# MASS SPECTROMETRIC CHARACTERIZATION OF ELASTIN PEPTIDES AND THE EFFECT OF SOLAR RADIATION ON ELASTIN

## Dissertation



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von

**Melkamu Getie Kebtie**

geboren am 02.10.1972 in Gundewoin, Äthiopien

Gutachter:

1. Prof. Dr. Dr. Reinhard Neubert
2. Prof. Dr. Michael Linscheid
3. PD Dr. Johannes Wohlrab

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This work is dedicated to
my brother the late Dr. Anteneh Getie
and
my sister the late Ayalnesh Getie

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## ABBREVIATIONS

| | |
|---|---|
| AGE | Glycation end products |
| amu | Atomic mass unit |
| APCI | Atmospheric pressure chemical ionization |
| API | Atmospheric pressure ionisation |
| CI | Chemical ionization |
| CID | Collision-induced dissociation |
| DC | Direct current |
| DE | Delayed extraction |
| DES | Desmosine |
| $DESU_{481}$ | Degradation product of DES with *m/z* 481.1 |
| $DESU_{497}$ | Degradation product of DES with *m/z* 497.1 |
| ECM | Extracellular matrix |
| EI | Electron ionization |
| ESI | Electrospray ionization |
| FAB | Fast atom bombardment |
| FT-ICR | Fourier transform-ion cyclotron resonance |
| HP | Hematoporphyrin |
| HPLC | High performance liquid chromatography |
| IDE | Isodesmosine |
| $IDEU_{481}$ | Degradation product of IDE with *m/z* 481.1 |
| $IDEU_{497}$ | Degradation product of IDE with *m/z* 497.1 |
| IOXD | Isooxodesmosine |
| LC | Liquid chromatography |
| MAGP | Microfibril-associated glycoprotein |
| MALDI | Matrix-assisted laser desorption ionization |
| MP | Multiple positions |
| mRNA | Messenger RNA |
| MS | Mass spectrometry |
| NMSC | Nonmelanoma skin cancer |
| OXD | Oxodesmosine |
| PD | Plasma desorption |

| | |
|---|---|
| PSD | Post-source decay |
| PTM | Post-translational modification |
| qTOF | quadrupole Time-of-flight |
| RER | Rough endoplasmic reticulum |
| RER | Rough endoplasmic reticulum |
| rf | Radio frequency |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RP | Reverse phase |
| TFA | Triflouroacetic acid |
| TOF | Time-of-flight |
| TSP | Thermospray |
| UV | Ultraviolet |

# 1. INTRODUCTION

Ultraviolet (UV) radiation can be both beneficial and harmful to normal human skin. The beneficial effects comprise killing pathogens on the skin, inducing vitamin D synthesis and treating certain skin diseases such as psoriasis vulgaris. The harmful effects of UV radiation are immune suppression, photoaging and, above all, skin carcinogenesis. The incidence of skin cancer has been increasing at an astonishing rate over the past several decades due to changes in leisure-time activity accompanied by an increased exposure to UV radiation. It is estimated that more than one million new cases of nonmelanoma skin cancer (NMSC) occur each year in the USA alone (Gloster and Brodland, 1996). The skin responds to sun exposure by tanning and thickening, both of which provide some protection from further damage by UV radiation. The degree of pigmentation in the skin and the ability to tan are important risk factors in skin cancer development, and the risk of NMSC is highest in people who sunburn easily and rarely tan (Gloster and Brodland, 1996).

Since most of the UV sunlight reaching the earth's surface (>95%) is in the deeply penetrating UVA (320–400 nm) region, solar radiation-induced photo-oxidative stress effectively reaches into the human dermis (Sander*, et al.*, 2002). The dermal elastic fibers increase and become amorphous, a characteristic feature of a disease known as solar elastosis. The clinical symptoms include dryness (roughness), irregular pigmentation, wrinkling, elastosis and dilation of pre-existing blood vessels creating small focal red lesions. These features are predominantly observed in fair-skinned white individuals with a history of ample past sun exposure and usually involve the face, neck, or extensor surface of the upper extremities.

Although UVB (290–320 nm) photons are much more energetic than UVA photons and are mostly responsible for sunburn, sun-tanning and photocarcinogenesis, UVA is also suspected of playing a substantial role in photoaging. Human skin exposed daily for one month to suberythemic doses of only UVA demonstrated epidermal hyperplasia, stratum corneum thickening and dermal inflammatory infiltrates with deposition of lysozymes on the elastic fibers (Lavker*, et al.*, 1995a; Lavker*, et al.*, 1995b). These changes suggest that frequent casual exposure to sunlight while wearing a UVB-absorbing sunscreen may eventually result in damage to dermal collagen and elastin in ways expected to produce photoaging. The mechanism behind such deleterious effect of solar radiation is not yet fully understood.

However, it is suggested that the process may be initiated by the photochemical reactions of endogenous light-absorbing molecules (chromophores), which, after light absorption, may react with cellular molecules or generates reactive species, such as free radicals, that subsequently alter cellular molecules, such as elastin and collagen.

Understanding the biochemical basis of such photochemical processes is required to design an appropriate method of intervention or therapy. The former calls for the availability of analytical methods to characterize these extracellular proteins, such as elastin at the molecular level. The heterogeneity of the elastic tissue composed of elastin and microfibrillar components, the small percentage of elastin in some tissues, such as the skin, the difficulty in extracting and isolating elastin, and the extreme insolubility of elastin neither in water nor in organic solvents make direct biochemical analysis of elastin particularly difficult. Such studies have been, therefore, mainly focused on complete hydrolysis of the protein in strong acidic environment and analysis of the resulting cross-linked amino acids, desmosine (DES) and its isomer isodesmosine (IDE), which are only found in elastin. This approach has been challenged by the difficulty in chromatographic separation of the amino acids with the analytical methods employed to date and the partial information that can be obtained about the entire elastin molecule only based on the cross-linked amino acids. Moreover, the deleterious effects of the strong acidic environment and the high temperature on elastin complicate the identification of alterations as the result of pathological disorders.

Therefore, the primary objective of this work was to develop analytical methods and employ them to investigate the influence of different wavelength radiation generated from artificial sources on the cross-linking of elastin, tropoelastin (the precursor of elastin), and peptides of elastin.

In the first part of this work, a study was performed to evaluate the effect of radiation on the elastin cross-links, DES and IDE. It was observed that exposure of the amino acids to radiation emitted from artificial sources in the presence of $Fe^{2+}$ and $H_2O_2$ produced a drastic oxidative effect on the amino acids. The degradation of the cross-links is of paramount importance, because the elasticity and strength of elastic fibers is maintained by the existence of these cross-links in the elastin molecule. Answering the question whether the mentioned degradation products are produced *in vivo* and if they produce any physiological alteration needs further study. Furthermore, evaluation of the effect of these endogenous and

environmental oxidative agents on the intact elastin molecule and elastic fibers is an essential component of the effort towards obtaining a more comprehensive understanding of the changes that occur on skin's extracellular matrix (ECM) as the result of intrinsic aging or photoaging. However, such studies will have to encounter the aforementioned difficulties of performing biochemical studies on elastin. We assume that extraction of elastin from a healthy and diseased or sun-protected and sun-exposed tissues and comparison of the molecular structures of the peptides resulting from enzymatic digestion of the protein could give a good insight into the biochemical changes that occur due to the mentioned photochemical processes or pathological conditions. Therefore, in the second part of the work, methods comprising complementary mass spectrometric techniques and peptide sequencing tools were employed to characterize peptides generated by digestion of human skin elastin and recombinant human tropoelastin with different enzymes (elastase, thermitase, pepsin and chymotrypsin). Finally, the methods were employed to evaluate the effect of UVB on the recombinant tropoelastin and its peptides as well as peptides of the human skin elastin. Treatment of tropoelastin with UVB in the presence of a photosensitizer, hematoporphyrin (HP), resulted in a significant degradation of tropoelastin, whereas the same treatment or replacement of the HP by ferrous sulfate did not show major changes in the peptides of tropoelastin or elastin.

# 2. BACKGROUND

## 2.1. Elastic fibers

### 2.1.1. Composition, structure and distribution

The elastic properties of many tissues are owing to the presence of highly branched elastic fibers in the extracellular space. The amount, distribution and structural organization of the elastic fibers vary in different organs. They are numerous in tissues, which undergo periodic or frequent stretching, such as aorta, lung, and elastic ligaments (Pasquali-Ronchetti and Baccarani-Contri, 1997). In normal human skin, the elastic connective tissue proteins represent a relatively small fraction of the total dermal proteins, collagen being the predominant component (Grant and Prockop, 1972; Uitto and Lichtenstein, 1976). They are organized into distinct morphologies in different tissues: small, rope-like networks in lung, skin and ligament; thin concentric sheets in blood vessels; and large three-dimensional honeycomb structures in elastic cartilage (Pasquali-Ronchetti and Baccarani-Contri, 1997).

Structural studies on the elastic fibers revealed a complex structure consisting 2 distinct components (Ross*, et al.*, 1977). The major component, which has an amorphous appearance, represents the elastin protein. The amorphous elastin is surrounded by distinct fibrillar structures, which are rich in acidic glycoproteins and are organized into 10 to 12 nm fibrils of beaded appearance (Cleary*, et al.*, 1981; Cotta-Pereira*, et al.*, 1976). This component represents the elastic fiber microfibrillar proteins called microfibrils. The relative concentrations of elastin and the microfibrillar component vary during embryonic development; the newly developed fibers in young embryos are composed exclusively of the microfibrillar component, but during development the proportion of elastin increases progressively and in a fully developed fiber more than 90% is elastin (Ross, *et al*., 1977). Microfibrils themselves are made up of at least five distinct proteins (Gibson*, et al.*, 1989), including two forms of the 350 kDa glycoprotein, fibrillin (Sakai*, et al.*, 1986; Zhang*, et al.*, 1994) and two microfibril-associated glycoproteins (MAGPs), MAGP-1 (Gibson*, et al.*, 1991) and MAGP-2 (Gibson*, et al.*, 1996). Several other components, such as lysyl oxidase (Kagan*, et al.*, 1986), proteoglycans (Baccarani-Contri*, et al.*, 1990), osteopontin (Pasquali-Ronchetti and Baccarani-Contri, 1997), emilin (Bressan*, et al.*, 1993), and fibulin-1 (Roark*, et al.*, 1995) are also reportedly present in the elastic fiber.

### 2.1.2. Biosynthesis of elastic fibers

Elastic fiber formation requires the coordination of a number of important processes including the intracellular transcription and translation of tropoelastin, intracellular further processing of the protein, secretion of the protein into the extracellular space, delivery of the tropoelastin monomers to sites of elastogenesis, alignment of the monomer units with previously deposited tropoelastin through associating microfibrillar proteins, and lastly, the conversion to the insoluble elastin polymer through the cross-linking action of lysyl oxidase (Mithieux and Weiss, 2005).

#### 2.1.2.1. Tropoelastin synthesis

*Structure of the elastin gene*

In the last decades, a number of investigators have characterized the structure of the gene coding for tropoelastin, the precursor of elastin, from several species including humans, cows, and rats (Bashir*, et al.*, 1989; Indik*, et al.*, 1987; Olliver*, et al.*, 1987; Pierce*, et al.*, 1990). These studies have revealed the presence of only a single elastin gene in the mammalian genome that, in most species, is composed of 36 exons distributed throughout approximately 40,000 bp of genomic DNA, has an intron:exon ratio of almost 19:1, and has an unusually high frequency of repetitive DNA sequences within many introns, particularly at the 3'-end. Some of these investigations have also demonstrated that hydrophobic and cross-linking domains of tropoelastin are encoded by separate, alternating exons, reflecting the protein structure.

Some variation exists amongst species with respect to the presence of exon homologues. The human gene, which is localized to chromosome 7 (Fazio*, et al.*, 1991), has 34 exons, compared with 36 in the bovine and rat elastin genes (Bashir*, et al.*, 1989; Yeh*, et al.*, 1989). Exons 34 and 35 of the latter species are absent from the human gene, while the human gene contains the unusual hydrophilic-encoding exon 26A, not described in any other species (Bashir, *et al*., 1989; Indik, *et al*., 1987).

*Tropoelastin mRNA expression*

The expression of tropoelastin mRNA and elastic fiber synthesis is highest in early development and occurs primarily within a limited period during development (Parks*, et al.*, 1988). The changes in elastin synthesis appear to be a consequence of a change in amount of

elastin mRNA, and a strong correlation exists between mRNA levels and tropoelastin synthesis (Burnett, *et al.*, 1982; Pollock, *et al.*, 1990; Sephel, *et al.*, 1987). Many potential control regions exist in the elastin gene including untranslated regions, intronic regions and promoter regions. Negative regulatory elements have been found in the first intronic region in the bovine gene in a region that is highly similar to the human gene (Manohar and Anwar, 1994). The promoter region of the human gene has been analyzed and found to contain both up-regulatory and down-regulatory elements (Rosenbloom, *et al.*, 1991). Up to eight different transcription start sites have been identified (Bashir, *et al*., 1989; Rosenbloom, *et al.*, 1993; Rosenbloom, *et al*., 1991), demonstrating that the elastin gene is under a complex control mechanism.

Age-dependence of the human elastin promoter coupled to the chloramphenicol acetyltransferase reporter gene has been demonstrated in mice *in vivo* (Hsu-Wong, *et al.*, 1994). In chick aorta cells, the decrease in elastin synthesis that occurs with age has been shown to result partly from the destabilization of mRNA (Johnson, *et al.*, 1995). Growth factors and hormones such as transforming growth factor β1 (Kahari, *et al.*, 1992; McGowan, *et al.*, 1997), insulin-like growth factor I (Wolfe, *et al.*, 1993), vitamin D (Pierce, *et al.*, 1992) and interleukin-1β (Mauviel, *et al.*, 1993) have all been shown to affect tropoelastin synthesis at either the promoter level or post-transcriptionally by affecting the stability of tropoelastin mRNA. In addition, there is evidence that tropoelastin may be under negative feedback autoregulation whereby accumulation of tropoelastin in the ECM space may inhibit the further production of tropoelastin mRNA (Foster and Curtiss, 1990).

