptides generated by MMP-7 and MMP-12 from elastin. With the help of these peptides, sites of hydroxylation in elastin were mapped and are shown in Fig. 3.6 by small and colored letter "**p**". As can be seen, at least 15 hydroxylation sites could be assigned, out of which 10 have not been reported before. The hydroxylation of Pro in elastin is contentious topic and only few reports have been dedicated to exhaustively quantify the extent of hydroxylation. In fact, the first work that comprehensively identified such modification sites in human skin elastin was reported only recently (Getie *et al.*, 2005; Schmelzer *et al.*, 2005). Thus, together with previously identified 30 sites (Getie *et al.*, 2005; Schmelzer *et al.*, 2005) the total number of potential Pro hydroxylation sites in human skin elastin is 40. This is out of a possible 86 Pro residues in elastin, considering the most frequent isoform in the human skin. Literature reports that 0–33 % of the

Pro in elastin are hydroxylated (Bergethon *et al.*, 1989; Schmelzer *et al.*, 2005). However, it was not possible to infer the extent of hydroxylation from the present data since not all Pro at the same position but in different elastin molecules are always hydroxylated. Partial modification is not uncommon for complex proteins such as elastin. Worthy of note, nonetheless, is that the majority of the modified Pro in elastin lie at the Y position in the -Gly-X-Y- repeats, which is consistent with the sequence specificity of the enzyme prolyl hydroxylase (Rhoads and Udenfriend, 1969).

***Table 3.2:*** *List of peptides produced from human skin elastin by the action of MMP-7 and -12. They possess hydroxyproline residues in their sequences.*

| | $M_r$. | Sequence | Residue |
|---|---|---|---|
| **by MMP-7** | 1789.0 | G.LGAGIpGLGVGVGVPGLGVGAG.V | 581-602 |
| | 3348.9 | G.LGAGIpGLGVGVGVPGLGVGAGVPGLGVGAGVPGFGAVPGA.L | 581-654 |
| | 3364.9 | G.LGAGIpGLGVGVGVPGLGVGAGVpGLGVGAGVPGFGAVPGA.L | 581-654 |
| | 3888.2 | Q.LRAAAGLGAGIPGLGVGVGVpGLGVGAGVPGLGVGAGVPGFGAVPGA.L | 575-654 |
| | 4511.5 | L.VGAAGLGGLGVGGLGVPGVGGLGGIPpAAAAKAAKYGAAGLGGVLGGAGQFpLG.G | 710-763 |
| **Released by MMP-12** | 867.4 | I.pGGVVGAGPAA.A | 683-693 |
| | 1081.6 | Q.LRAAAGLGAGIp.G | 575-586 |
| | 1090.5 | A.FAGIpGVGPFGG.P | 186-197 |
| | 1109.6 | G.LGVSAGAVVPQp.G | 121-132 |
| | 1263.7 | A.GVLpGVGGAGVPGVp.G | 272-286 |
| | 1277.6 | I.PGVGpFGGPQPGVP.L | 190-203 |
| | 1334.8 | G.LGAGIpGLGVGVGVPG.L | 581-596 |
| | 1363.7 | A.LGGVGIpGGVVGAGPAA.A | 677-693 |
| | 1378.8 | G.LGVpGVGGLGGIppAA.A | 723-738 |
| | 1504.9 | G.LGAGIpGLGVGVGVPGLG.V | 581-598 |
| | 1535.8 | K.YGARpGVGVGGIPTYG.V | 383-398 |
| | 1665.9 | A.FAGIpGVGPFGGPQPGVP.L | 186-203 |
| | 1736.9 | G.AFAGIpGVGPFGGPQPGVP.L | 185-203 |
| | 2096.1 | A.FAGIpGVGPFGGPQPGVPLGYP.I | 186-207 |
| | 3348.9 | G.LGAGIpGLGVGVGVPGLGVGAGVPGLGVGAGVPGFGAVPGA.L | 581-654 |
| | 3583.3 | G.VGAGGFPGFGVGVGGIpGVAGVPGVGGVPGVGGVPGVGISPEA.Q | 399-441 |

*$M_r$, Calculated monoisotopic mass; p, hydroxyproline*

The role of HyP in elastin has not been fully understood although some reports show that HyP plays a minimal role during elastic fiber formation. For instance, the inhibition of Pro hydroxylation affected neither secretion of tropoelastin nor the oxidation of Lys residues nor did it affect the incorporation of tropoelastin into elastic fibers (Mecham, 1991; Narayanan *et al.*, 1977; Narayanan *et al.*, 1978; Rosenbloom and Cywinski, 1976). This has led

to the speculation that the synthesis of tropoelastin and procollagen at the same area in the endoplasmic reticulum may be the source of HyP in elastin. Under this circumstance, prolyl hydroxylase may act on some of the Pro residues in elastin (Rosenbloom, 1982; Uitto, 1979). However, the view of coincidental hydroxylation needs careful examination. There are reports which indicate over-hydroxylation of Pro has an effect on the stability of the tropoelastin secondary structure and the coacervation process with eventual consequence on the elastic fiber synthesis (Davidson *et al.*, 1997; Tinker and Rucker, 1985; Urry *et al.*, 1979; Vrhovski and Weiss, 1998).

## B. Cross-linking

The present study has also aimed at devising a MS-based method for the characterization of the cross-linking pattern in the mature elastin. Bovine neck ligament elastin was digested by pancreatic elastase (enzyme-to-substrate ratio 1:50) in 1 mM Tris at pH 7.5 for 24 h and the resultant peptides were separated by HPLC using an XTerra C-18 column (150 x 3.9 mm 5µm, Waters, Watford, UK). A methanol-water solvent system of A (20 % methanol containing 0.05 % formic acid) and B (80 % methanol containing 0.05 % formic acid) was used. The gradient follows: 0 to 75 % B in 25 min, to 100 % B in the next 10 min and remain at 100 % B for 15 min to return to 100 % A within 10 min. The elution profile of non-linear peptides was mapped by collecting fractions of every minute and analyzing the presence of cross-linkers in each step. In this context, non-linear peptides are those peptides which are modified by the presence of cross-linkers, mainly desmosine. The cross-linkers content at each step was determined after complete hydrolysis of peptides under boiling 6 N HCl for 36 h. A high proportion of desmosine-containing peptides was eluted between 15 and 25 min. Thus, this region of the chromatogram was further enriched with care to include only as few peptides as possible. Some fractions (particularly those $F_{19\text{-}20}$, $F_{20\text{-}21}$, and $F_{21\text{-}22}$; numbers in subscript are elution times in minutes) were then exhaustively investigated using complementary MS. The linear peptides were sequenced with the help of a combination of nanoESI-qTOF MS and MALDI-TOF/TOF MS, while the cross-link-containing candidates were further studied using LTQ-Orbitrap MS (Thermo Fisher Scientific, Bremen, Germany). The accurate masses were determined and the corresponding fragment spectra were acquired. As an example, the spectra of MS and MS/MS

experiments of a triply charged cross-linked peptide at *m/z* 855.4797 (derived from $F_{19\text{-}20}$) are shown in Figs. 3.8 and 3.9.

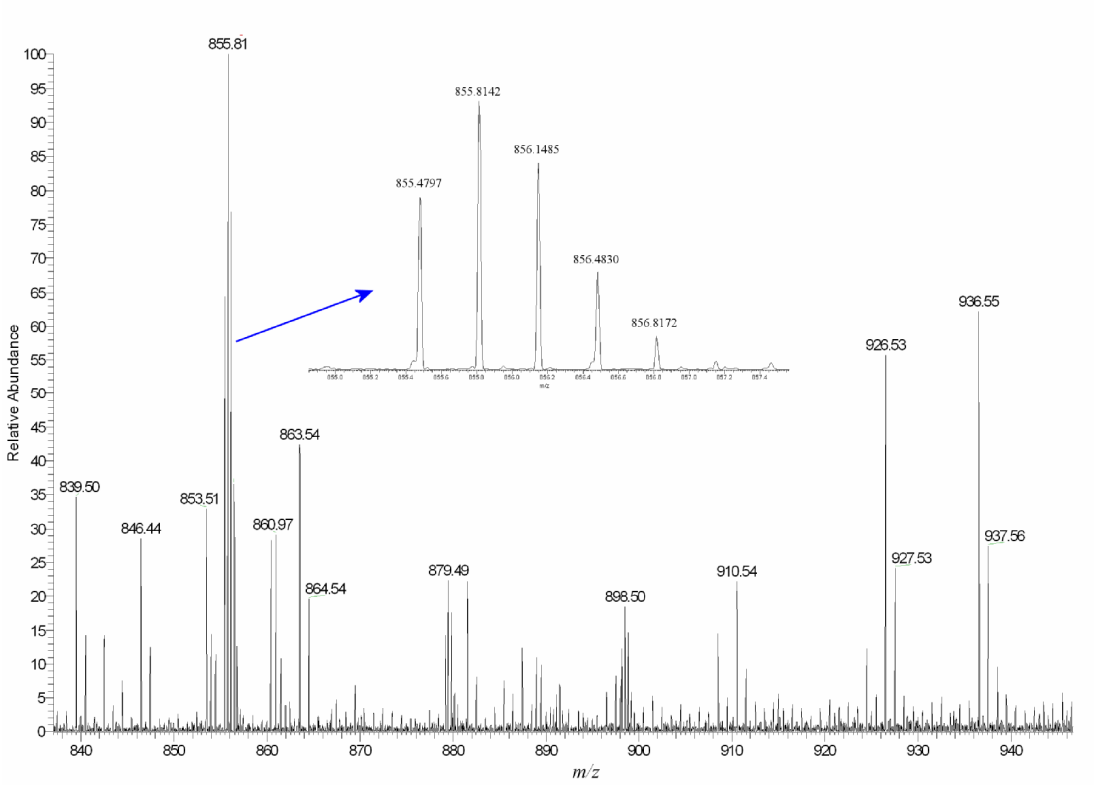



***Fig 3.8:*** *A positive ion full scan spectrum of pancreatic elastase digest of bovine elastin acquired using an orbitrap MS. The spectrum is magnified in the inset to show the isotope cluster of the triply charged species at m/z 855.4797.*

Using the accurate masses, candidate peptides were searched in the sequence from bovine tropoelastin isoform 1 (SwissProt accession number P04985-1) with the help of algorithms based on General Protein Mass Analysis for Windows (GPMAW), version 8.00 (Lighthouse Data, Odense, Denmark, http://www.gpmaw.com) (Peri *et al.*, 2001) and the software Links which is part of the Collaboratory for MS3D program (Sandia National Laboratories, Livermore, CA, https://ms3d.ca.sandia.gov:11443/cms3d/portal) (Schilling *et al.*, 2003). For searching purpose, previous knowledge on the cleavage specificities of pancreatic elastase in elastin was considered (Schmelzer, 2007). Up to five missed cleavages were allowed and the maximum error permitted was 5 ppm. Moreover, the hydroxylation of Pro and the changes in masses of peptides because of cross-linking were also taken into account. In this regard, lysinonorleucine and allysine aldol lead to mass reductions of

17.0266 Da and 20.0738 Da, respectively with respect to two Lys residues, while desmosine reduces by 58.1350 Da with respect to four Lys residues. Once candidate peptides were identified, matching of the experimental and the theoretical fragment spectra was performed using GPMAW and MS2Links, which is part of the Collaboratory for MS3D algorithms. All possible fragment types (a, b, c, x, y, and z) as well as losses of $-H_2O$, $-NH_3$, and -CO were considered during matching.

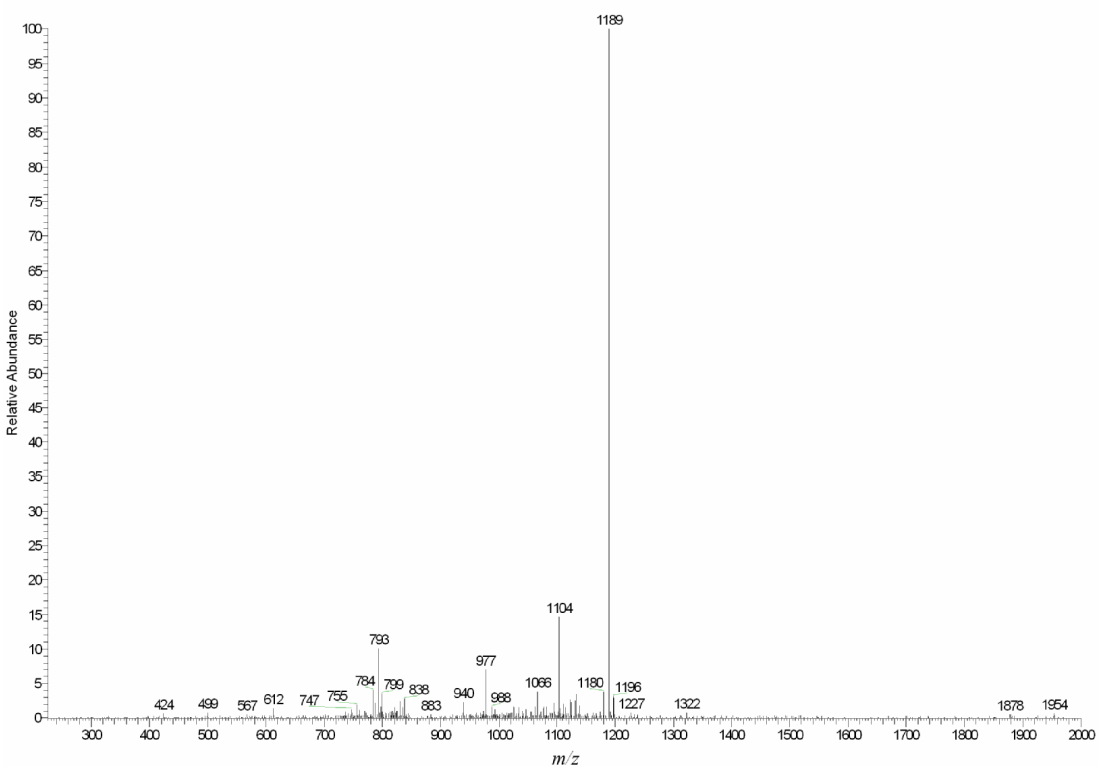



***Fig 3.9:*** *Fragment spectrum of a triply-charged peptide at m/z 855.4797 from a pancreatic elastase digest of bovine elastin. Acquisition was performed using LTQ-MS at 35 eV CID.*

Table 3.3 lists the lysinonorleucine- and desmosine-containing candidates for the peptide detected at *m/z* 855.4797. Previous modeling and direct investigation on elastin identified that tetra-functional cross-links, namely desmosine and isodesmosine can be formed in the region containing Lys residues separated by two or three Ala residues (Brown-Augsburger *et al.*, 1995; Foster *et al.*, 1976; Gerber and Anwar, 1975; Miao *et al.*, 2005). In contrast, Lys residues in Pro-rich regions, for instance the peptides shown in asterisks in Table 3.3, cannot form an α-helix due to steric constraints imposed by the presence of multiple Pro

residues and formation of desmosine is unlikely (Brown-Augsburger *et al.*, 1995; Tamburro, 2009; Vrhovski and Weiss, 1998). Thus, based on this and additional factors such as mass accuracy and matching of the fragment spectra, desmosine-containing candidates were considered to be unlikely for a peptide at *m/z* 855.4797. The accurate precursor mass determined using orbitrap MS has also enabled to make further comparison based on the elemental composition predicted using the monoisotopic mass (2563.4172 Da). Thus, 26 compounds were predicted within 5 ppm error margin and the compound with the composition $C_{119}H_{190}O_{31}N_{32}$ corresponds with candidate I (4 ppm error), while $C_{120}H_{190}O_{32}N_{30}$ corresponds to the elemental compositions of candidates II and III (0.4 ppm error). Candidates II and III have very similar sequences except for the position of one Ala residue on the first peptide sequence.