*Alternative splicing of the elastin gene*

A significant variation in nucleotide sequence and size of both mRNA and cDNA of tropoelastin has been observed within a species (Baule and Foster, 1988; Fazio, *et al.*, 1988a; Pierce, *et al*., 1990). These variable cDNAs were shown to be the result of alternative splicing of tropoelastin mRNA. Two types of alternative splicing have been demonstrated; a complete excision of an entire exon, as seen with human exons 22, 23 and 32 or the excision of a portion of an exon, as is the case for human exon 26A (Indik, *et al*., 1987). At least 11 human tropoelastin splice variants have been identified with some exons shown to be subject to alternative splicing: exons 22, 23, 24, 26A, 32 and 33 (Boyd, *et al.*, 1991; Fazio, *et al*.,

1988a; Fazio*, et al.*, 1988b; Indik, *et al*., 1987). Exon 23 is a cross-linking domain while exons 22, 24, 32 and 33 are hydrophobic domains (Vrhovski and Weiss, 1998).

*Translation and secretion*

The intracellular biosynthesis of elastin polypeptides follows the same basic principles as the formation of other mammalian proteins. Elastin is synthesized by smooth muscle cells, endothelial and microvascular cells, chondrocytes and fibroblasts, and the translation of the mRNA molecules coding for elastin polypeptides takes place in the rough endoplasmic reticulum (RER) of these cells (Uitto, 1979; Vrhovski and Weiss, 1998). Polypeptide chains are released into the lumen of the RER with the release of the signal peptide (Saunders and Grant, 1984). Tropoelastin is secreted to the plasma membrane via secretory vesicles (Saunders and Grant, 1985). Inhibition of secretion of tropoelastin experimentally accumulated in the RER and Golgi apparatus results in intracellular degradation of the accumulated protein by cysteine proteases (Davis and Mecham, 1996). Before secretion, intracellular tropoelastin is, therefore, likely to be chaperoned by a 67-kDa elastin binding protein (galactolectin), which prevents intracellular self-aggregation and premature enzymatic degradation of the protein (Hinek and Rabinovitch, 1994). This association lasts until the complex is excreted into the extracellular space where the companion chaperone interacts with galactosugars of the microfibrils, dramatically decreasing its own affinity for the tropoelastin molecule, which then be released locally. The 67 kDa chaperone is recyclable and is a constitutive part of the elastin cellular receptor (Debelle and Tamburro, 1999). The elastin-binding protein binds tropoelastin primarily at the VGVAPG peptide site of human tropoelastin (Mecham*, et al.*, 1989).

### 2.1.2.2. Elastic fiber assembly

Elastogenesis is a highly complex process, which consists of all events leading to the construction of a functional elastin within the elastic fiber. It starts inside the cell by specifically targeting and transporting the tropoelastin molecule to sites of fiber formation on the cell surface (Debelle and Tamburro, 1999). Before any elastin is deposited, microfibrils are secreted into the extracellular space close to the cell surface. The microfibrillar component works as a scaffold for the deposition of elastin and serve to align the tropoelastin molecules into the correct orientation for subsequent cross-linking (Mecham, 1991). The

molecular basis of how tropoelastin binds microfibrils during elastic fiber formation remains unclear, and it is the concern of current research in this field.

The formation of a specific transglutamase crosslink between fibrillin-1 and tropoelastin may act to covalently stabilize the newly deposited tropoelastin on the microfibrils (Rock*, et al.*, 2004). This in turn is facilitated by the binding of MAGP-1 with tropoelastin (Brown-Augsburger*, et al.*, 1996; Clarke and Weiss, 2004). When this binding is missing, the fiber integrity is severely altered, as noted in lamb ductus arteriosis, where a truncated tropoelastin is present and does not form fibers (Hinek and Rabinovitch, 1993). Calcium-dependent multiple binding sites were observed between tropoelastin and MAGP-1. The interaction of the N-terminal part of the MAGP-1 with the highly conserved C-terminal end of tropoelastin was particularly emphasized.

The only two Cys residues confined to the C-terminus of tropoelastin have been demonstrated to form an intrachain disulfide bond and molecular modeling has predicted the formation of a hairpin loop giving rise to a positively charged pocket with the C-terminal RKRK sequence at the end (Brown*, et al.*, 1992). It was speculated that this pocket could provide a non-covalent binding site for the highly acidic microfibrils. It has been confirmed experimentally that tropoelastin can indeed bind to the microfibrillar protein MAGP-1 (Bashir*, et al.*, 1994; Brown-Augsburger*, et al.*, 1994); the binding being localized to the C-terminus of tropoelastin and the N-terminal half of MAGP-1 (Brown-Augsburger, *et al*., 1996; Clarke and Weiss, 2004). Both proteins appear to require an intact secondary structure, which has been cited as evidence that binding is not simply ionic (Brown-Augsburger, *et al*., 1994). Transglutaminase-mediated cross-linking of MAGP-1 to tropoelastin might then covalently lock this association (Brown-Augsburger, *et al*., 1994). Blocking MAGP-1 or the C-terminus of tropoelastin with antibodies reduces elastin accumulation in the ECM (Brown-Augsburger, *et al*., 1994), indicating that the interaction between tropoelastin and MAGP-1 is an important step in elastic fiber formation.

Fibrillin-1, a large cysteine-rich multidomain glycoprotein, polymerizes in the extracellular space in a head-to-tail manner as parallel bundles of six to eight molecules to form microfibrils that provide a force-bearing structural framework for fibrillogenesis (Gibson, *et al*., 1996; Kielty*, et al.*, 2002). Mutations in fibrillin-1 result in Marfan syndrome, a heritable disease associated with severe aortic, ocular, and skeletal defects due to defective elastic

fibers (Dietz*, et al.*, 1994). Mutations in the second fibrillin gene (fibrillin-2) cause a related disorder, congenital contractural arachnodactyl (Lee*, et al.*, 1991).

In a study aimed at screening the entire fibrillin-1 molecule for sites of interaction with tropoelastin and the major microfibril-associated molecule MAGP-1, Rock *et al*. (Rock, *et al.*, 2004) have recently discovered high affinity interactions between tropoelastin and two overlapping fibrillin-1 fragments encoded by central fibrillin-1 sequences (exons 18-25 and 24-30) indicating that this region of fibrillin-1 is the major tropoelastin binding site. A further interaction of moderate affinity between tropoelastin and a proline-rich region of fibrillin-1 fragment encoded by exons 9-17 has been identified. The principal MAGP-1 binding site is suggested to be on fibrillin-1 sequence encoded by exons 1-8. These authors proposed the following model of elastic fiber formation (fig. 2.1).

I. Microfibrils are first formed by linear and lateral fibrillin-1 interactions.
II. MAGP-1 then associates onto microfibril bead surfaces via an interaction with N-terminal fibrillin-1 domains.
III. Tropoelastin is then deposited on an interbead region adjacent to the beads through strong interactions with the fibrillin-1 central sequence and subsequently becomes cross-linked to fibrillin-1. Tropoelastin and MAGP-1 may then interact on microfibrils.
IV. Further deposition of tropoelastin to microfibril-bound tropoelastin and MAGP-1 followed by lysyl oxidase cross-linking
V. Formation of mature elastic fiber

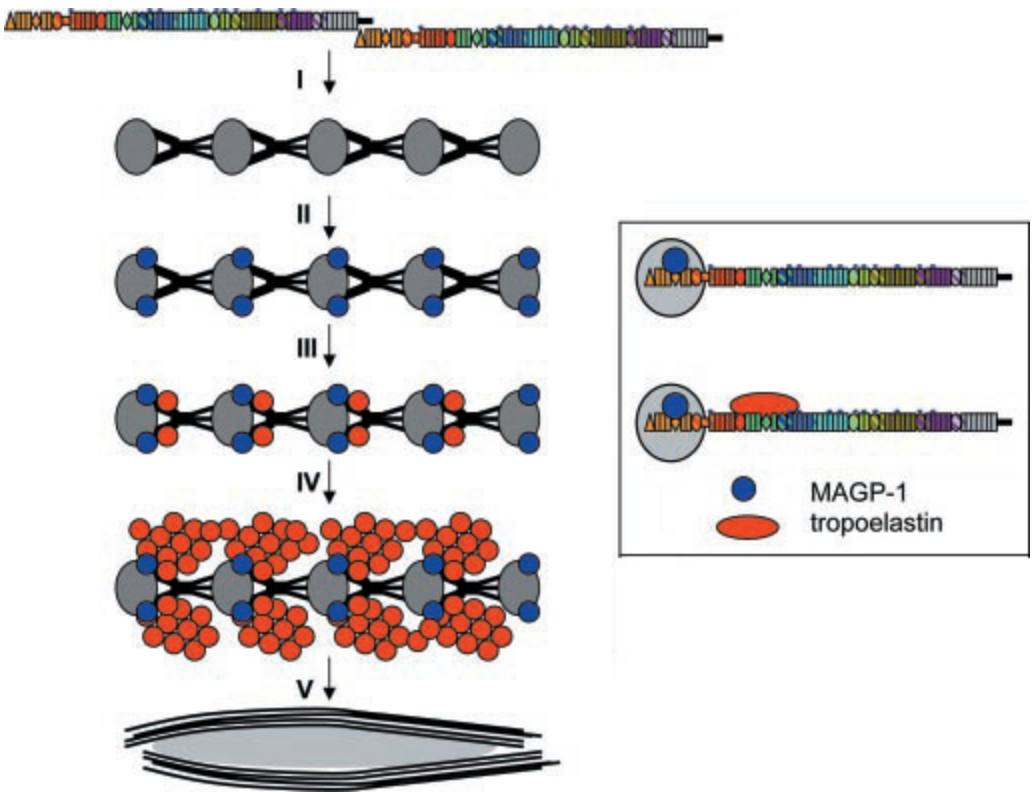



Figure 2.1: Model of elastic fiber formation in the extracellular space modified after Rock *et al*. (Rock, *et al.*, 2004).

### 2.1.2.3. Coacervation and cross-linking of tropoelastin

Cross-linking of tropoelastin molecules is preceded by the association and alignment of the molecules bringing together each cluster of four positively charged lysine side chains necessary for cross-linking. Coacervation is thought to be the molecular mechanism through which aligning and concentrating can occur (Urry, 1978). Inappropriate coacervation may be detrimental to fiber formation and it appears that many different molecules may influence the coacervation of tropoelastin. The interaction of glycosaminoglycans with tropoelastin (Fornieri, *et al.*, 1987), the binding of lipids by tropoelastin (Vrhovski, *et al.*, 1997), and overhydroxylation of Pro residues (Urry, *et al.*, 1979) are suggested to reduce the ability of tropoelastin to coacervate. Cross-linking of tropoelastin is greatly reduced at low temperatures when tropoelastin is not coacervated (Narayanan, *et al.*, 1978). It was suggested that at low temperature, water forms a clathrate-like structure around the hydrophobic regions of tropoelastin, keeping the protein unfolded (Mithieux and Weiss, 2005). With increasing temperature, the ordered clathrate water is disrupted, rendering the hydrophobic domains,

such as the oligopeptide repetitive sequences GVGVP, GGVP, and GVGVAP, free to fold and interact with other hydrophobic segments.

The initial step of the cross-linking reaction, which renders tropoelastin insoluble, is an oxidative deamination of lysine residues by $Cu^{2+}$ dependent lysyl oxidase to produce allysine, also known as α-amino adipic δ-semialdehyde. All subsequent reactions are spontaneous and involve the condensation of closely positioned lysine and allysine residues to produce cross-links such as allysine aldol, lysinonorleucine, merodesmosine, and tetrafunctional cross-links unique to elastin, such as desmosine and isodesmosine (Noblesse*, et al.*, 2004; Reiser*, et al.*, 1992; Vrhovski and Weiss, 1998). Desmosine and isodesmosine are thought to result from two different pathways (fig. 2.2).

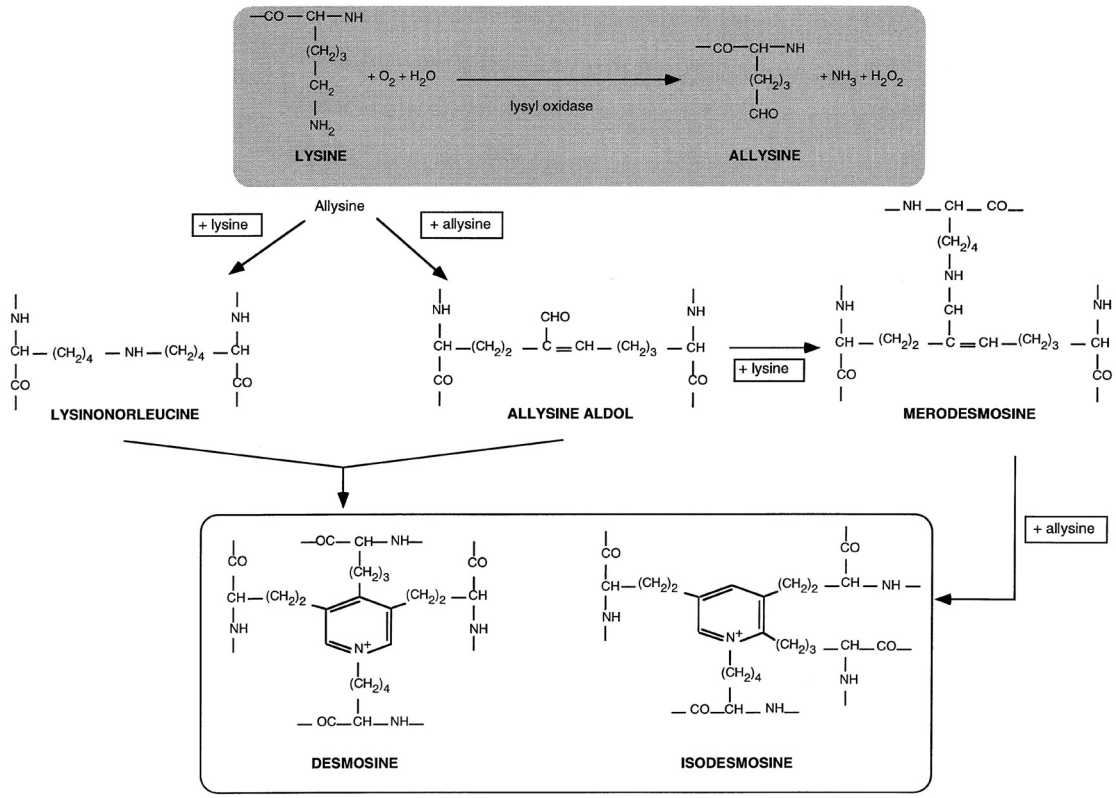



Figure 2.2: Structure and route of formation of elastin cross-links (Vrhovski and Weiss, 1998).