***Table: 3.3:*** *Candidate cross-linked peptides for a triply charged peptide detected at m/z 855.4797. Accurate mass was measured using orbitrap MS.*

| Candidate peptides | | Residue | *Mr.* | Error (ppm) | Elemental composition |
|---|---|---|---|---|---|
| **Lysinonorleucine** | | | | | |
| (I) | GVGG**K**PPKPFGGAL | 714–727 | | | |
| | GV**K**PKAQVGAGAFA | 185–198 | 2563.4274 | 4.0 | $C_{119}H_{190}O_{31}N_{32}$ |
| (II) | A**K**SAA | 543–547 | | | |
| | GPFGGQQPGLPLGYPI**K**APKL | 204–224 | 2563.4162 | 0.4 | $C_{120}H_{190}O_{32}N_{30}$ |
| (III) | AA**K**SA | 542–546 | | | |
| | GPFGGQQPGLPLGYPI**K**APKL | 204–224 | 2563.4162 | 0.4 | $C_{120}H_{190}O_{32}N_{30}$ |
| **Desmosine/Isodesmosine** | | | | | |
| | GPGV**K**PA**K**PGVGGL* | 57–70 | | | |
| | GVGGLGPGV**K**PA**K**PGV* | 52–67 | 2563.4034 | 5 | $C_{119}H_{188}O_{32}N_{31}$ |

* *Peptides from Pro-rich regions*

*Mr. Monoisotopic mass, Lys residues involved in cross-linking are shown in red*

*NB: No allysine aldol-containing candidate was found within 5 ppm error margin*

Furthermore, matching of the experimentally acquired tandem mass spectra with the theoretically generated fragment spectra of the candidates revealed that candidate II has several fragments that matched within an acceptable error margin of the MS instruments as is shown in Table 3.4 for qTOF MS. MS/MS experiments using LTQ and subsequent detection of fragment ions using orbitrap resulted in the reduction of intensities; however, the detected fragments matched with better mass accuracy (within 2 ppm error). Thus, putting

the evidence together suggests that the triply-charged peptide at *m/z* 855.4797 could be constituted by two peptides containing lysinonorleucine cross-link between $K^{544}$ (domain 25) and $K^{520}$ (domain 12). However, further confirmation of these results is necessary and can be carried out with additional experiments such as by conducting $MS^n$ for selected ions (e.g., the doubly-charged fragment at *m/z* 1188) or investigating the peptide using other modes of fragmentations, for instance, electron capture dissociation (ECD) helps to make extensive fragmentation by keeping PTMs intact (Sinz, 2006).

***Table 3.4:*** *Matched fragments of a triply-charged peptide (m/z 855.466) with the theoretically generated fragment spectra of the cross-linked peptide (candidate II in Table 3.3). Tandem mass spectrometric measurements were carried out using qTOF MS.*

| Measured *m/z* | Charge | Error (ppm) | Fragment | Ion* |
|---|---|---|---|---|
| 1066.106 | 2 | 10 | A**K**SAA–QQPGLPLGYPI**K**APKL | α/y16-$H_2O$ |
| 1103.660 | 2 | 25 | A**K**SAA–GQQPGLPLGYPI**K**APKL | α/y17 |
| 1122.589 | 2 | 30 | A**K**SAA–GGQQPGLPLGYPI**K**APKL | α/z18 |
| 1132.175 | 2 | 27 | A**K**SAA–GGQQPGLPLGYPI**K**APKL | α/y18 |
| 1139.141 | 2 | 16 | A**K**SAA–GPFGGQQPGLPLGYPI**K**AP | α/a19 |
| 1188.704 | 2 | 24 | A**K**S–GPFGGQQPGLPLGYPI**K**APKL | a3/β |
| 1196.640 | 2 | 28 | A**K**SAA–FGGQQPGLPLGYPI**K**APKL | α/y19-$H_2O$ |

α *Represents full length peptide 1 (AKSAA)*

B *Represents peptide 2 (GPFGGQQPGLPLGYPIKAPKL)*

* *Fragment ions of peptides 1 and 2 are separated by forward slash*

The nature of cross-linking in elastin is such that up to four Lys residues can be involved to form cross-links. The matter becomes even more complicated by the fact that more than two chains can be cross-linked by more than one polyfunctional cross-linker as has been identified for pig aortic elastin (scheme shown in Fig. 3.10) (Brown-Augsburger *et al.*, 1995). This pattern of cross-linking obviously yields a peptide with complex fragment spectra that can be difficult to interpret using common search engines employed to assign bi-functionally cross-linked peptides. This can be one of the reasons why cross-linking in mature elastin has not been investigated using MS so far. In this study, for reasons of complex fragmentation it was not possible to unequivocally assign tandem mass spectra of some modified peptides. Therefore, the attempt to sequence cross-linked peptides from elastin in the future needs to be supported by versatile and smart algorithms, which are capable of interpreting complex fragmentation patterns. Peptide characterization can also be facilitated by taking certain measures. For example, acquisition of precursor and frag-

ment ions of cross-linked peptides with high mass accuracy helps in limiting the combinatorial possibilities of candidate peptides. Targeting peptides of higher charge states (when ionized by ESI) can also be used to further filter out linear peptide candidates. Cross-linked peptides have at least two N-termini, which may support the formation of multiply-charged ions when ionized by ESI.

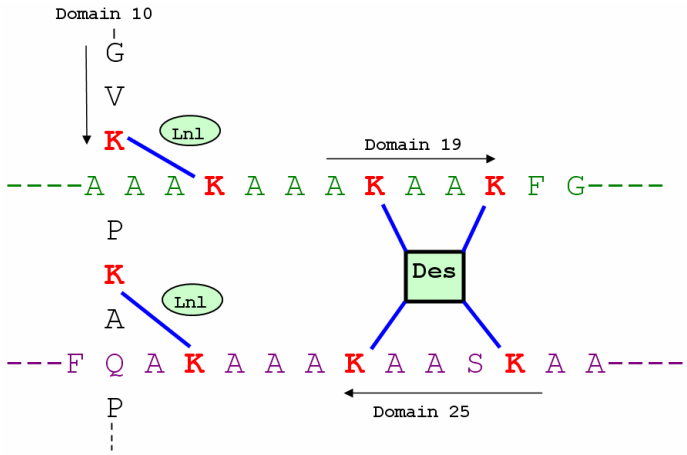



***Fig. 3.10:*** *Scheme of multifunctional cross-links formation that connects three bovine tropoelastin monomers. Desmosine (Des) is formed by four Lys residues from domain 19 and 25, while two lysinonorleucines (Lnl) connect domain 10, 19, and 25 (adapted from (Brown-Augsburger et al., 1995)).*

Furthermore, although cross-linker-guided fractionation of the elastin digest (the method adopted by this study) has helped to concentrate modified peptides, there was still a high degree of co-elution of non-modified peptides. Since linear peptides may decrease the signal intensity of modified peptides by ion suppression effect, this can create another challenge on MS-based sequencing of cross-linked peptides. Therefore, selective and affinity-based enrichment is another area, where further investigation should be directed. In this regard, a stepwise digestion of elastin beginning with less aggressive elastases can be one starting step towards concentrating cross-linked peptides. Weaker elastases help to remove peptides from non-cross-linked regions and subsequent elastase treatment will yield higher concentration of cross-link-containing peptides. Overall, based on the lesson gained from the present study a workflow that can be implemented to identify cross-linked peptides from elastin digest using MS is suggested and summarized in Fig. 3.11.

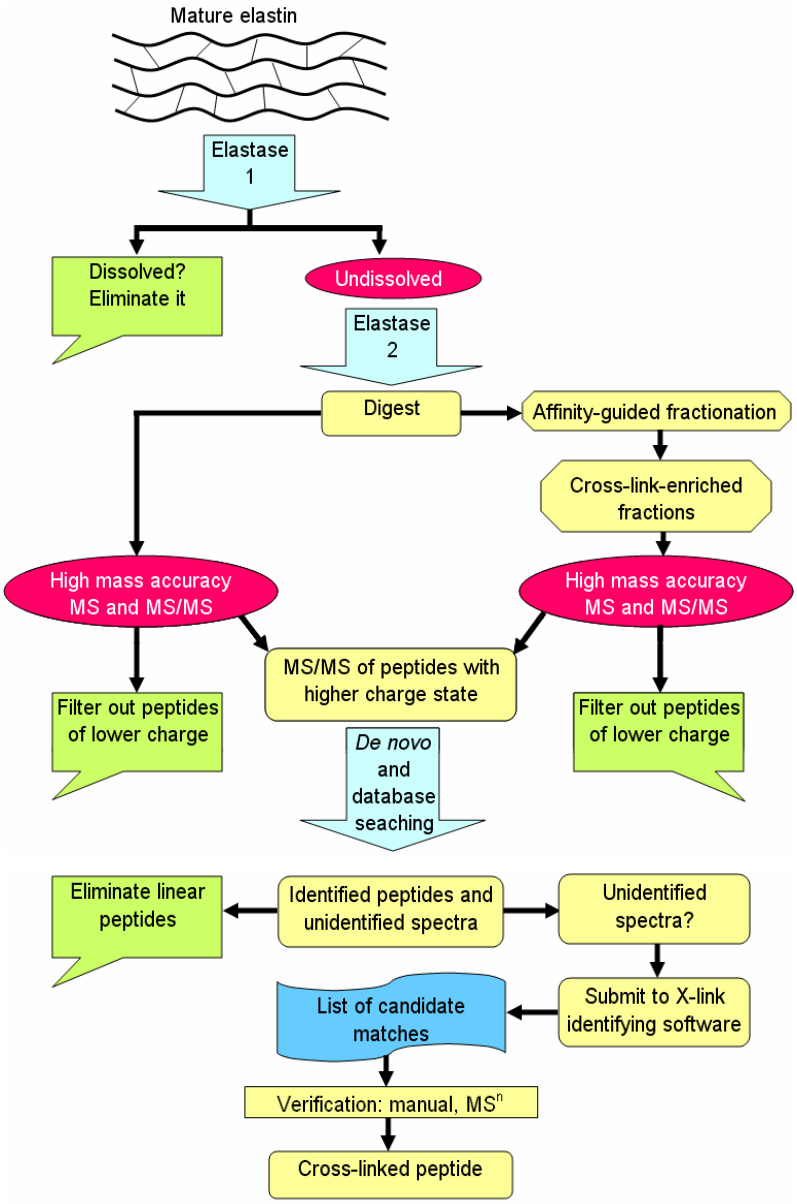



***Fig. 3.11:*** *A potential workflow for identification of cross-linked peptides from elastin using MS.*

## C. Domains subject to alternative splicing (Appendices 6.3, Taddese *et al.*, 2008 and 6.4, Taddese *et al.*)

Multiple elastin variants exist in tissues and one of the causes is alternative splicing of domains from the primary elastin transcript (Hirano *et al.*, 2001). Several domains are known to be spliced out in human elastin but specific domains such as domains 26A and 32 have been identified to be a subject of frequent excision in human skin (Hirano *et al.*, 2001).

The influence of the resulting isoforms on the physiological function of the final protein is not fully documented. However, some reports indicate that splice variants may play a role in disease processes. For example, in a recent report Chen and coworkers identified an increase in domain 26A-containing elastin in photoaged skin (Chen *et al.*, 2009). The authors suspected that domain 26A-containing elastin could contribute to the development of solar elastosis by changing the elastic fibers network. The present study tried to gain an overview on the domain composition of human skin elastin. In this regard, a maximum effort was exerted to identify peptides from the regions, which have been claimed to be alternatively spliced out.

Domain 26A is known to be unique to the human gene and expressed only under few circumstances (Hirano *et al.*, 2001). Both MMP-7 and MMP-12 are shown to cleave in residues encoded by domains 25–27. Thus, sequencing of the peptides released from the region closer to domain 26A confirmed the absence of residues encoded by exon 26A. For example, peptides generated by MMP-12 in the region are shown in Fig. 3.12. These peptides have sequences with N-termini that start at particular residues in domain 26 and stretch up to some residues in domain 27, excluding residues from domain 26A. This finding is in agreement with similar reports on human skin elastin (Getie *et al.*, 2005; Schmelzer *et al.*, 2005). Exon 32 is another example for frequent splicing in human skin elastin (Hirano *et al.*, 2001; Zhang *et al.*, 1999). In this work, 14 peptides from the region close to domain 32 were sequenced and all of them were found to possess residues from exon 32. Similarly, several peptides containing residues encoded by exons 24, 30 and 33 were identified. However, no peptide could be found from the region encoded by exons 22, 23 and 24A. Another interesting observation was made with regard to the highly conserved C-terminal domain of elastin. Residues from domain 36 have been reported to be either lost or highly modified during maturation of elastin (Broekelmann *et al.*, 2008). As discussed in the previous sections, both MMP-7 and MMP-12 cleaved preferentially in the C-terminal region of elastin. Thus, it was possible using these enzymes to investigate the availability of domain 36-derived residues in mature elastin. Several peptides from this region were sequenced; however, only 3 amino acids (out of a total of 14 residues encoded by exon 36) could be identified. This finding seems to agree with the previous observation, which suggested that the entire sequence of domain 36 can rarely (0.2 %) be incorporated in mature elastin (Broekelmann *et al.*, 2008).

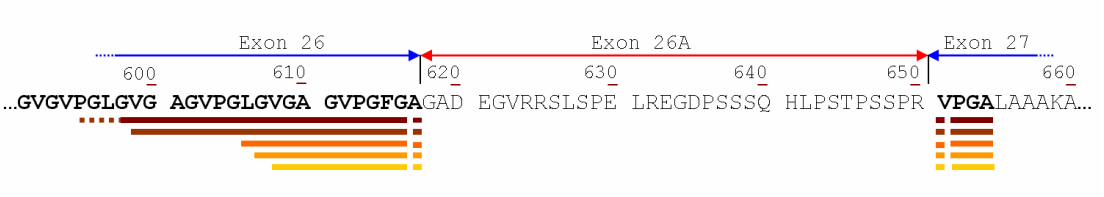



***Fig. 3.12:*** *Peptides released from insoluble human skin elastin by MMP-12. The peptides have sequences that start from exon 26 and then stretch in to exon 27, excluding residues from exon 26A.*

Moreover, during sequencing of peptides released from elastin it was found that one of the peptides could not be assigned to any of the 11 isoforms of tropoelastin in Uni-ProtKB/TrEMBL database with the accession number P15502. However, it could be assigned to a hypothetical sequence identified from human cDNA. This protein is identified in the database with the accession number Q8N2G0 and it contains 472 amino acids. The peptide sequenced from human skin elastin belongs to one polymorph of the elastin gene products. It contains an A-to-G polymorph in exon 20, which has been reported to be caused by conversion of the codon AGT (codes for Ser) to GGT (codes for Gly) at amino acid position 422 (Tromp *et al.*, 1991). The allele frequencies of such change is reported to be 36 % for A allele and 64 % for G allele (Hanon *et al.*, 2001). In human skin elastin, the residue Gly instead of Ser at position 422 could be identified (shown below). A similar observation was also made in a previous study on human skin elastin (Schmelzer, 2007). The functional significance of this type of change has not been well understood. However, it has been suggested to have significance on the carotid artery elasticity, especially on aged patients (Hanon *et al.*, 2001)

P15502: G.VGAGGFPGFGVGVGGIPGVAGVP**S**VGGVPGVGGVPGVGISPEA.Q
Q8N2G0: G.VGAGGFPGFGVGVGGIPGVAGVP**G**VGGVPGVGGVPGVGISPEA.Q

## 3.6 The catalytic domain of MMP-12 can bind and cleave collagens type I and III (Appendix 6.5, Taddese *et al, 2010.*)

Native collagens are resistant to many proteolytic enzymes and their degradation under inflammatory conditions requires the involvement of MMPs. A number of MMPs have been described to possess collagenolytic properties (Chung *et al.*, 2004; Lauer-Fields *et al.*, 2002; Lauer-Fields *et al.*, 2000). However, the role of MMP-12 in degrading collagen I has

not been addressed fully. Considering the abundance of collagen I in the ECM and the upregulation of MMP-12 under several progressive inflammatory conditions, it is necessary to establish the functional role of MMP-12 on collagen I catabolism (Nenan *et al.*, 2005). Based on this premise, the present work aimed to elucidate the degradation process of collagen I in the presence of MMP-12. It should be recalled, however, that MMP-12 has a unique feature of processing itself to a final active form (22 kDa) by releasing its hemopexin-like domain after initial activation to 45 kDa from a full length (54 kDa) (Shapiro, 1999; Shapiro *et al.*, 1993). This final active form has been reported to be physiologically relevant. For instance, in a tissue affected by aneurysm a significantly higher amount of the processed MMP-12 (22 kDa) has been identified in areas of extensive degradation (Curci *et al.*, 1998). Thus, this study utilized the truncated form of MMP-12 (physiologically active form) to investigate its binding and cleavage capacity against collagen I.