Other than understanding the chemical mechanism of cross-link formation, little is known about how tropoelastin monomers interact one another to form the functional mature elastin. This is largely due to the highly insoluble nature of elastin, making it difficult to analyze.

Two types of cross-linking domains exist in tropoelastin: those rich in alanine (KA) and those rich in proline (KP). Within the KA domains, lysine residues are typically found in clusters of two or three amino acids, separated by two or three alanine residues. Molecular modeling and structural analysis show these regions to be α-helical structure, which has the effect of positioning two lysine sidechains on the same side of the helix (Brown-Augsburger*, et al.*, 1995; Gray*, et al.*, 1973). This conformation has been suggested to be critical to the formation of desmosines. Modeling studies have shown that allysine aldol and dehydrolysinonorleucine will fit on the α-helix without distortion with one interesting restriction; allysine aldol can only be accommodated when the precursor lysines are separated by three alanines while dehydrolysinonorleucine can form between lysines separated by either two or three residues (Gray, *et al.*, 1973). In human tropoelastin, the KA domains are encoded by exons 6, 15, 17, 19, 21, 23, 25, 27, 29, and 31. The KP domains are encoded by exons 4, 8, 10, and 12. In these KP domains, the lysine pairs are separated by one or more proline residues and are flanked by prolines and bulky hydrophobic amino acids (Brown-Augsburger, *et al.*, 1995). Desmosines and isodesmosines have not been found in association with KP domains. This could be attributed to the presence of multiple proline residues, which impose steric constraints that would not allow the formation of α-helical structure. There is little information describing how many tropoelastin molecules could be involved in the crosslink formation. However, one elastin cross-linking domain has been identified that joins three tropoelastin domains identified as 10, 19, and 25 (Brown-Augsburger, *et al*., 1995). The only two KA domains that contain three lysine residues are encoded by exons 19 and 25. Two lysines from each exons form a desmosine crosslink in an antiparallel arrangement of the exons, while the remaining lysine on each chain forms a lysinonorleucine crosslink with two lysine residues present on the KP domain encoded by exon 10.

### 2.2. Elastic fibers in the skin

The skin is a complex organ composed of two main tissues: epidermis and dermis. The epidermis, a stratified and differentiated epithelium, is responsible for the barrier function of the skin. The dermis is a connective tissue in which cells are embedded in their own and the abundant ECM, which is mainly composed of collagen and many macromolecules such as elastin. Elastic fibers are responsible for skin elasticity (Gibson, *et al.*, 1989), and collagen fibers for its tensile strength. The progressive disorganization of elastic fibers and their

replacement by collagen fibers are major manifestations of skin aging. In addition, burns or deep wounds result in scarring with loss of elasticity.

The dermal elastic system consists of a meshwork of loose and wide branched fibers, which are larger and more represented in the reticular dermis, and which split repeatedly, becoming smaller as they ascend into the middle papillary dermis, where they form an arcade projected towards the dermal-epidermal junction. In this region, elastic fibers loose their elastin content and become almost exclusively composed of microfibrillar glycoproteins (Pasquali-Ronchetti and Baccarani-Contri, 1997).

### 2.2.1. Skin aging

Aged human skin appears loose and sagging with reduced resilience and elasticity compared to young skin. Aging of the skin is thought to consist of two processes taking place simultaneously. The first process is intrinsic, chronologic aging and similar perhaps to aging of other tissues. The second process is photoaging, an environmentally induced remodeling of the dermis that arises as a result of repeated exposure of skin to sunlight. It is always rather difficult to distinguish the real age-related modifications from those induced by exogenous factors, including ultraviolet light and minor traumatic injuries (Pasquali-Ronchetti and Baccarani-Contri, 1997). In both cases, however, the age-related alterations affect all dermal components (Fornieri*, et al.*, 1989). Varani *et al.* have shown decreased procollagen gene expression and increased expression of genes encoding several matrix metalloproteinases as the result of intrinsic aging and photoaging (Varani*, et al.*, 1998).

Elastin is produced early in life, and then appears to be stable and to have a turnover, which approaches the life span (Shapiro*, et al.*, 1991). In a transgenic mouse line, the activity of the elastin promoter was found to increase during postnatal development and, in skin, to reach a peak at 3 months of age, then to decrease (Hsu-Wong, *et al.*, 1994). Therefore, the age-related modifications extensively described in the elastic fibers of all organs and tissues may be largely interpreted as result of progressive degradation of a protein polymer that has been produced early in life.

Scanning electron microscopy showed that, in humans, the elastic meshwork grows largely undistorted during postnatal growth, where fibers seem to enlarge according to the growth of

tissue. Later, in adults and old subjects, elastic fibers gradually become tortuous, frayed and porous (Imayama and Braverman, 1989). Moreover, it is well documented that solar radiation induces a series of progressive structural and biochemical changes in the elastic fibers of exposed skin (Engel*, et al.*, ; Uitto, 1986), such as overproduction and basophilic degeneration up to destruction of the elastin meshwork in dermis (Bouissou*, et al.*, 1988; Brien, 1985; Frances and Robert, 1984) as well as in superficial vessels (O'Brien and Regan, 1991).

The physiological changes that occur on elastic fibers during aging and development were precisely described by Pasquali-Ronchetti and Baccarani-Contri (Pasquali-Ronchetti and Baccarani-Contri, 1997). In fetal skin, the elastin appears around the seventh month of gestation as thin strands of amorphous material associated with bundles of about 10-20 wide microfibrils. At birth, the elastic fibers in the reticular dermis are very small, and comprise a core of amorphous elastin surrounded by a discontinuous coat of microfibrils. Up to 10 years, the elastic fiber diameter tends to grow to its final size by further deposition of amorphous elastin, whereas the number of surrounding microfibrils decreases. The amorphous elastin seems to represent the major component of the fiber, whereas microfibrils are rare. Starting of the fourth decade of life, electron-dense materials accumulate within skin elastic fibers in an age-dependent manner. In very old subjects, these materials seem to have disappeared, leaving behind holes, which give to the fiber a sieve appearance. A concomitant phenomenon is the appearance of isolated elastic fibers, which do not exhibit the typical amorphous ultrastructural organization but seem to consist of interwoven filaments (Robert*, et al.*, 1988).

A few studies designed to distinguish actinic damage from chronological aging of skin elastic fibers demonstrated that the alterations were similar, but were most pronounced in sun-exposed skin (Bouissou, *et al*., 1988; Braverman and Fonferko, 1982). The age-associated alterations consisted of swelling of fibers, granular degradation of elastin, with the appearance of lacunae within the elastic fibers, filled by osmiophilic materials (Braverman and Fonferko, 1982). This latter phenomenon has been described as being mostly associated with actinic damage (Bouissou, *et al*., 1988). Rather interestingly, in the dermis of sun-exposed skin, inflammatory cells (macrophages and mast cells) were often observed. Therefore, although this has not yet been proved, lysosomal enzymes or mast cell products might contribute to the severe elastin damage in sun-exposed skin. On the other hand, elastin seems to accumulate in the superficial photoaged skin; in fact, the morphologic changes in

sun-damaged skin have been shown to be associated with accumulation of material having the staining properties of elastin, known as solar elastosis (Gilchrest, 1989).

The cellular mechanisms leading to solar elastosis are not understood and, indeed, controversial findings concerning the synthesis of elastic fibers during solar elastosis have been reported. Several reports have demonstrated that elastic fibers deposited during solar elastosis consist of the same components as normal elastic fibers. In response to UVA and/or UVB radiation, keratinocytes secrete many mediators that could stimulate fibroblast synthetic activity, and some of them, e.g., tumor necrosis factor b, interleukin-1b, and interleukin-10, have been shown to increase the promoter activity of the elastin gene, steady-state mRNA levels, and elastin accumulation (Kahari, *et al*., 1992; Mauviel, *et al*., 1993; Reitamo*, et al.*, 1994). Whereas Bernstein *et al* (Bernstein*, et al.*, 1994) have noted increased elastin mRNA levels in sun-damaged skin, others (Schwartz*, et al.*, 1995; Werth*, et al.*, 1997) have reported no difference in steady-state levels of elastin mRNA during solar elastosis.

Although ROS are widely accepted as crucial mediators of UV phototoxicity, the exact mechanism of their generation in irradiated skin is poorly understood and seems to be dependent on the presence of non-DNA chromophores acting as photosensitizers (Sander, *et al*., 2002). Photosensitization occurs as a consequence of initial formation of excited states of chromophores and their subsequent interaction with substrate molecules (type I photoreaction) or molecular oxygen (type II photoreaction) by energy and/or electron transfer (Foote, 1991). Various chromophores (e.g., riboflavin, porphyrins, nicotinamide adenine dinucleotide phosphate, ECM proteins, etc.) contained in human skin have been proposed as endogenous UV sensitizers of photo-oxidative stress (Dalle Carbonare and Pathak, 1992; Kipp and Young, 1999; Scharffetter-Kochanek*, et al.*, 1997; Wondrak*, et al.*, 2003). The skin-damaging effects of UVA appear to result from type II photoreactions in which UVA or near-UV radiation in the presence of certain photosensitising chromophores leads to the formation of reactive oxygen species (Dalle Carbonare and Pathak, 1992).

Upon UV photo-excitation human skin reveals a strong tissue auto-fluorescence indicative of the presence of various fluorescent UV chromophores, which in the dermis are mainly attributed to ECM protein bound heterocyclic cross-links of enzymatic and nonenzymatic origin (Tian*, et al.*, 2001). Fluorescence excitation measurements on a 6-week-old hairless mouse skin *in vivo* revealed three maxima at 295, 340, and 360 nm. It was suggested that the

dominant excitation maximum at 295 nm originating in the epidermis is attributable to tryptophan, and the remaining two excitation maxima predominantly originating in the dermis are attributed to the presence of ECM protein fluorophores (Kollias*, et al.*, 1998). Investigations have demonstrated that certain cross-link chromophores called advanced glycation end products (AGE) accumulate on dermal ECM proteins in human skin (Dyer*, et al.*, 1993; Jeanmaire*, et al.*, 2001) and may play an active part as mediators of skin phototoxicity acting as potent sensitizers of photo-oxidative stress (Masaki*, et al.*, 1999; Wondrak*, et al.*, 2002a; Wondrak*, et al.*, 2002b). However, the observation that a significant amount of ROS is formed upon solar simulated light radiation of ECM proteins (collagen and elastin) not modified with AGE (Wondrak, *et al*., 2003) made the importance of the AGE doubtful.

### 2.2.2. Pathology

In a series of pathological conditions, either inherited or acquired, dermal elastic fibers can be severely affected. Such conditions include pseudoxanthoma elasticum, cutis laxa, and Marfan syndrome.

*Pseudoxanthoma elasticum*

Pseudoxanthoma elasticum is a relatively rare disorder affecting tissue rich in elastic fibers. Although most cases of the disease appear to be inherited, the precise mode of transmission through generations in many families is difficult to establish due to delayed age of onset and variable expression of the pseudoxanthoma elasticum phenotype (Christiano and Uitto, 1994). It is characterized by progressing mineralization and fragmentation of the elastic fibers (Pasquali-Ronchetti*, et al.*, 1986) and accumulation in the mid dermis (Christiano*, et al.*, 1992). The skin on the involved areas becomes thickened and leathery, forming yellowish plaques (Uitto, 1979). Typical skin lesions may contain relatively small, 1-3 mm yellowish papules that give the affected area a “plucked chicken skin” and the skin later on becomes lax, redundant and inelastic (Uitto, 1979). Although the elastic fibers of the affected tissue are clearly abnormal, the underlying mutuations and etiologic mechanisms remain largely unknown. Findings indicated, however, that mineralization of the elastic fibers is caused by secretion of a series of glycoproteins with high affinity for calcium ions by dermal fibroblasts and their deposition within the fibers (Pasquali-Ronchetti and Baccarani-Contri, 1997).

*Cutis laxa*

Cutis laxa is an elastin related disorder, which induces the loss of elastin and elastic fibers in the cutaneous and other connective tissue compartments. It can occur in both genetic and acquired forms and exhibits a considerable heterogeneity in its clinical manifestations (Uitto, 1979). For example, in its most severe, perinatal form, the elastic fibers are almost undetectable in the skin and internal organs, leading to the early death of the patient. However, other cutis laxa phenotypes only lead to a mild wrinkling of the skin.

Cutis laxa is characterized by extreme loose and redundant skin, which frequently forms sagging folds on the face (Uitto and Lichtenstein, 1976). The major histopathologic feature of cutis laxa is the degeneration of elastic fibers in the dermis or other connective tissues due to very scarce or almost absent elastin. The disorder can be caused by different molecular defects: impaired synthesis of tropoelastin due to a variety of genetic defects affecting one of the steps of the synthetic pathway of this protein, increased degradation by abnormal activity of elastin-degrading enzymes (Fornieri*, et al.*, 1994; Giro*, et al.*, 1985), or decreased cross-linking of elastin due to decreased activity of lysine oxidase (Byers*, et al.*, 1980). The latter is suggested to be associated with lysyl oxidase deficiency due to impaired copper metabolism (Pasquali-Ronchetti*, et al.*, 1994). More recently, another genetic form of cutis laxa was successfully correlated to the elastin gene (Zhang*, et al.*, 1999). In this case, a single base deletion in exon 30 leads to the missense translation of the C-terminal part of the molecule. As a consequence, the encoded tropoelastin lacks the C-terminal region.