Collagen I was incubated in the presence of ctD MMP-12 under the conditions described in appendix 6.5. To characterize the profile of degradation, samples were frequently taken during the incubation period and qualitatively characterized with the help of MALDI-TOF MS. As shown in Fig. 3.13, peptides could already be detected in solution within 30 min of incubation. After 24 h, at least 90 % of the initial collagen was dissolved by MMP-12 and no intact collagen could be detected after 30 h incubation. The profile of peptides generated after 24 h is shown in Fig. 1 (appendix 6.5). To ensure collagens' triple helicity all experiments were performed at 33°C, a temperature well below the melting point of collagen. The triple helicity at 33 °C was tested and confirmed by incubating collagen in the presence of trypsin. Resistance of collagen I to the action of trypsin has been used as a test for stability of the triple helix (Kafienah *et al.*, 1998; Lee *et al.*, 1992). Moreover, the influence of other buffer components particularly dimethyl sulphoxide (DMSO) on the stability of collagen was checked by conducting separate experiments in the absence of these reagents. Taken together, the test procedure ensured the triple helicity of collagen under the experimental setup. Therefore, the evidence that the ctD of MMP-12 alone can bind and cleave collagen I is compelling.

With the help of nanoHPLC coupled to nanoESI-qTOF MS or offline with MALDI-TOF/TOF MS, the susceptible sites were mapped and it was found that MMP-12 cleaved

the typical collagenase sites in α-1 (-Gly$^{775}$-Ile$^{776}$-) and in α-2 (-Gly$^{775}$-Leu$^{776}$-) type I collagen. Besides, it cleaved at several other sites and all these cleavages were characterized and shown in Figs. 2 and 3 (appendix 6.5). Overall, MMP-12 cleaved at least at 53 sites in the α-2 chain, while 42 such sites could be identified in the α-1 chain.

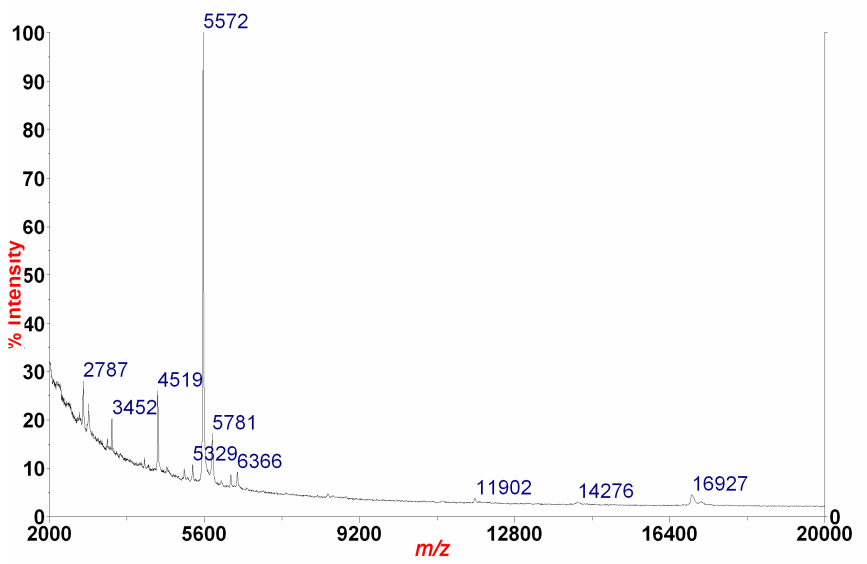



***Fig. 3.13:*** *Positive ion MALDI mass spectrum of the MMP-12 digest of human skin collagens (enzyme-substrate ratio 1:100) after 30 min incubation at 33 °C, showing early appearance of peptides released into solution.*

During sequencing of the released peptides some of the unequivocally identified sequences were assigned to collagen type III. These peptides were considered to be originated from type III collagen that was co-isolated with collagen type I. This is not unexpected because type III collagen exists in close association and is perhaps bound to collagen type I in the skin (Epstein and Munderloh, 1978; Friess, 1998). The overall cleavages caused by MMP-12 in collagen III are summarized in Fig. 4 (appendix 6.5). Similar to cleavages in collagen I, MMP-12 cut the typical collagenase site at -Gly$^{775}$-Ile$^{776}$- and multiple other linkages in collagen III.

Based on the overall cleavages mapped in collagens the residue specificities of MMP-12 have been characterized and summarized in Table 1. In general, the cleavage and residue specificities of MMP-12 are consistent with the preferences of other collagenases. The primed positions are poor in Pro/HyP residues, while the unprimed are rich in Pro/HyP.

Moreover, MMP-12 frequently cleaved -Gly-Leu- bonds. A detailed account of the individual subsite preferences is provided in appendix 6.5.

Furthermore, although the extensive hydroxylation of Pro in collagens is widely known, the modification patterns in human collagens have not been characterized so far (Nerenberg *et al.*, 2008; Nerenberg and Stultz, 2008). With the help of MS, it was possible to accurately map 16 and 26 Pro hydroxylation sites in α-1 and α-2 type I collagens, respectively. Similarly, 15 modification sites could be identified in collagen type III. The positions of modifications are summarized in the respective collagen sequences in Figs. 2–4 in appendix 6.5.

Hydroxyproline is known to provide collagens with the resistance to numerous proteases mainly by stabilizing their triple helical domains (Gelse *et al.*, 2003). In fact, the explanation of one of the proposed collagenolytic mechanisms entirely depends on the structure of collagens. According to this hypothesis, collagens exhibit chain flexibility in the region close to the typical collagenase cleavage site and a dynamic equilibrium between a folded (native) and partially unfolded (vulnerable) states exists. The chain flexibility depends highly on the Pro hydroxylation pattern (Nerenberg and Stultz, 2008). Therefore, collagenases attack collagens on the pre-formed vulnerable form (Nerenberg *et al.*, 2008; Nerenberg and Stultz, 2008). The findings of the present study are in agreement with the notion of two states of collagens. For example, in type I collagen the Pro at Y-position of the first Gly-X-Y triplet N-terminal to the typical collagenase site was hydroxylated in the α-2 chain, while it was not hydroxylated in the α-1 chain. Other Pro residues close to the scissile bond were also identified to be either partially or not hydroxylated. Hence, under these circumstances collagen I may exist in both partially unfolded and native states, where the unfolded form is vulnerable to the attack by MMP-12, as also described in the work of Nerenberg and Stultz. Fig. 3.14 illustrates the folding structure of collagen I in the region of the typical cleavage site under the conditions described by the present work (Nerenberg and Stultz, 2008). Hydrogen bonds exist in the native state of collagen (a and b), while in the vulnerable state hydrogen bonds seem to be disrupted (interchain distance shown in orange) (c and d). In the latter case, the α-2 chain exists in the partially unfolded state (Nerenberg *et al.*, 2008; Nerenberg and Stultz, 2008). Therefore, these results seem to be

consistent with the explanation of the recently proposed collagenolysis mechanism (Nerenberg *et al.*, 2008; Nerenberg and Stultz, 2008).

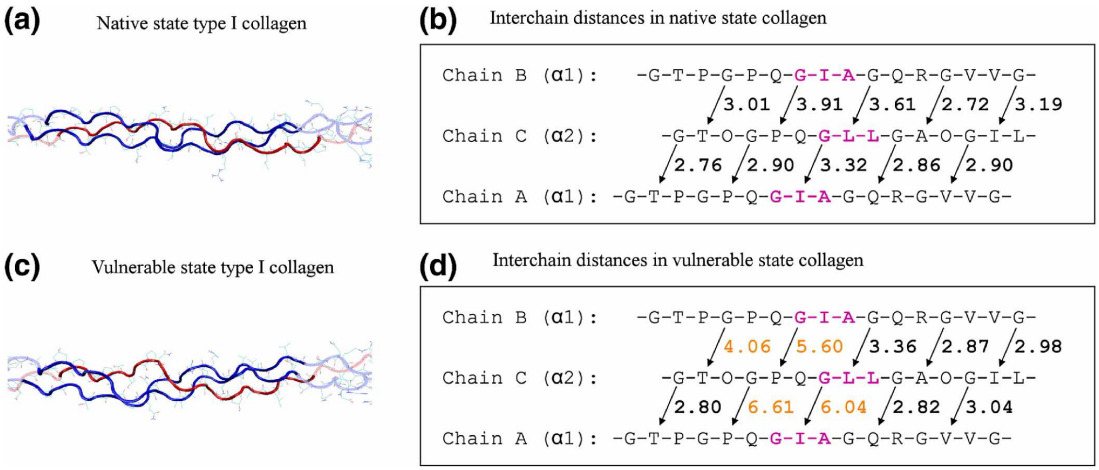



***Fig. 3.14:*** *Representative folding structures (a, c) and the respective interchain distances (b, d) of the native state and unfolded state of collagen I in the vicinity of the typical cleavage site. The Pro in the Y position of the immediate Gly-X-Y triplet upstream to the scissile bond is hydroxylated in the α-2 chain but not in α-1 chain. The α-2 chain is colored red (a & c) and the residues containing the scissile bond are shown in magenta (adopted from (Nerenberg and Stultz, 2008)).*