*Marfan syndrome*

Marfan syndrome is a heritable connective tissue disorder manifested by defects in the dermal, skeletal, ocular, and cardiovascular systems (Godfrey, 1994). It is inherited as an autosomal dominant trait, with an incidence of nearly one per 10,000 people without gender or ethnic predilection. Life expectancy is significantly reduced, and more than 85% of patients die of cardiovascular complications. Defects in fibrillin are now known to cause the manifestations of this disease (Dietz, *et al*., 1994). Immunofluorescence studies of skin sections and dermal fibroblast cultures were the first to show this association.

## 2.3. Biochemical methods for elastin analysis

Although its role in the elastic property of elastic fibers is an essential element in the physiological function of connective tissues, elastin is a relatively less studied protein as compared to other human tissue proteins. The difficulty of performing biochemical studies explains the limited amount of work in this field. The heterogeneity of the elastic tissue composed of elastin and microfibrillar components, the small percentage of elastin in the skin, the difficulty in extracting and isolating elastin, the extreme insolubility of elastin neither in water nor in organic solvents are some of the problems encountered. Elastin is often defined as the residual protein obtained when all other components of connective tissue have been eliminated by drastic biochemical methods. Increasing interest in the biochemistry of elastin is derived from the realization that marked changes in the structure of the protein can be encountered in various acquired diseases, such as arteriosclerosis and pulmonary emphysema, and also during normal aging of the animal tissues (Sandberg, 1976; Urry, 1978). Aberrations in elastic fibers are also encountered in inherited diseases affecting the skin (Hashimoto and Kanzaki, 1975; Pasquali-Ronchetti*, et al.*, 1981).

A limited number of studies were performed in order to measure the amount of elastin in skin and other connective tissues. Such biochemical analyses are based on the assumption that elastin is a very resistant protein which can be isolated from tissues by drastic chemical treatment or the amount of which may be evaluated by the desmosine content of the whole tissue. The first approach can be satisfactorily applied to ‘‘normal’’ elastin, as it is known that it resists to backbone cleavage by conditions by which all other biological constituents are removed. However, this approach cannot be applied to aged or pathological elastin, which could be already partially degraded and probably susceptible to chemical treatments. Moreover, the quantification of desmosines by radioimmunoassay (Uitto*, et al.*, 1983) or by high performance liquid chromatography (HPLC) may underestimate immature elastin or pathological elastin in which, for some reason, lysyl oxidase activity is reduced (Fornieri, *et al*., 1987; Pasquali-Ronchetti, *et al*., 1994). The limitations of elastin analysis mentioned above call for alternative analytical methods devoid of such drawbacks. Qualitative and quanitative characterization of elastin on the basis of enzymatically-produced peptides was considered in this study.

## 2.4. LC-MS of peptides and proteins

The identification and measurement of peptides and proteins particularly at the nanoscale level has become a critical area of research. Most traditional approaches for the analysis of proteins have involved separation by one- or two-dimensional poly-acrylamide gel electrophoresis (1D or 2D-PAGE). Even though this technique is an excellent quantitative tool, it is often inadequate for the separation of complex protein mixtures. In addition, it is very difficult to automate, is labor intensive, and requires a very experienced analyst to read the gel fingerprints. For these reasons, liquid chromatography (LC), coupled with mass spectrometry (MS) has become the technique of choice. It can either be applied to the analysis of the actual 2D spots to improve the resolution of the separation or be used for the direct analysis of the protein mixture. LC-MS is an analytical technique that couples high-resolution chromatographic separation with sensitive and specific mass spectrometric detection. This includes HPLC-MS, capillary electrophoresis-MS (Banks, 1997; von Brocke*, et al.*, 2001) and capillary electrochromatography-MS (Verheij*, et al.*, 1991).

MS is a chemical analysis technique that is used to measure the mass of unknown molecules by ionizing, separating and detecting ions according to their mass-to-charge ratios ($m/z$). The utilization of mass spectrometry to investigate biological processes dates back to the late 1930's and early 1940's with the use of stable isotopes and isotope ratio mass spectrometers (Ratner*, et al.*, 1943; Schoenheimer*, et al.*, 1937). The last twenty years has seen a dramatic increase in the capabilities of MS. At the beginning of this period, the invention of fast atom bombardment (FAB) (Barber*, et al.*, 1981), plasma desorption (PD) and thermospray (TSP) that permitted the production of gas phase ions from charged and polar compounds without prior chemical derivatization enabled easier analysis of involatile and thermally unstable molecules, especially those of biological interest. These new ionization techniques made high mass macromolecules amenable to mass spectrometric analysis and they opened the room for the development of other ionization techniques, such as matrix-assisted laser desorption/ionization (MALDI) and ESI, applicable for the analysis of even bigger molecules, such as polypeptides and proteins (Lim and Lord, 2002). The remarkable advances in analyzer technology and electronics accompanied this development, so that some of today's most powerful mass spectrometers are relatively small and easy to use.

ESI has had a tremendous commercial impact over the last few years on the use of mass spectrometry in biological research (Smyth, 2003). It was the first method to extend the useful mass range of instruments to well over 50,000 amu. Although introduced in its present form in 1984 (Yamashita and Fenn, 1984), a major discovery took place almost unnoticed in 1968, when Malcolm Dole and coworkers were able to bring macromolecules into the gas-phase at atmospheric pressure (Dole*, et al.*, 1968). Building on Dole *et al.*'s ideas, Fenn and co-workers developed electrospray as a true interface for mass spectrometry (Yamashita and Fenn, 1984). Since then, a wide range of biomolecules has been investigated by ESI.

Figure 2.3 shows the steps involved in the formation of ions by ESI. Briefly, liquid sample introduced from the LC or syringe pump then enters the ESI needle, to which a high voltage is applied. The needle sprays the sample solution into very fine droplets that retain electrical charges at their surface. As the droplets fly through a region of dry gas at atmospheric pressure, solvent from the droplets evaporates, thereby concentrating the number of charges on a smaller area. The solvent evaporates completely, leaving a multiply charged analyte molecules. The mist of charged molecules then hits a heated capillary skimmer that leads to the ion optics and mass analyzer.

A significant advance in the ESI technique was the introduction of nano-ESI, which utilizes a very low solvent flow rate and consequently a very small sample volume. The signal-to-noise ratio of the technique is enhanced compared with standard ESI and a small aliquot of the sample (e.g., 1 μL of $10^{-6}$M) will spray for ca. 30 minutes, thus enabling a peptide mass map and several MS/MS sequence tag analyses to be performed on single minimal sample introduction without chromatographic separation (Karas*, et al.*, 2000). Moreover, advantages, such as a lower susceptibility to salt contamination and a more uniform response to chemically very different analytes such as peptides and oligosaccharides (Karas, *et al*., 2000), and high sensitivities down to the low attomol range (Valaskovic*, et al.*, 1995) have been reported.

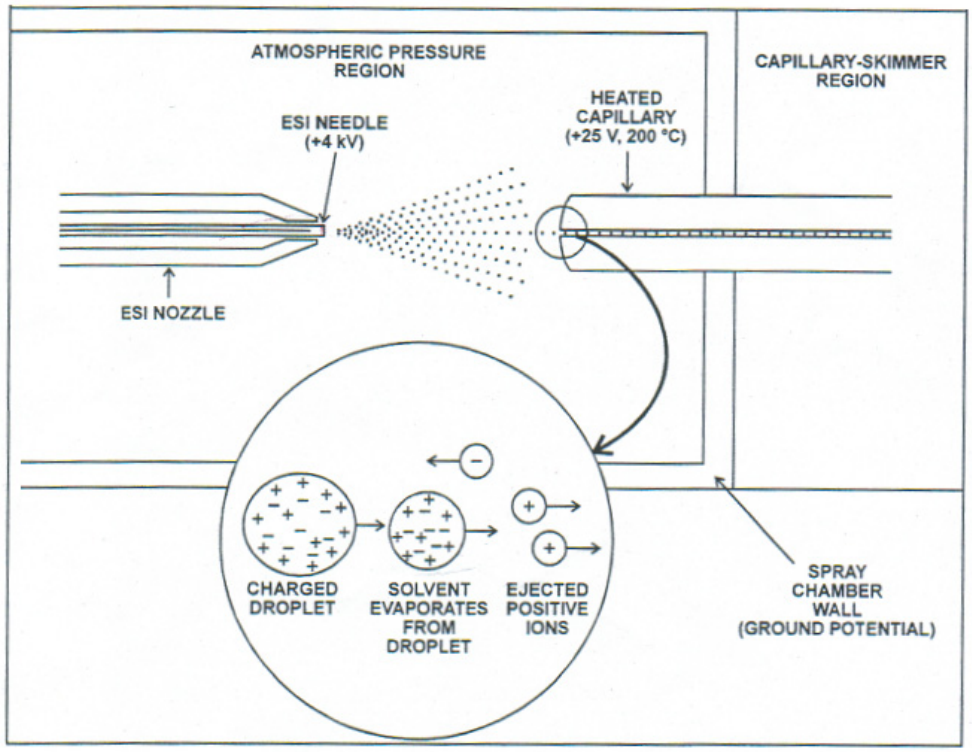



Figure 2.3: ESI process in the positive polarity mode (after Finnigan LCQ MS detector hardware manual)

A major breakthrough in the analysis of biomolecules came in 1988 with the introduction of MALDI (Karas and Hillenkamp, 1988; Tanaka*, et al.*, 1988), a technique that is capable of detecting molecules over 300,000 amu. The technique involves embedding the analyte in a solid matrix, which absorbs laser radiation, typically in the UV region. Upon drying the matrix-analyte mixture, the analyte molecules are intercalated into the matrix crystals. A number of matrices including α-cyano-4-hydroxy cinnamic acid, 2,5-dihydroxybenzoic acid, and sinapinic acid are commonly used in peptide and protein analysis.

An important development to improve the mass resolving power of MALDI-MS is the implementation of delayed extraction (DE) (Bahr*, et al.*, 1997; Brown and Lennon, 1995; Whittal and Li, 1995), a method based on the idea of "time lag energy focusing" originally described in 1955 by Wiley and McLaren (Wiley and McLaren, 1955). In conventional MALDI instruments, the ions generated by the laser beam near the surface of the sample probe are extracted by a dc potential. In DE mode, a short time delay is inserted between the laser ionization and ion extraction events. Within the delay time, ions will spread out into the

extraction gap owing to their initial velocities, thus spreading out in space and acquiring different total kinetic energies, when accelerated by the delayed extraction. Application of the appropriate pulse voltage provides the energy correction necessary to simultaneously detect all ions of the same *m*/*z* regardless of their initial energy. The initially less energetic ions closer to the voltage source receive more energy (from the pulsed potential) than the initially more energetic ions further from the source at the time the pulse is applied. An energy/spatial correction is thus provided such that all ions of the same mass/charge reach the detector plane simultaneously.

## 2.5. Peptide and protein sequencing

### 2.5.1. Edman degradation

To sequence a protein some years back, a substantial amount had to be purified and a technique known as Edman degradation had to be used. This method, which was developed by Pehr Edman (Edman, 1950), relies on the identification of amino acids that have been chemically cleaved in a stepwise fashion from the amino terminus of the protein and requires much expertise. Phenylisothiocyanate reacts with the uncharged terminal amino group of the peptide to form a phenylthiocarbamyl derivative. Under mildly acidic conditions, a cyclic derivative of the terminal amino acid is liberated leaving the intact peptide shortened by one amino acid. The hydrolysis reaction results in a rearrangement of the released N-terminal residue to a phenylthiohydantoin derivative, which can be identified by chromatographic procedure. Often no sufficiently long or unambiguous peptide sequence could be assigned and the method fails completely if the protein is acetylated at its amino terminus or is otherwise blocked to the Edman reaction, which requires a free amino terminus.

### 2.5.2. Tandem mass spectrometry

During the 1990s, tandem MS displaced Edman degradation significantly, because it is much more sensitive and can fragment the peptides in seconds instead of hours or days (Wilm*, et al.*, 1996). Furthermore, MS does not require proteins or peptides to be purified to homogeneity, it has no problem of identifying blocked or otherwise modified proteins, and the sample amount necessary for analysis is usually less than 1 pmol to obtain a high quality mass spectrum (Mendes*, et al.*, 2004). In the last few years, further breathtaking

technological advances have established MS not only as the definitive tool to study the primary structure of proteins, but also as a central technology for the field of proteomics.

A desirable tandem mass spectrometer in peptide sequencing should provide high mass accuracy for both precursor and product ions, high resolution, simple selection of precursor ions, precise tuning of the collision energy, and a much simplified calibration procedure. The fragmentation ions produced by tandem MS can be separated into two classes. If, after the peptide fragments along the amino acid backbone, the charge stays on the N-terminal fragment, then the ion is designated an "a", "b", or "c" ion depending on which particular bond is cleaved in the peptide chain (fig. 2.4). Conversely, if, after the peptide fragments, the charge remains on the C-terminal fragment, the ions are labeled "x", "y", or "z" ions.

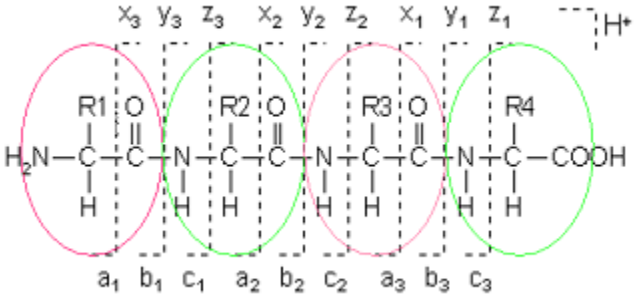



Figure 2.4: Peptide fragment ions in MS/MS

Peptide sequencing by tandem mass spectrometry begins with the acquiring of fragment spectra of the peptides in a mixture of enzymatically digested protein using different approaches, such as LC-MS/MS, nano-ESI-MS/MS, and MALDI-PSD-TOF MS. Deducing peptide sequences from the raw MS/MS data is slow and tedious when it is done manually. Instead, the most popular approach is to convert the resulting mass spectra into mass list and to subject it to peptide sequencing either by matching with protein databases (Creasy and Cottrell, 2002; Mann and Wilm, 1994; Perkins*, et al.*, 1999; Yates*, et al.*, 1995) or by *de novo* sequencing (Ma*, et al.*, 2003; Shevchenko*, et al.*, 1997; Taylor and Johnson, 2001). Even more accurate results could be obtained by coupling both methods when the protein of interest exists in databases.