Taken together, the present work provided the first comprehensive and conclusive results confirming that the catalytic domain of MMP-12 alone can bind and degrade types I and III collagens. Areas susceptible to cleavages were exhaustively mapped and peptides generated as a result of the collagenolysis were sequenced. Furthermore, hydroxylation patterns were extensively characterized. These findings can help to advance the knowledge on the degradation processes taking place in the ECM. They also help to explain reported observations such as a recent finding by Nabha *et al.*, who have shown the MMP-12 dependent processing of collagen I by prostate cancer cells (Nabha *et al.*, 2008). Similar results have also been reported by Sarkar and coworkers. They identified the involvement of MMP-12 in regulating glioma invasiveness by degrading collagen I (Sarkar *et al.*, 2006). Finally and most importantly, the results of the present work can stimulate other *in vivo* studies to fully describe the physiological relevance of MMP-12-mediated collagenolysis.

# 4 SUMMARY

In the scope of this thesis, MS-based analytical methods were developed to investigate the molecular basis of the degradation of tropoelastin, elastin and collagens under the influence of MMPs. The degradation processes and the resultant products were exhaustively characterized using complementary MS methods based on multiple analyzers and ionization techniques. The mass analyzers typically used were quadrupole ion trap, qTOF, and TOF/TOF and the ionization techniques were ESI, nanoESI and MALDI. This work is one of the few attempts that utilized the unique advantages of MS to provide comprehensive and new insights in the degradation processes involving the aforementioned ECM components and MMPs. A detailed account on the findings of this work has been provided and the following is a brief summary.

## a) Characterizing the degradation of tropoelastin and elastin

Tropoelastin was identified to be very vulnerable to the actions of MMP-7, -9, and -12, with remarkable susceptibility to MMP-12 attack. Immediate cleavages in tropoelastin produced characteristic polypeptides including compounds of 32 kDa, 43 kDa, 44 kDa, 48 kDa, 53 kDa, and 56 kDa. These intermediate polypeptides were subsequently cleaved into smaller masses. Mapping of the susceptible regions in tropoelastin revealed that domains closer to either N- or C-termini of tropoelastin were particularly subjected to proteolytic attacks. In contrast, the linkages encoded by exons in the central region of tropoelastin sequence were found to be resistant. The degradation of elastin was also characterized and it was found that MMP-12 cleaved in at least 76 sites in elastin and released at least 95 peptides ranging in lengths from 4 to 51 amino acids. In the case of MMP-7, at least 54 peptides with lengths ranging from 5 to 87 residues were sequenced and 60 cleavage sites were mapped. Domains 2, 3, 5, 6, 26, and 30–33 were identified to be subjects of frequent attacks by MMP-7 and -12, while few or no cleavage could be detected in other domains. As a possible consequence of cross-linking, moreover, some of the cleavages identified in tropoelastin could not be detected when elastin was exposed to MMPs.

To gain insight on the specificities of the three MMPs, the overall cleavage sites were analyzed with respect to residues interacting with the $S_4$–$S_4$` subsites of the catalytic domains

of the three enzymes. It was possible to describe similarities and identify several important differences in the actions of the three MMPs against elastin and tropoelastin. The study also further tried to explain observed differences by molecular modeling using the 3D crystal structures of MMPs in complex with peptide substrates. It was, for example, possible to explain why the $S_1$` subsite of MMP-12 accommodated charged residues like Lys and bulky residues such as Ser, Lys, and Arg better than the other two MMPs. Overall, by precisely mapping cleavages with the help of MS, this study identified MMP-susceptible regions in both mature elastin and its monomer tropoelastin. It also provided comparative evidence on the residue preferences of MMPs. The information generated through this study can be used in many ways, for example, to describe MMP-associated molecular changes in the ECM.

**b) Generation of cryptic bioactive peptides from tropoelastin and mature elastin**

The three MMPs were confirmed to produce several potentially bioactive peptides from tropoelastin with MMP-12 releasing the highest number of matrikines. Sequencing confirmed that some of the potential matrikines contain multiple bioactive motifs. Previous reports have suggested that the presence of multiple motifs confer peptides with higher chances of initiating bioactivity. Further investigation of the potential role of MMPs in releasing the potent ligand VGVAPG from domain 24 confirmed that MMP-12 has the capacity to produce small fragments by cleaving at multiple sites in this region. However, further *in vitro* and *in vivo* screening is necessary to establish the role and biological relevance of these peptides.

**c) An overview on the primary structure of elastin**

MS-based method was also developed and used to gain information on the cross-linking pattern and PTMs in mature human skin elastin. It was, for instance, possible to probe and map the exact sites of at least 15 hydroxylated Pro residues, from which 10 sites are reported for the first time. Together with those identified previously, the total number of likely Pro hydroxylation sites in human skin elastin is 40 (out of a total of 86 Pro). However, since partial hydroxylation was confirmed to be common in elastin it was not possible to quantitatively estimate the extent of hydroxylation.

Furthermore, to get an overview on domains reported to be subjects of frequent splicing, peptides released from these regions were exhaustively screened. It was possible to unequivocally confirm the absence of residues encoded by exon 26A. All peptides sequenced from the region closer to this domain were found to be devoid of residues from domain 26A. In contrast, all residues encoded by exons 24, 30, 32 and 33 were confirmed to be present in the skin elastin sequence. However, no peptide could be identified in the region close to domains 22, 23 and 24A. Moreover, it is interesting to note that although several peptides could be sequenced in the region encoded by exon 36, it was possible to recover only 3 residues out of a possible 14. Residues of domain 36 have been reported to be either highly modified or lost during elastin maturation.

**d) The ctD of MMP-12 alone can efficiently cleave triple helix collagen I and III**

The second part of this thesis was dedicated to the development of MS-based methods to explore the role of MMP-12 in the degradation of collagens. With the help of these methods, it was possible to unequivocally confirm that the catalytic domain of MMP-12 alone can bind and cleave bonds in collagens type I and III. The susceptible sites in collagens were exhaustively mapped and it was identified that MMP-12 could cleave at the typical collagenase cleavage site. MMP-12 could also cut at multiple other sites. Moreover, since information on the hydroxylation patterns of Pro and Lys in human collagens are not available, MS was used extensively to probe and identify hydroxylation sites including in the region closer to the typical collagenase cleavage site. In collagen I, for example, the pattern of hydroxylation was confirmed to be in agreement with the possibility of co-existence of local native and partially unfolded states. Under such a condition, MMP-12 can attack bonds from the unfolded or relaxed chain. Taken together, the evidence presented by this work is the first major report that conclusively showed the degradative role of MMP-12 on collagen I. The results cannot only help to explain certain disease processes, but also stimulate further *in vivo* studies to define the physiological relevance of MMP-12-mediated collagen destructions.

## ZUSAMMENFASSUNG

Im Rahmen dieser Arbeit wurden massenspektrometrische Methoden entwickelt, um den durch MMPs hervorgerufenen Abbau von Tropoelastin, Elastin sowie zwei Kollagenen auf molekularer Ebene zu untersuchen. Der Abbauvorgang bzw. die dabei entstehenden Peptide wurden durch den Einsatz komplementärer massenspektrometrischer Techniken eingehend charakterisiert. Als Massenanalysatoren kamen Ionenfallen, qTOF sowie TOF/TOF und als Ionisationstechniken ESI, nanoESI sowie MALDI zum Einsatz. Diese Arbeit ist eine der wenigen, die sich die außergewöhnlichen Möglichkeiten der Massenspektrometrie zu Nutze macht, um neue Einblicke in die Degradationsvorgänge der zuvor genannten ECM-Bestandteile durch MMPs zu erhalten. Im Folgenden sollen die Ergebnisse dieser Untersuchungen noch einmal thematisch zusammengefasst werden.