*LC-MS/MS*

In this method, the peptides that are generated by protein digestion are injected onto an HPLC column that is directly coupled to a mass spectrometer. The peptides are eluted from the column using a solvent gradient of increasing organic content, so that the peptide species elute in order of their hydrophobicity. As the mass spectrometer can distinguish the peptides by their masses, there is no need to separate them into non-overlapping chromatographic peaks and usually many peptides arrive at the end of the column at any given time. The signal intensity in the mass spectrum is directly proportional to the analyte concentration, so the peptides are eluted in as small a volume as possible.

Having determined the *m*/*z* values and the intensities of all the peaks in the spectrum, the mass spectrometer then proceeds to obtain fragment ion spectra. In this process, a particular peptide ion is isolated, energy is imparted by collisions with an inert gas (such as nitrogen molecules, or argon or helium atoms), and this energy causes the peptide to break apart. A mass spectrum of the resulting fragments - the tandem MS (also called MS/MS or $MS^2$) spectrum - is then generated. In MS terminology, the species that is fragmented is called the 'precursor ion' and the ions in the tandem-MS spectrum are called 'product ions'.

*Nano-ESI MS/MS*

For many applications, where on-line coupling with HPLC is not needed, the high flow rates typically invoked by syringe pumps are neither required nor applicable, if low sample volumes are to be investigated. Dilution of sample to obtain a sufficiently large sample volume usually does not solve the problem, since satisfactory results are obtained in a concentration range between $10^{-5}$ and $10^{-7}$ mol/L, depending on the kind of analyte and the purity of the sample. Only in special cases, lower concentrations have been analyzed successfully. Hence, when only low volumes and amounts of sample are available, the logical consequence is to reduce the flow rate as low as possible, and this is one of the ideas underlying the concept of nano-ESI (Wilm and Mann, 1996). In the mid-1990s, Wilm and Mann (Wilm and Mann, 1994) introduced nanospray to the MS community, and Valaskovic *et al*. pioneered the development of nanospray ionization emitters (Valaskovic, *et al*., 1995).

In nano-ESI glass capillaries are used as spray capillaries which are drawn out at one end either by a mechanical or a laser puller to give orifices of only 1-10 µm in diameter. For sufficient conductivity the capillaries are coated with conductive material, e.g. gold, or a thin

metal wire is inserted into the capillary. The capillaries are loaded from the back with only 1-5 µL of sample solution. The spray needle is adjusted under stereomicroscopic or video camera control directly in front of the counter electrode orifice. The distance between needle and orifice is only 0.5 to 2 mm. No liquid feed system is used; the dispersion of the liquid and the generated flow rates of about 20-50 nL/min are exclusively due to the forces of the electrical fields when voltages between 500 and 1000 V are applied. Some backpressure to the spray capillary by a gas-tight syringe is usually applied and is helpful in initiating the flow. Spray capillaries are only used once, which gives the additional advantage that contamination and memory effects are avoided (Karas, *et al*., 2000).

Nano-ESI provides many advantages over conventional ESI for the analysis of macromolecules, including decreased sample consumption, lower flow rate, and increased sensitivity (Valaskovic*, et al.*, 1996). The high molecule-ion intensity is a prerequisite for structure elucidation with MS/MS experiments. Some problems associated with nano-ESI are that the handling of the glass capillaries (sample loading, opening procedure of the capillary) requires some training, as well as the spatial adjustment. Moreover, nano-ESI needles vary in quality and reliability and one has to be aware of absorption effects to the glass walls.

*MALDI-PSD-TOF MS*

PSD analysis is an extension of MALDI MS that allows one to observe and identify structurally informative fragment ions from decay taking place in the field free region after leaving the ion source. After leaving the ion source, all ions have the same nominal kinetic energy, most of them are still unfragmented precursor molecular ions and they have already acquired internal energy by various mechanisms (gas-phase collisions, laser irradiation, thermal mechanisms, etc.). During their flight through the field-free drift region they have a long time available for post-source decay into product ions. These product ions still have basically the same velocity as their precursor ions but now they have a much lower kinetic energy owing to their lower mass. The kinetic energy of the product ions is a measure of their mass. In linear instruments, PSD ions are detected at the same time as their precursors and, therefore, cannot be mass analyzed. The ion reflector, in classical time-of-flight instruments used as a device for flight time compensation of initial energy distributions, is used here as an energy analyzer and thus as a mass analyzer for PSD ions. Owing to their mass-dependent kinetic energies, PSD ions are reflected at different positions within the reflector and thus have mass-dependent total flight times through the instrument (Spengler, 1997).

*Conversion of MS/MS raw data to mass list*

In tandem MS identification of peptides, the fragment ion spectra derived from peptides should either be matched with theoretical spectra calculated from known DNA or protein sequences, or be used for automated *de novo* sequencing using special software tools. Algorithms used for this purpose usually have their own input formats and are not able to read the proprietary binary file formats of the mass spectrometer manufacturers. They usually demand conversion of the MS/MS raw data to peak list. There are some software programs, such as Mascot Distiller, MassLynx with its ad-on program MaxEnt, and wiff2dta (Boehm*, et al.*, 2004) that are capable of creating high quality peak lists from each mass spectrum. Although they use different approaches to detect and pick a signal as a true peak, all of these algorithms have common aims, such as removal of noises to subsequently reduce data and selection of peaks of the monoisotopic mass. In this work, Mascot Distiller and MassLynx were used to create peak lists for the MS/MS spectra produced.

### 2.5.3. *De novo* sequencing

Theoretically, each peptide fragment in a series differs from its neighbor by one amino acid (fig. 2.4). It is, therefore, possible to determine the amino-acid sequence by considering the mass difference between neighboring peaks in a series. Determining the sequence in this way is called *de novo* sequencing. A number of algorithms and software packages have been reported for the deduction of protein sequences from MS/MS data (Chen*, et al.*, 2001; Ma*, et al.*, 2003; Taylor and Johnson, 1997; Taylor and Johnson, 2001). A powerful software, PEAKS, which extracts amino acid sequence information from MS/MS data with or without the use of databases was used in this work (Ma*, et al.*, 2003).

*De novo* sequencing may not be possible for the majority of spectra obtained because of the following reasons (Peng and Gygi, 2001).

1. More than one ion series is usually present (i.e. sequencing the peptide from the N- and C-termini simultaneously),
2. Ion series are rarely complete,
3. Fragment ions are present in varying abundances, often with associated losses of water and/or ammonia, and
4. Some amino acids have very similar or identical molecular masses (e.g. isoleucine and leucine, glutamine and lysine, etc.).

For these reasons, database-searching algorithms have become an indispensable tool for the interpretation of tandem mass spectra.

### 2.5.4. Database searching

A decade ago, it has been realized that the peptide-sequencing problem could be converted to a database-matching problem, which would be much simpler to solve. The reason database searching is easier than *de novo* sequencing is that only an infinitesimal fraction of the possible peptide amino-acid sequences actually occur in nature. A peptide-fragmentation spectrum might, therefore, not contain sufficient information to unambiguously derive the complete amino-acid sequence, but it might still have sufficient information to match it uniquely to a peptide sequence in the database on the basis of the observed and expected fragment ions. A limitation of database searching compared to *de novo* sequencing is that large-scale proteomic experiments should only be carried out using organisms that have had their genome sequenced, so that all the possible peptides are known.

There are several different algorithms that are used to search sequence databases with tandem-MS-spectra data, and they have names such as PeptideSearch (Mann and Wilm, 1994), Sequest (Eng*, et al.*, 1994), and Mascot (Perkins, *et al*., 1999).

PeptideSearch makes use of the fact that fragmentation spectra usually contain at least a small series of easily interpretable sequence (Mann and Wilm, 1994). This series constitutes an amino acid tag. The lowest mass in the series contains information about the distance, in mass units, to one terminus of the peptide, and the highest mass contains information about the distance to the other peptide terminus. Together, the peptide-sequence tag consists of three parts (the amino-terminal mass, a short amino-acid sequence and the carboxy-terminal mass). This construct can be matched against sequences in the database and, if desired, the peptide that is identified can be made to comply with the cleavage event of the proteolytic enzyme used.

The software algorithm Sequest matches a peptide sequence with a tandem mass spectrum using the following steps (Eng, *et al*., 1994):

1. Peptides with molecular masses matching that of the peptide ion sequenced in the tandem mass spectra are extracted from a protein database;
2. Each peptide is given a preliminary score by examining the number of predicted fragment ions from the database peptide that match the acquired fragment ions in the tandem mass spectrum; and
3. The top 500 best matching peptides undergo a more rigorous ion-matching algorithm that generates a cross-correlation score. A list of peptides with good correlation is returned to the user with the top-scoring peptide being considered the best candidate.

Mascot search engine calculates the theoretically predicted fragments for all the peptides in the database and matches them to the experimental fragments in a top-down fashion, starting with the most intense b- and y-ions (Perkins, *et al*., 1999). It is called probability-based matching, since it calculates the probability that the number of fragment matches is random. The negative logarithm of this number multiplied by 10 is the identification score.

## 3. RESULTS AND DISCUSSION

### 3.1. Evaluation of the effect of radiation on two elastin cross-links, desmosine and isodesmosine (Appendix 6.1: Getie *et al*. 2003)

Some reports have suggested that during aging there are changes in the amino acid composition of elastin, so that newly synthesized molecules are rich in polar amino acids (Sandberg, 1976). At the same time, the total content of elastin may increase while the extent of cross-linking decreases, which is proposed to be due to oxidative stress (Fujimoto, 1982; Pasquali-Ronchetti and Baccarani-Contri, 1997; Watanabe*, et al.*, 1996). These changes lead to the stiffening and loss of elasticity of elastic fibers. The mechanism and causes are still not well defined. However, it is assumed that ROS produced as the result of solar radiation or oxidative reactions are among the potential causal factors in aging (Harman, 1956; Harman, 1981). Recently, Umeda and co-workers (Umeda*, et al.*, 2001) reported that hydroxyl radicals derived from Fenton reaction between transition metal ions, e.g., $Fe^{2+}$ and $Cu^{2+}$, and $H_2O_2$ play a significant role in the generation of oxodesmosine (OXD) from DES. A study was, therefore, performed to evaluate the effect of the Fenton reaction on the two cross-linked amino acids of elastin in the absence or presence of radiation generated from artificial sources. Solutions of DES or IDE (0.1 mg/ml) with or without $FeSO_4$ (10 μM) and $H_2O_2$ (1.5 mM) were prepared in distilled water. The samples were then treated and analyzed as described in Appendix 6.1.

Fig. 3.1 shows the positive ion ESI-ion trap mass spectrum of a solution of DES, $Fe^{2+}$, and $H_2O_2$ irradiated by the lamp that produces a sun-like spectrum (300 W) for 6 h. A monoisotopic ion peak at *m*/*z* 526.1, which is consistent with an elemental composition of $C_{24}H_{40}N_5O_8$ containing a quaternary ammonium ion, and two more peaks at *m*/*z* 497.1 and 481.1 were observed. A similar result was found for a solution containing IDE, $Fe^{2+}$, and $H_2O_2$, and treated in the same manner. Also incubation of DES as well as IDE solutions in the presence of $Fe^{2+}$ and $H_2O_2$ gave rise to similar spectra. On the other hand, both incubation and irradiation of the two solutions in the absence of $Fe^{2+}$ and $H_2O_2$ resulted in a single peak spectrum at *m*/*z* 526.1, indicating no observed alteration in the stability of the amino acids.

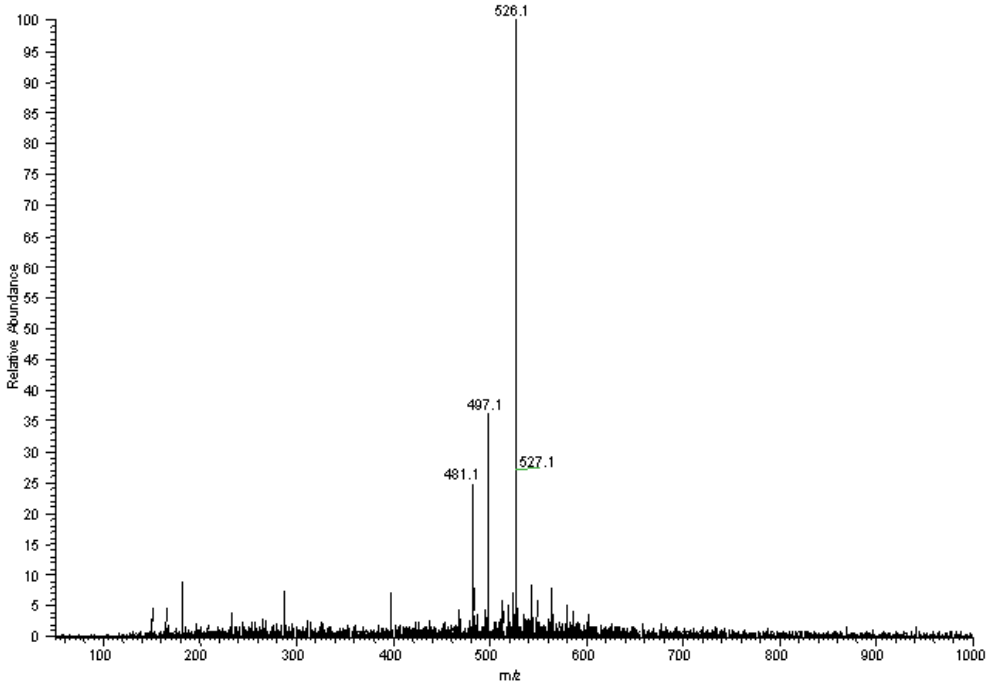
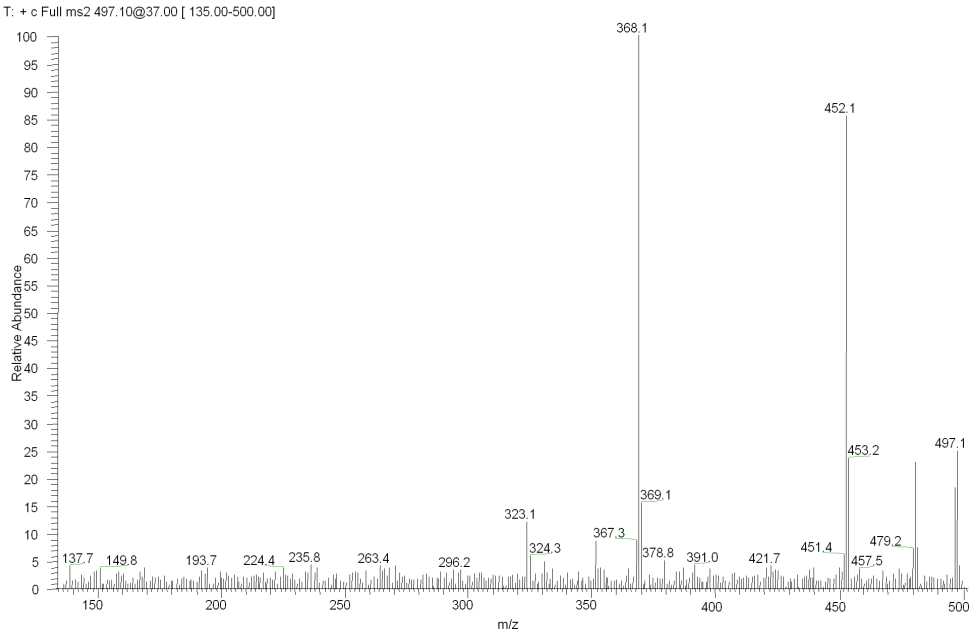



Figure 3.1: Mass spectrum of DES and its radiation induced degradation products

Umeda and co-workers (Umeda, *et al*., 2001) documented that OXD (mol. mass 495) was formed from DES as the result of oxidative stress while their experiment did not show the formation of isooxodesmosine (IOXD) from IDE. In contrast, our results demonstrated that Fenton reaction alone or accompanied by irradiation with a sun-like spectrum, UVA or UVB produced compounds of mol. mass 496, one mass higher than that reported by the authors, both from DES and IDE. ESI-qTOF MS experiments of irradiated and incubated solutions of DES and IDE revealed protonated molecular ion peaks at 497.2621, consistent with elemental compositions of $C_{23}H_{37}N_4O_8$, with standard errors of 1.86 ppm each. Supplemented by tandem MS experiments using ESI-ion trap, it was possible to postulate the structures of the oxidation products that provided peaks at *m*/*z* 497.1 and 481.1 (Appendix 6.1). The oxidation of IDE would most likely follow a similar scheme. The oxidation products are arbitrarily designated as $DESU_{497}$ and $DESU_{481}$, for oxidation products of DES that gave peaks at *m*/*z* 497.1 and 481.1, respectively, and $IDEU_{497}$ and $IDEU_{481}$ for oxidation products of IDE.

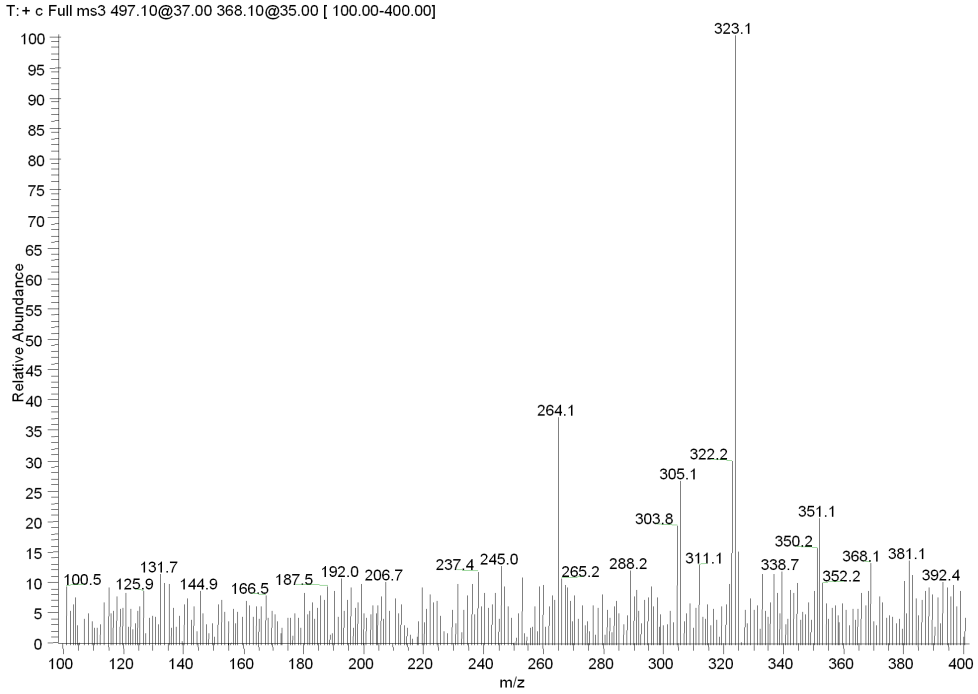
The suggested structures of the oxidation products contain an aldehyde group, which does not exist in the structure of Umeda *et al*. The presence of this aldehyde group was confirmed using Purpald®, a reagent that specifically reacts with aldehydes (Hopps, 2000), according to the method previously described by Jacobsen and Dickinson (Jacobsen and Dickinson, 1974).

Formation of a deep purple colour within 2 minutes upon addition of Purpald® proved the assumption that an aldehyde functional group is present on the chemical structure of at least one of the degradation products (Appendix 6.1). The tandem mass spectra of $DESU_{497}$ (fig. 3.2) revealed subsequent losses of $NH_3$ followed by CO confirming the presence of three unsubstituted amino groups and three unsubstituted carboxyl groups (Appendix 6.1). The tandem MS fragmentation pattern of $IDEU_{497}$ was exactly the same as that of $DESU_{497}$.

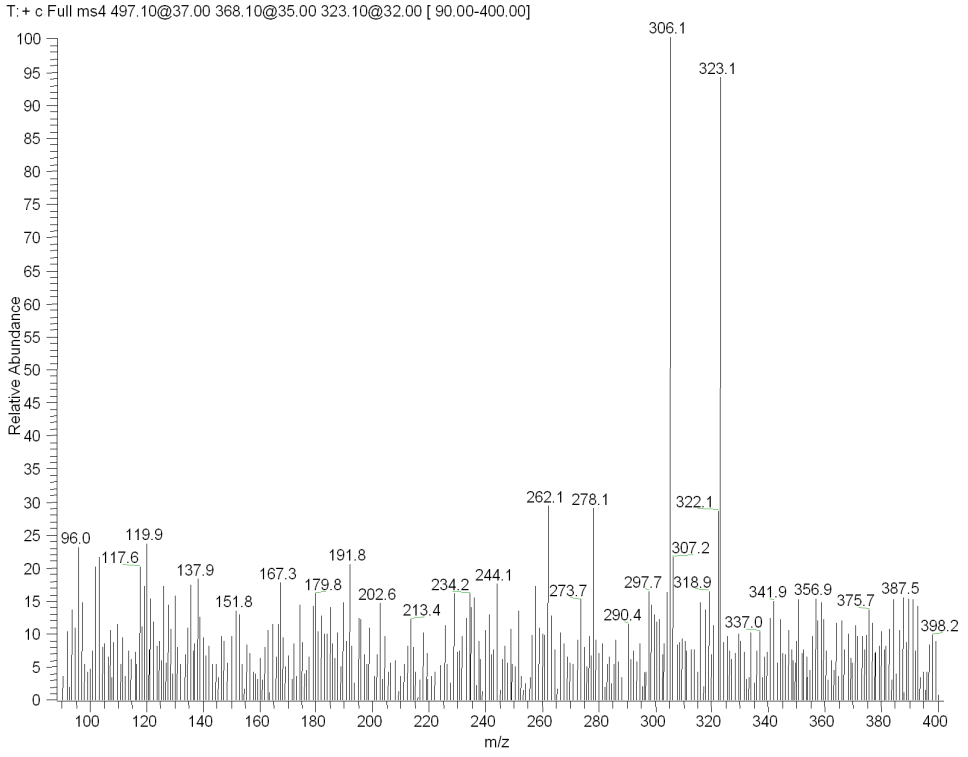



**$MS^2$ spectrum**

It has been observed that, following incubation of the solutions of DES and IDE at 37 °C in the presence of $FeSO_4$ and $H_2O_2$, there was a slight time dependent reduction in the concentration of the amino acids and a slight increase in the amount of products having *m/z* 497.1 and 481.1. Exposure of the solutions to IR radiation over a period of 8 h does not alter their concentrations. On the other hand, upon exposure with the Vitalux lamp, there was a high time dependent reduction in DES as well as IDE concentrations (down to 40 and 50%, respectively in 8 h time) and a high time dependent increase in the amounts of $DESU_{497}$ and $IDEU_{497}$. However, the amounts of $DESU_{481}$ and $IDEU_{481}$ were not significantly increased (Appendix 6.1).




**MS$^3$ spectrum**




**MS$^4$ spectrum**

Figure 3.2: The MS$^2$, MS$^3$, and MS$^4$ spectra of $DESU_{497}$

Exposure to UVA radiation resulted in a significant dose dependent decrease in the concentrations of DES and IDE and an increase in the concentrations of their degradation products (Appendix 6.1). Exposure to UVB radiation of doses 10 times less than that of UVA radiation resulted in a more drastic degradation of the cross-links. Since approximately one third of UVA radiation and about 10% of UVB radiation incident on Caucasian skin penetrates to the dermis (Bruls*, et al.*, 1984), it is essential to consider that prolonged exposure of the skin to solar radiation certainly could have a damaging effect to the elastin content of the skin.

### 3.2. Characterization of human skin elastin peptides (Appendix 6.2 and 6.3: Getie *et al.* 2005a, Schmelzer *et al.* 2005)

Characterization of the elastin structure is very useful in helping to understand the biochemical basis of several pathological conditions, including solar elastosis, emphysema, aneurysm and atherosclerosis, in which the mechanical and elastic properties of tissues are altered. However, due to its extreme insolubility in water or any organic solvent, such studies have been hampered. Biochemical studies on elastin have focused mainly on complete hydrolysis of the protein in a strongly acidic environment and analysis of the resulting cross-linked amino acids, desmosine and its isomer isodesmosine, which are only found in elastin (Kaga*, et al.*, 2003; Salomoni*, et al.*, 1991). This approach has been challenged, among other things, by the incomplete chromatographic separation of the amino acids with the analytical methods employed to date and by the insufficient information that can be obtained about the entire elastin molecule only based on the cross-linked amino acids. Extraction of elastin from a healthy and diseased tissue and comparison of the primary structures of the peptides resulting from enzymatic digestion of the protein could provide a good insight into the biochemical changes that occur due to the pathological conditions mentioned. Accomplishment of this task requires the identification of the resulting peptides by determining their primary sequences with consideration of posttranslational modifications.

Currently, determination of the amino acid sequence of peptides resulting from enzymatic digestion or chemical hydrolysis of proteins is done most often by employing tandem mass spectrometry. Therefore, in this study, the amino acid sequences of some of the peptides resulting from enzymatic digestion of human skin elastin with elastase, thermitase, or pepsin were determined by tandem MS using ion trap or qTOF analyzers and the resulting mass

spectra were sequenced by database matching or combination of *de novo* sequencing and database matching.

Fig. 3.3 shows peptides identified from the elastase digest of human skin elastin mapped on human tropoelastin sequence (Accession number: A32707). Over 100 peptides were identified, making the sequence coverage 58.8%. As shown in fig. 1 of Appendix 6.3 (Schmelzer *et al*. 2005), 89 and 72 peptides were identified from the thermitase and pepsin digests, respectively, the sequence coverage being 44.2 and 48.8%. Combining the results of the three enzymes, the sequence coverage was 74.1%.

It can be seen from fig. 3.3 and fig. 1 of Appendix 6.3 that almost no peptide was identified in regions consisting of stretches of lysine separated by two or three alanine residues such as AAAKAAKAA. This is due to the fact that the lysine molecules in tropoelastin are modified to form polyfunctional cross-links such as desmosine, isodesmosine, lysinonorleucine, merodesmosine, and cyclopentenone (Akagawa and Suyama, 2000; Bedell-Hogan*, et al.*, 1993; Reiser*, et al.*, 1992; Rosenbloom*, et al.*, 1993). Sequencing peptides containing such modifications was not possible by the approach employed in this study. Alanine was detected in the sample indicating that some or most of the alanine residues in these regions could have been cleaved out.

Analyses of tropoelastin cDNAs indicated that there is significant variation within a species in nucleotide sequence and size of both the isolated mRNA and cDNA (Baule and Foster, 1988; Fazio*, et al.*, 1988a; Fazio*, et al.*, 1988b; Pierce*, et al.*, 1990). cDNAs were found to differ only in the presence or absence of specific domains corresponding to entire exons or segments of exons. These variable cDNAs were shown to be the result of alternative splicing of tropoelastin mRNA. Several human tropoelastin splice variants have been identified with some exons shown to be subject to alternative splicing (Boyd, *et al*., 1991; Fazio, *et al*., 1988a; Indik, *et al*., 1987). In an effort to investigate which of the splicing variants are present in our human skin elastin sample, we developed a database, which considers the aforementioned variants of elastin.

It has been previously reported that human skin elastin does not contain amino acid sequences expressed by two exons: exon 22 (residues 453-481) and exon 26A (residues 618-650) (Fazio, *et al*., 1988a). The absence of amino acids expressed by exon 26A could be

unambiguously confirmed by the absence of amino acids identified from this region and the presence of the two peptides, GVPGFGAVPG ($M_r$ 856.44) and GAGVPGFGAVPG ($M_r$ 984.50), in the elastase digest; AV**p**GAL (Mr 542.31) in the thermitase digest; and GAVPGAL (Mr 583.33) in peptic digest, which share amino acids expressed by exons before and after exon 26A. Contrary to what has been published earlier by Fazio *et al*., the identification of three peptides (residues 461-482 and 478-488 in peptic digest and residues 462-467 in thermitase digest), which share amino acids from exon 22 indicated that this exon existed in the human skin elastin sample under investigation. The presence of the 6 amino acids exon (residue 501-506) in human skin elastin (Fazio, *et al*., 1988a) was confirmed by the existence of three peptides in the elastase digest: NLAGL (residue 504-508), AGLVPGVGVAPGVGVAPG (residue 506-523), and AGLVPGVGVAPGVGVAPGVG (residue 506-525), which share amino acids expressed by the exon.