### a) Charakterisierung des Abbaus von Tropoelastin und Elastin

Für Tropoelastin konnte gezeigt werden, dass es sehr angreifbar gegenüber MMP-7, -9 und im Besonderen durch MMP-12 ist. In der Anfangsphase des Abbaus von Tropoelastin wurden charakteristische Polypeptide von 32 kDa, 43 kDa, 44 kDa, 48 kDa, 53 kDa sowie 56 kDa erzeugt. Diese Zwischenprodukte wurden im Folgenden weiter gespalten. Die Zuordnung der für den Abbau anfälligen Bereiche Tropoelastins ergab, dass insbesondere Domänen des N- und C-Terminus Tropoelastins proteolytisch gespalten wurden. Für die zentralen Bereiche des Substrats wurde hingegen gefunden, dass sie relativ beständig sind. Der Abbau von Elastin wurde ebenfalls charakterisiert, wobei 76 Spaltstellen sowie die Freigabe von 95 Peptiden mit Längen zwischen 4 und 51 Aminosäureresten nachgewiesen werden konnten. Im Falle von MMP-7 wurden 54 Peptide mit Längen zwischen 5 und 87 Aminosäureresten und insgesamt 60 Spaltstellen identifiziert. Die Domänen 2, 3, 5, 6, 26 und 30 bis 33 wurden als Bereiche identifiziert, in denen MMP-7 und -12 besonders häufig spalteten, wohingegen in den anderen Domänen wenige oder teilweise gar keine Spaltungen festgestellt werden konnten.

Um Einblick in die Spaltspezifitäten der drei MMPs zu erlangen, wurden die Spaltstellen mit Hinblick auf die Interaktionen der substratseitigen Aminosäurereste mit den Subsites $S_4$–$S_4$` der katalytischen Domänen der drei MMPs analysiert. Dadurch war es möglich,

Ähnlichkeiten aber auch einige wichtige Unterschiede in dem Abbauverhalten der drei MMPs gegenüber Elastin und Tropoelastin zu beschreiben. Ferner wurde versucht, Unterschiede in den Spaltspezifitäten aufzuklären, indem die dreidimensionalen Kristallstrukturen der MMPs im Komplex mit ausgewählten peptidartigen Substraten grafisch analysiert wurden. Dadurch ließ sich beispielsweise nachvollziehen, warum die Subsite $S_1$` der MMP-12 geladene Aminosäurereste wie Lysin und große Reste wie Ser, Lys und Arg besser als andere MMPs akzeptiert. Die Rekonstruktion der Spaltstellen aus den identifizierten Peptiden erlaubte zudem die Identifikation von Bereichen in Elastin und seinem Monomer, die durch die MMPs hydrolysiert werden. Die daraus erhaltenen Informationen können vielfältig eingesetzt werden, z.B. um molekulare Veränderungen in der ECM zu beschreiben, die in Verbindung mit MMPs stehen.

**b) Entstehung von potentiell bioaktiven Peptiden aus Tropoelastin und reifem Elastin**

Für alle drei MMPs konnte gezeigt werden, dass sie verschiedene, potentiell bioaktive Peptide erzeugen, wobei im Falle der Inkubation von Elastin mit MMP-12 die höchste Anzahl von Matrikinen freigegeben wurde. Die Sequenzierung dieser Peptide zeigte weiterhin, dass einige Peptide mehrere bioaktive Motive beinhalteten. Dies ist insofern besonders interessant, da aus vorangegangenen Arbeiten anderer Autoren hervorgeht, dass das Vorhandensein mehrerer solcher Motive die Wahrscheinlichkeit erhöht, dass diese Peptide über biologische Aktivitäten verfügen. In weiteren Experimenten sollte überprüft werden, ob die eingesetzten MMPs der Fähigkeit besitzen, den potenziellen Liganden VGVAPG aus Domäne 24 freizusetzen. Es konnte gezeigt werden, dass MMP-12 im Gegensatz zu den beiden anderen MMPs dazu in der Lage ist, da Spaltungen in dieser Domäne nachgewiesen werden konnten. Allerdings sind weitere *in vitro*- und *in vivo*-Experimente erforderlich, um die Bedeutung und biologische Relevanz dieser Peptide zu klären.

**c) Überblick über die Primärstruktur von Elastin**

Außerdem wurden auf Massenspektrometrie basierende Methoden entwickelt und verwendet, um Informationen über das Quervernetzungsmuster und posttranslationale

Modifikationen in reifem Elastin der menschlichen Haut zu erhalten. So war es beispielsweise möglich, die exakten Positionen von mindestens 15 hydroxlierten Prolinresten zu untersuchen und zu bestimmen, von denen 10 Positionen zum ersten Mal ermittelt wurden. Zusammen mit denjenigen, die vorher identifiziert wurden, ergibt sich eine Gesamtzahl von 40 potenziellen Hydroxylierungspositionen in menschlicher Haut (bei insgesamt 86 Prolinen). Aufgrund partieller Hydroxylierung, die typisch für Elastin ist, war es allerdings nicht möglich, das Ausmaß der Hydroxylierung quantitativ zu schätzen.

Um zu prüfen, welche der für alternatives Spleißen bekannten Domänen auf Proteinebene vorhanden sind, wurde gezielt nach Peptiden gesucht, die deren An- bzw. Abwesenheit belegen können. Durch solche Peptide war es beispielsweise möglich, nachzuweisen, dass die durch Exon 26A kodierte Domäne ausgespleißt war. Im Gegensatz dazu konnten alle Aminosäurereste, die durch die Exons 24, 30, 32 und 33 kodiert werden, im untersuchten Hautelastin nachgewiesen werden. Es konnten jedoch keine Peptide gefunden werden, die das Vorhandensein der Domänen 22, 23 oder 24A belegt hätten. Weiterhin ist es erwähnenswert, dass zwar einige aus Domäne 36 stammende Peptide gefunden werden konnten, diese aber insgesamt lediglich 3 der 14 Aminosäurereste abdeckten. Für Aminosäurereste der Domäne 36 wurde in der Literatur berichtet, dass sie entweder modifiziert oder während der Elastinreifung verloren gehen.

**d) Die katalytische Domäne der MMP-12 ist in der Lage, tripelhelikales Kollagen I und III zu spalten**

Der letzte Teil dieser Arbeit widmete sich der Entwicklung MS-basierter Methoden um die Rolle der MMP-12 beim Abbau von Kollagenen zu beleuchten. Mit Hilfe dieser Methoden war es möglich, eindeutig nachzuweisen, dass die katalytische Domäne der MMP-12 allein dazu befähigt ist, an die Kollagene vom Typ I und III zu binden und diese zu spalten. Die Spaltstellen in den Kollagenen wurden eingehend untersucht und es konnte gezeigt werden, dass MMP-12 sowohl an den für Kollagenase typischen als auch an zahlreichen anderen Positionen hydrolysiert. Weiterhin wurden erstmalig zahlreiche Positionen von Hydroxylierungen an Pro und Lys identifiziert, insbesondere von solchen, die sich in unmittelbarer Nähe typischer Spaltstellen befinden. In Kollagen I wurde beispielsweise für die Anordnung der Hydroxylierungen gefunden, dass diese mit möglichen lokalen,

teilweise entfalteten Bereichen einhergehen können. Dies könnte dazu führen, dass MMP-12 leichter an Aminosäurereste der entfalteten oder relaxierten Kette binden kann. Die Ergebnisse dieser Studie können bei der Aufklärung von Degradationsvorgängen Kollagens behilflich sein, bei denen die MMP-12 im Zusammenhang mit pathologischen Zuständen eine physiologische Relevanz zu haben scheint.

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# 6 APPENDIX (LIST OF PUBLICATIONS)

**6.1** **Samuel Taddese,** Anthony S. Weiss, Günther Jahreis, Reinhard H.H. Neubert, Christian E.H. Schmelzer (2009*). In vitro* degradation of human tropoelastin by MMP-12 and the generation of matrikines from domain 24*, Matrix Biology, 28: 84-91*.