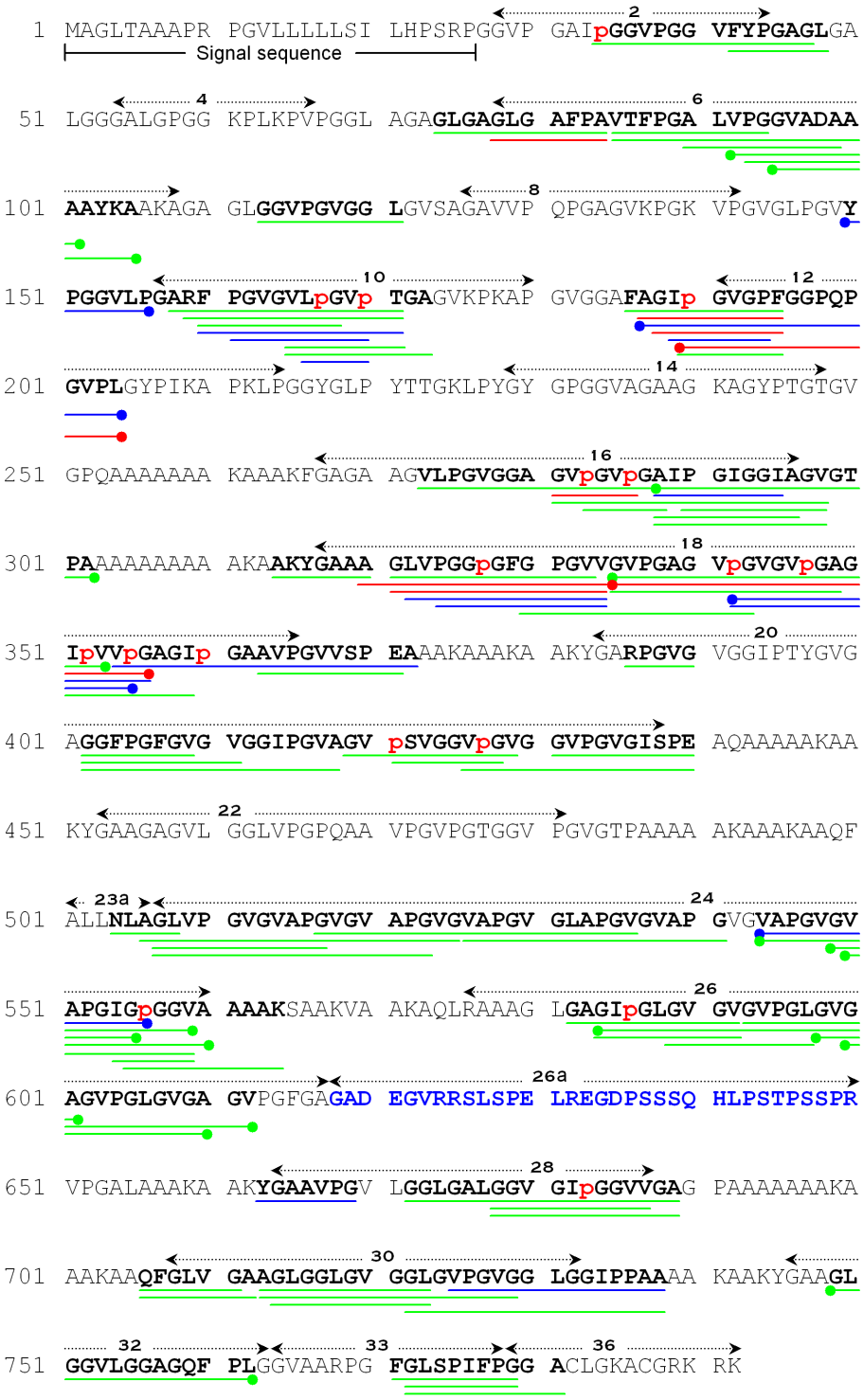



Figure 3.3: Peptides identified from the elastase digest of human skin elastin mapped on human tropoelastin sequence (Accession number: A32707). Peptides identified after analysis using the LC-ESI (bright green), nano-ESI (blue) and both (red) instruments are underlined in solid lines. Sequences extending to the next lines are underlined in oval arrow lines. **p** refers to proline residues with a potential for hydroxylation.

### 3.3. Identification of the potential sites of proline hydroxylation in human skin elastin

(Appendix 6.2 and 6.3: Getie *et al*. 2005a, Schmelzer *et al*. 2005)

One of the interesting features of protein characterization with MS is the ability to determine posttranslational modifications. Hydroxylation of Pro residues in tropoelastin by the enzyme prolyl hydroxylase is reported to occur in some animals to a varying degree (Dunn and Franzblau, 1982; Sandberg*, et al.*, 1969; Smith*, et al.*, 1972; Uitto*, et al.*, 1976). The function of this modification is, however, not yet understood. Whereas Pro hydroxylation plays a critical role in the synthesis and secretion of a related protein, procollagen, and for its complete maturation to insoluble collagen (Uitto and Lichtenstein, 1976), its presence is not required for the synthesis and secretion of tropoelastin (Rosenbloom and Cywinski, 1976). It has been reported that the existence of hydroxyproline in elastin may be a coincidental feature of the fact that the precursors of elastin and collagen are synthesized in the same region of the endoplasmic reticulum and the prolyl hydroxylase, which exists there, hydroxylates some of the Pro residues occurring in the tropoelastin polypeptides (Uitto, 1979). On the other hand, there are reports, which suggest that cross-linking and the formation of elastin from tropoelastin is reduced by overhydroxylation. For example, Urry and co-workers have shown that a synthetic polymer pentapeptide $(Val\text{-}Pro\text{-}Gly\text{-}Val\text{-}Gly)_n$, which is capable of coacervating at 37 °C similar in a mechanism to tropoelastin, requires a higher temperature to coacervate when some of the Pro residues are replaced by hydroxyproline residues (Urry, *et al*., 1979). The degree of such a modification in human elastin is little investigated. Therefore, in this work, an attempt was made to determine the extent of Pro hydroxylation and the locations of potentially hydroxylated Pro residues in the human skin elastin sample. For this purpose, the protein was digested with three enzymes; elastase, thermitase or pepsin and the results obtained by analyzing the resulting peptides were combined to determine the hydroxylation sites. The combined use of Mascot search engine and *de novo* sequencing enabled the unequivocal assignment of hydroxylation sites.

Several Pro residues were found to exist in a hydroxylated form in peptides of human skin elastin. Fig. 3.3 and fig. 1 of Appendix 6.3 depict the Pro residues existent in a hydroxylated form at least in one of the peptides identified from the proteolytic digest of any of the enzymes used; Pro molecules with a potential for hydroxylation are designated by a red colored small letter **p** in the human tropoelastin sequence (Accession number A32707). Over 30 Pro residues, where hydroxylation could take place were identified, covering about 40%

of the Pro molecules in the sequenced regions of the protein. The observation that some Pro residues exist in a hydroxylated and non-hydroxylated form in a single peptide indicated the possibility of partial hydroxylation of the Pro residues in the tropoelastin molecule. Therefore, the actual extent of hydroxylation, which is difficult to estimate from this experiment, is presumably less than 40%. Table 3.1 shows the list of such peptides containing Pro residues and their hydroxylated counterparts. Despite their higher masses, the peptides containing hydroxylated Pro were consistently observed 1 to 2 min ahead of their non-hydroxylated counterparts in the LC-MS chromatograms. This is in a good agreement with the general understanding that, under the chromatographic conditions employed in this experiment, if all other variables remain constant, the more hydrophilic peptides are eluted earlier than the less hydrophilic peptides.

The hydroxyproline/proline ratios, as determined from the relative LC-MS chromatogram peak areas of the hydroxylated to non-hydroxylated peptide pairs, differ region wise (fig. 3.4), indicating regional variation in the extent of Pro hydroxylation by prolyl hydroxylase. In order to appreciate the consistency of these ratios for a specific peptide, comparison was made between peptides that existed in the digests of at least two enzymes. All results unequivocally confirmed the absence of significant difference ($P<0.05$). The results of three such peptides present in the elastase and thermitase digest are shown in fig. 3.4.

### 3.4. Complementary mass spectrometric techniques to achieve complete sequence coverage of recombinant human tropoelastin (Appendix 6.4: Getie *et al*. 2005b)

As mentioned above, analyses of tropoelastin cDNAs resulted in the identification of several human tropoelastin splice variants. Tropoelastin is also known to undergo posttranslational modification, particularly hydroxylation of the Pro residues and cross-linking of the lysine residues. Several pathological conditions are associated to elastic fibers, which mainly comprises of elastin, the matured form of tropoelastin. Understanding the structural changes arising from these physiological and pathological modifications requires the availability of data showing the primary structure of the proteins before and after the modifications. Such data could be obtained by proteolytic digestion of the proteins in order to produce peptides that have proper molecular size for mass spectrometric characterization and subsequent sequencing. A good insight into the molecular changes would be achieved if this kind of approach enables characterization of the protein with full sequence coverage.

Table 3.1: List of peptides containing hydroxylated Pro residues and their non-hydroxylated counterparts

| Residue No.[a] | Peptide sequence | Molar mass | RT[b] | Enzyme | | |
|---|---|---|---|---|---|---|
| | | | | Elastase | Thermitase | Pepsin |
| 113-121 | GGVPGVGGL | 711.39 | 21.1 | | x | x |
| | GGVpGVGGL | 727.39 | 20.0 | | | |
| 190-195 | PGVGPF | 572.30 | 30.2 | x | x | |
| | pGVGPF | 588.29 | 29.0 | | | |
| 187-195 | AGIPGVGPF | 813.44 | 30.6 | x | x | |
| | AGIpGVGPF | 829.43 | 29.1 | | | |
| 272-287 | GVLPGVGGAGVPGVPG | 1288.71 | 32.5 | x | | |
| | GVLPGVGGAGVpGVPG | 1304.71 | 30.6 | | | |
| 281-293 | GVPGVPGAIPGIG | 1089.62 | 29.4 | | x | |
| | GVPGVpGAIPGIG | 1105.61 | 28.3 | | | |
| 321-334 | GLVPGGPGFGPGVV | 1208.66 | 32.3 | x | | |
| | GLVPGGpGFGPGVV | 1224.65 | 30.4 | | | |
| 335-356 | GVPGAGVPGVGVPGAGIPVVPG | 1809.01 | 32.0 | x | | |
| | GVPGAGVpGVGVPGAGIPVVPG | 1825.01 | 31.0 | | | |
| 582-592 | GAGIPGLGVGV | 895.51 | 29.6 | x | | x |
| | GAGIpGLGVGV | 911.51 | 28.5 | | | |
| 584-592 | GIPGLGVGV | 767.45 | 29.2 | x | | |
| | GIpGLGVGV | 783.45 | 27.5 | | | |
| 672-689 | GGLGALGGVGIPGGVVGA | 1406.79 | 31.8 | x | | |
| | GGLGALGGVGIpGGVVGA | 1422.78 | 30.4 | | | |
| 678-689 | GGVGIPGGVVGA | 938.52 | 24.5 | x | x | |
| | GGVGIpGGVVGA | 954.51 | 22.0 | | | |

[a] Residue number on the tropoelastin sequence in PIR database with the accession number: A32707

[b] Retention time on the basis of LC-MS experiment: Nucleosil 120-5 C18 (125 x 2) column, linear gradient mobile phase (5-60% of acetonitrile in water, both containing 0.1% of formic acid), flow rate: 0.2 mL/min

Several complementary ionization techniques of MS, such as ESI and MALDI, which -when used together - lead to the identification of more peptides than can be identified by either technique alone have been documented (Baldwin*, et al.*, 2001; Bodnar*, et al.*, 2003; Heller*, et al.*, 2003; Medzihradszky*, et al.*, 2001; Stapels and Barofsky, 2004; Stapels*, et al.*, 2004). The complementarity between ESI and MALDI is thought to be due to preferences of the two ionization techniques for certain classes of amino acids. MALDI is reported to preferentially ionize basic residues (Cohen and Chait, 1996; Krause*, et al.*, 1999; Zhu*, et al.*, 1995) or aromatic peptides (Stapels and Barofsky, 2004), while ESI might be better employed in the analysis of hydrophobic peptides (Cech and Enke, 2000).

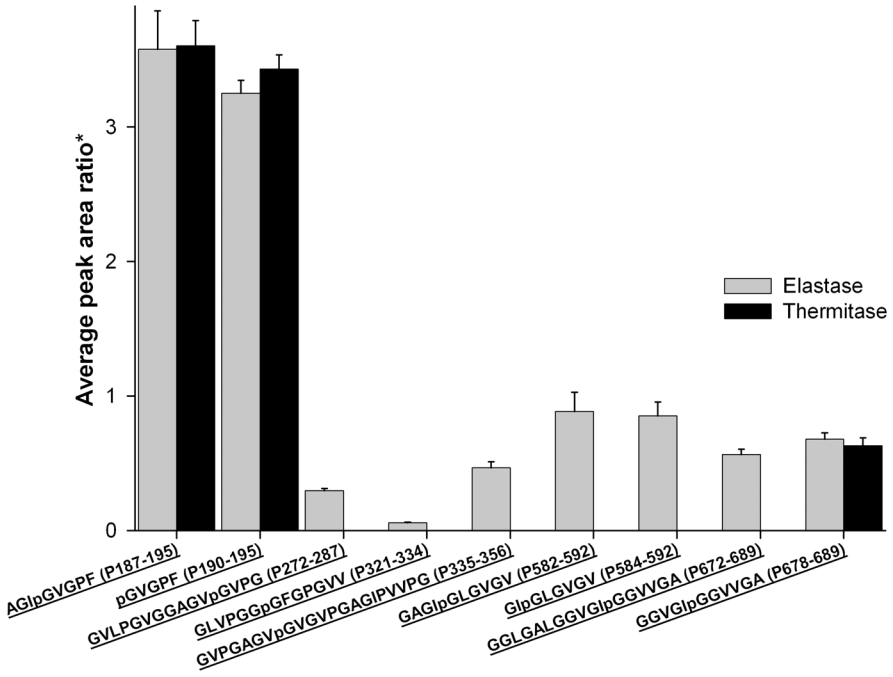



Figure 3.4: The extent of Pro hydroxylation in some peptides resulting from digestion of human skin elastin with elastase in relation to the co-existing non-hydroxylated forms. Comparison is made with the thermitase digest for peptides that also existed both as hydroxylated and non-hydroxylated forms in the digest. The hydroxylated Pro is designated with a small letter "**p**" in the sequences.
*The average LC-MS chromatogram peak area ratio of the hydroxylated to non-hydroxylated peptide.