**6.2** A. Heinz, M. Jung, W. Sippl, **S. Taddese,** C. Ihling, A.S. Weiss, R.H.H. Neubert, and C.E.H. Schmelzer (2010). Degradation of tropoelastin by matrix metalloproteinases: cleavage site specificities and release of matrikines, *(Febs Journal, in press).*

**6.3** **Samuel Taddese,** Anthony S. Weiss, Reinhard H.H. Neubert, Christian E.H. Schmelzer (2008). Mapping of macrophage elastase cleavage sites in insoluble human skin elastin, *Matrix Biology, 27: 420-428.*

**6.4** **Samuel Taddese,** Reinhard H.H. Neubert, Christian E.H. Schmelzer (2009). Insights into the degradation of human elastin by matrylysin-1, *(soon to be submitted).*

**6.5** **Samuel Taddese,** Michael Jung, Christian Ihling, Reinhard H.H. Neubert, Christian E.H. Schmelzer (2010). MMP-12 catalytic domain recognizes and cleaves at multiple sites in human skin collagen type I and type III. *Biochimica et Biophysica Acta-Protein and proteomics, 1804: 731-739.*

## I Contact information

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## II Personal information

| | |
|---|---|
| Date of Birth | 20/02/1978 |
| Place of Birth | Ethiopia |
| Citizenship | Ethiopian |
| Sex | Male |
| Marital status | Not married |

## III Education

| | |
|---|---|
| 1983 – 1990 | Elementary and Junior Secondary School, Ethiopia |
| 1991 – 1994 | ACSS, High School, Ethiopia |
| 1995 – 2000 | B. Pharm, School of Pharmacy, Addis Ababa University (AAU), Ethiopia |
| 2002 – 2004 | MSc in Pharmaceutics, School of Pharmacy, AAU, Ethiopia. |
| Sept. 06 – Present | PhD candidate, under the supervision of Prof. Dr. Dr. Reinhard Neubert at the Martin-Luther University, Halle-Wittenberg, Germany. |

## IV Employment history

| | |
|---|---|
| 2000 – 2001 | Graduate Assistant with main responsibility of handling laboratory sessions for undergraduate students, School of Pharmacy, AAU, Ethiopia |
| 2002 – 2004 | Assistant Lecturer; handling lab sessions, teaching courses including dosage forms and formulations, School of Pharmacy, AAU, Ethiopia |
| 2004 – 2006 | Lecturer, teaching courses mainly Biopharmaceutics and Pharmacokinetics, School of Pharmacy, AAU, Ethiopia |

## V Awards:

- Aklilu Lemma Foundation, financial award (September 1999- August 2000), Ethiopia
- Medal, best student of the year upon graduation, School of Pharmacy, AAU, August 2000, Ethiopia
- Scholarship, Katholisher Akademischer Auslaender Dienst (KAAD), Sept, 2006 to present, Germany

## VI Publications:

### A. Articles

1. **Samuel Taddese,** Anthony S. Weiss, Reinhard H.H. Neubert, Christian E.H. Schmelzer (2008). Mapping of macrophage elastase cleavage sites in insoluble human skin elastin. *Matrix Biology, 27: 420-428.*
2. **Samuel Taddese,** Anthony S. Weiss, Günther Jahreis, Reinhard H.H. Neubert, Christian E.H. Schmelzer (2009*). In vitro* degradation of human tropoelastin by MMP-12 and the generation of matrikines from domain 24, *Matrix Biology, 28: 84-91.*
3. **Samuel Taddese,** Michael Jung, Christian Ihling, Reinhard H.H. Neubert, Christian E.H. Schmelzer (2010). MMP-12 catalytic domain recognizes and cleaves at multiple sites in human skin collagen type I and type III, *BBA protein and proteomics, 1804: 731-739*
4. **Samuel Taddese,** Reinhard H.H. Neubert, Christian E.H. Schmelzer (2009). Insights into the degradation of human elastin by matrylysin-1, *(soon to be submitted).*
5. A. Heinz, M. Jung, W. Sippl, **S. Taddese,** C. Ihling, A.S. Weiss, R.H.H. Neubert, and C.E.H. Schmelzer (2010). Degradation of tropoelastin by matrix metalloproteinases: cleavage site specificities and release of matrikines, *(Febs Journal, in press).*
6. **Taddese S,** Asres K and Gebre-Mariam T. (2003). Formulation study of topical antimicrobials containing the extracts of *Alchemilla pedata* and *Maesa lanceolata*, *Eth. Pharm. J, 21:13-24.*
7. **Taddese S,** Asres K and Gebre-Mariam T. (2003). *In vitro* antimicrobial activities of some selected topically applied medicinal plants of Ethiopia. *Eth. Pharm. J, 21:39-46.*
8. **Samuel Taddese,** (2004). Screening for antimicrobial and anti-inflammatory activities and, formulation studies on the extracts of selected medicinal plants topically applied in Ethiopia. AAU (MSc. thesis).

### B. Oral and poster presentations

1. **Taddese S.,** Neubert R.H.H., Schmelzer C.E.H. Degradation of human skin elastin by a major elastinolytic matrix metalloproteinase, *4th Polish-German Symposium*, Halle (Saale), Germany, 2007 (Poster).
2. **Taddese S.,** Neubert R.H.H., Schmelzer C.E.H. Mass spectrometric characterization of the elastolytic properties of human macrophage elastase (MMP-12) on human skin elastin, *55th ASMS Conference*, Indianapolis, IN, USA, 2007 (Poster).
3. **Taddese S.,** Weiss A.S., Neubert R.H.H., Schmelzer C.E.H. Susceptibility of tropoelastin to macrophage elastase (MMP-12) degradation. *41. Jahrestagung der DGMS*, Giessen, Germany, 2008 (Poster).
4. **Taddese S.,** Weiss A.S., R.H.H. Neubert, Schmelzer C.E.H. Inhibition of MMP-12 elastolytic property using low molecular mass peptides. *6th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology*, Barcelona, Spain, 2008 (poster).
5. **Taddese S.,** Neubert R.H.H., Schmelzer C.E.H. Investigation of the protein elastin using different MS methods, *Expert seminar with Addis Ababa University Alumni*, Halle (Saale), Germany, 2008 (Oral presentation).

6. Christian E. H. Schmelzer, **Samuel Taddese,** Ute Richter, Anthony S. Weiss, Reinhard H.H. Neubert. Susceptibility of tropoelastin and elastin to degradation by matrix metalloproteinases, *5th Elastin European meeting*, Madrid, Spain, 2008 (Oral presentation).
7. **Samuel Taddese,** Günther Jahreis, Reinhard H.H. Neubert, Christian E.H. Schmelzer. Generation of matrikines from exon 24-derived sequence of elastin by matrix metalloproteinases, *42. Jahrestagung der DGMS*, Konstanz, Germany, 2009 (Poster).
8. A. Heinz, M. Jung, **S. Taddese,** C. Ihling, A.S. Weiss, R.H.H. Neubert, and C.E.H. Schmelzer. Specificity studies of three elastinolytic matrix metalloproteinases, *42. Jahrestagung der DGMS*, Konstanz, Germany, 2009 (Poster).
9. Jung, M., **Taddese, S.,** Ihling, C., Neubert, R.H.H., Schmelzer, C.E.H. Untersuchungen zum proteolytischen Abbau von Hautkollagenen durch MMP-12, *42. Jahrestagung der DGMS*, Konstanz, Germany, 2009 (Poster)
10. **S. Taddese,** R.H.H. Neubert, and C.E.H. Schmelzer, Pattern of proline hydroxylation in elastin, *5th Polish-German Symposium*, Poznan, Poland, 2009 (Poster).
11. A. Heinz, M.C. Jung, **S. Taddese,** C. Ihling, A.S. Weiss, R.H.H. Neubert, C.E.H. Schmelzer, Using complementary mass spectrometric techniques to determine the cleavage site specificity of elastinolytic proteases. *5th Polish-German Symposium*, Poznan, Poland, 2009 (Poster).
12. **S. Taddese,** A. Heinz, M. Jung, W. Sippl, C. Ihling, A.S. Weiss, R.H.H. Neubert, and C.E.H. Schmelzer. Insights into the cleavage process of elastin and its precursor by matrix metalloproteinases. *Gordon research Conference, Elastin Elastic fibers*, UNE, Biddeford, ME, USA, 2009 (Poster).

## VII Professional membership

Ethiopian Pharmaceutical association

Deutsche Gesellschaft für Massenspektrometrie (DGMS)

## DECLARATION

I, the undersigned, declare that this dissertation is solely my own work and no part of it has been submitted to other Universities or Higher Learning Institutions. In addition, all sources of materials used in this dissertation have been duly acknowledged.

Halle, 2010-01-04 .....

Samuel. Taddese Mindaye