ESI has mainly been used to sequence peptides separated by HPLC (Cech and Enke, 2001) while MALDI has been used to mass fingerprint mixtures of peptides (Aebersold and Mann, 2003; Mann and Talbo, 1996). This division of use came about because ESI can easily be coupled on-line to receive the eluate from an HPLC column, and MALDI can normally be used to analyze mixtures of peptides without resorting to separation. Separation of peptides prior to MALDI, however, reduces ion suppression (Kratzer*, et al.*, 1998) and leads to higher signal-to-noise ratios and greater sensitivity (Preisler*, et al.*, 2000).

In this study, tandem MS experiments were performed on the peptides of recombinant human tropoelastin using LC-ESI-ion trap, nano-ESI-qTOF and offline LC-MALDI-TOF MS in

order to exploit their complementarity towards obtaining full sequence coverage of the protein. The resulting fragment spectra were processed with software tools to create monoisotopic masses for subsequent peptide identification by database searching and *de novo* sequencing.

### 3.4.1. Characterization of the peptic digest

A total of 100 peptides possessing between 4 and 54 amino acids were identified from the peptic digest. Fig. 1 of Appendix 6.4 shows the sequence of the recombinant human tropoelastin on which the identified peptides are indicated. The sequence coverage excluding the signal sequence was 86.7%. Despite the relatively fewer number of peptides identified by the nano-ESI instrument, the sequence coverage (72.1%) was far greater than that of peptides detected by ESI (48.4%). This can be attributed to the increase in the length of the amino acid chain of the peptides detected by the former instrument.

### 3.4.2. Characterization of chymotrypsin digest

The tropoelastin was also digested with chymotrypsin and analyzed with nano-ESI-qTOF-MS-MS and MALDI-PSD. In the latter case, the peptide mixture was fractionated by off-line HPLC prior to introduction into the MALDI instrument. This step helps to minimize the occurrence of overlapping fragment spectra as the result of the introduction of more than one peptide at a time during PSD analysis using the timed ion selector. The peptides produced by chymotrypsin digestion, detected by nano-ESI and MALDI MS instruments and identified by *de novo* sequencing and database matching are depicted on the tropoelastin sequence as shown in fig. 2 of Appendix 6.4. A total of 49 peptides (20 by MALDI, 38 by nano-ESI and, 9 of which by both methods) with the number of amino acids ranging from 6 to 59 were sequenced. Despite their number being half the number of peptides identified from the pepsin digest, the overall sequence coverage of the peptides observed from the chymotrypsin digest (84.7%) was comparable. Taking into consideration the peptides identified by nano-ESI analysis alone, it was necessary to sequence 57 peptides from the pepsin digest in order to achieve sequence coverage of 72.1%, whereas a relatively higher coverage (78.9%) was attained by only 38 peptides from the chymotrypsin digest. This is attributed to the residue specificity of this enzyme as compared to pepsin, resulting in the formation of longer peptides.

### 3.4.3. Overall sequence coverage

In general, combining the results obtained from the digests of the two enzymes analyzed by the three instruments, sequence coverage of 94.4% was achieved. The sequence coverage could have been higher than reported if some detected ions with *m/z* corresponding to individual amino acids, such as Ala, Lys, Leu/Ile, Phe, Arg, and Tyr, and whose positions cannot be unequivocally determined in the protein sequence had been considered. The presence of the Ala and Lys in the samples is particularly important, because most of the region not covered by the peptides identified is rich in these residues. This is one of the very few attempts made to achieve complete sequence coverage of proteins by analyzing their enzymatically-produced peptides. Tropoelastin is one of the most internally repetitive human proteins known, so it represents an interesting model protein for this class of molecules that are still amenable to sequencing by MS analysis.

In order to attain sequence coverage of this extent, a comprehensive identification of the peptides produced by digestion of the protein with two enzymes, pepsin and chymotrypsin, with instruments of three complementary ionization techniques of MS, namely ESI, nano-ESI, and MALDI and *de novo* sequencing as well as database searching was made. As has been demonstrated earlier, the complementarity of these methods is attributed to preferential ionization of certain residues by any one of the methods. For studies involving the characterization of tropoelastin with significant sequence coverage, digestion of the protein with chymotrypsin and analysis of the peptides using MALDI and nano-ESI seems the best and timesaving approach. However, matured elastin is not adequately digestible with chymotrypsin; thus digestion requires the use of low substrate specific enzymes such as elastase, thermitase and pepsin.

**3.5. Cleavage sites of the enzymes used** (Appendix 6.2 - 6.4: Getie *et al*. 2005a, Schmelzer *et al*. 2005, Getie *et al*. 2005 b)

Another important observation made in this work was the determination of the cleavage sites of the enzymes used. On the basis of the cumulative results obtained from human skin elastin and recombinant tropoelastin, it was possible to determine the cleavage sites of the enzymes used. The cleavage sites of elastase, thermitase, pepsin and chymotrypsin observed on human skin elastin and recombinant tropoelastin are listed in Table 3.2.

C-terminal cleavage by elastase predominantly on the amino acids Gly, Val, Leu, Ala and Ile, but also to a lesser extent on Phe and Pro was previously reported (Mecham*, et al.*, 1997). Our experiment confirmed this and revealed C-terminal cleavage on two more amino acids, namely Glu and Arg. It has also been observed that thermitase and pepsin mostly cleaved at the C-terminals of three of the top four dominant amino acids, i.e., Gly, Ala, and Val. They cleaved at the C-terminals of Phe and Leu to a similar degree as well. Pepsin occasionally cleaved at the C-terminals of other amino acids: Ile, Asp, Tyr, Gln, Ser, and Thr., too. The results of pepsin are in agreement with previous reports (Schmelzer*, et al.*, 2004; Wilkins*, et al.*, 1997). Chymotrypsin is known to hydrolyze peptide bonds specifically at the C-terminals of Tyr, Phe, Trp, and Leu (Chan*, et al.*, 1986; Szabo*, et al.*, 1986). Met, Ala, Asp, and Glu are also cleaved at a lower rate. Similarly, in this study, we observed that chymotrypsin cleaved predominantly at Tyr, Phe, and Leu residues and, rarely, at Ala, Ile, Thr, and Gly. Several peptides that were a result of cleavage at the C-terminals of Lys and Arg residues were found in the chymotrypsin digest. Since cleavage at these sites is characteristic of trypsin (Olsen*, et al.*, 2004), contamination of the enzyme with traces of trypsin was anticipated and subsequently confirmed by MALDI-MS experiment.

Table 3.2: Cleavage sites of the proteases used on human skin elastin (Heln) and recombinant tropoelastin (bold – frequent cleavage sites; italics – occasional cleavage sites)

| Enzyme | Cleavage sites (C-terminal) | |
|---|---|---|
| | Heln | Tropoelastin |
| Elastase | **G**, **V**, **A**, **L**, **I**, *F*, *P*, *E*, *R* | NS |
| Thermitase | **G**, **V**, **A**, **L**, **F**, *P*, *T*, *E*, *Q*, *K* | NS |
| Pepsin | **G**, **V**, **A**, **L**, **F**, *Y*, *S*, *T*, *I*, *D* | **G**, **V**, **A**, **L**, **F**, *Y*, *S*, *T*, *I*, *D*, *Q* |
| Chymotrypsin | NS | **Y**, **F**, **L**, *A*, *I*, *T*, *G* |

NS – not studied

### 3.6. Effect of UVB on recombinant human tropoelastin and human skin elastin peptides

Tropoelastin was dissolved in 1 mM Tris buffer, pH 8.5, at a concentration of 250 µg/ml and a 200 µL solution was placed in a 48-well microplate, then exposed to UVB (250 mJ/cm$^2$) alone or in the presence of hematoporphyrin (HP) (40 mM) or ferrous sulfate (10 µM) using an equipment that emits specific wavelength ranges of UVB (280–350 nm) radiation (Veith import export, Westerau, Germany). The sample was then analyzed using MALDI MS or digested by chymotrypsin in 1 mM Tris buffer, pH 8.5, before analysis using LC-ESI MS. For the MALDI MS experiment, sinapinic acid was used as a matrix. A 200 µL solution of

peptides of the recombinant tropoelastin or human skin elastin produced by digesting the proteins by chymotrypsin was also exposed to UVB under similar conditions and analyzed using LC-MS. In all cases, untreated samples were used as controls. All experiments were performed in triplicate.

The MALDI mass spectra of the tropoelastin before and after exposure to UVB looked alike, showing peaks at 30237.91 ($z$ = 2) and 60571.17 ($z$ = 1). No considerable difference was observed between the intensity of the peaks of the mass spectra from tropoelastin exposed to UVB in the presence of ferrous sulfate and the untreated tropoelastin (fig. 3.5). Non-irradiated tropoelastin sample containing the photosensitizer, HP, showed comparable intensity mass spectrum. On the contrary, both peaks (from singly and doubly charged tropoelastin) almost disappeared after treatment of the protein with UVB in the presence of HP, indicating a high degree of degradation. Detectable degradation products with molecular weight less than 70 kDa did not accompany the decrease in tropoelastin peak intensity. This observation is in a good agreement with the report of Hayashi *et al*. (Hayashi*, et al.*, 1998), who found a similar result by treating tropoelastin with UVA (2 mW/cm$^2$). These authors, contrary to the HP treated sample, detected degradation products of molecular weight 45, 30 and 15 kDa, by exposing the tropoelastin to UVA in the presence of copper sulfate, which generates hydroxyl radicals when exposed to radiation. Our trial with ferrous sulfate, which as well induces formation of hydroxyl radicals, did show neither a substantial decrease in tropoelastin nor formation of degradation products.

The degradation of tropoelastin by the combined effect of UVB and HP was further confirmed by enzymatically digesting the protein and analyzing the resulting peptides. Comparison of the LC-MS peak areas of the peptides before and after treatment of the tropoelastin with UVB in the presence HP indicated a dramatic decrease in the amount of all of the peptides analyzed due to treatment (fig. 3.6). HP in the presence of UV radiation (UVA or UVB) is known to produce singlet oxygen (Hayashi, *et al*., 1998), which could most likely be responsible for the degradation of tropoelastin.

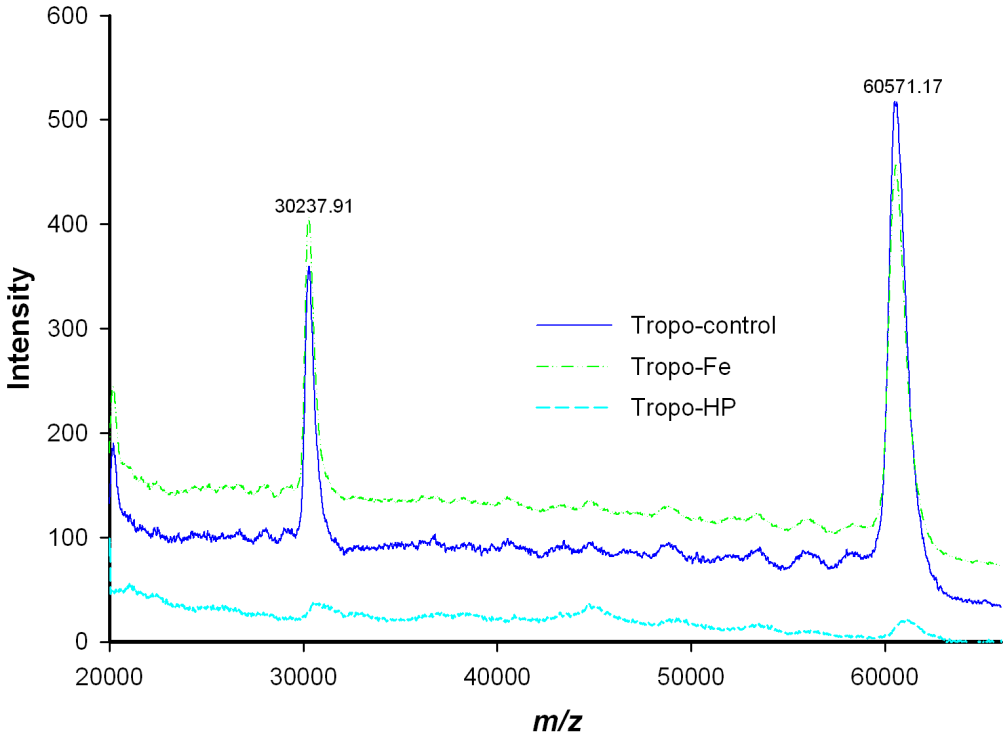



Figure 3.5: MALDI mass spectra of recombinant human tropoelastin before (solid line) and after exposed to UVB (250 mJ/cm$^2$) in the presence of ferrous sulfate (dash-dot-dot line) or hematoporphyrin (medium dash line).

Treatment of the already produced peptides of the tropoelastin or the human skin elastin under similar conditions did not show any detectable quantitative change (figs. 3.7 and 3.8). Comparison of the mass fingerprint of the LC-MS chromatograms before and after treatment did not reveal any sign of formation of degradation products in all cases. At this point, this result cannot be adequately explained. The decrease in the amount of tropoelastin peptides when the tropoelastin was treated intact followed by digestion could seem to be associated to the potential inhibition of enzymatic activity by HP. However, this assumption fails to explain the dramatic decrease in the peak intensity of the tropoelastin MALDI mass spectrum observed after exposure of the protein to UVB and HP (fig 3.5).

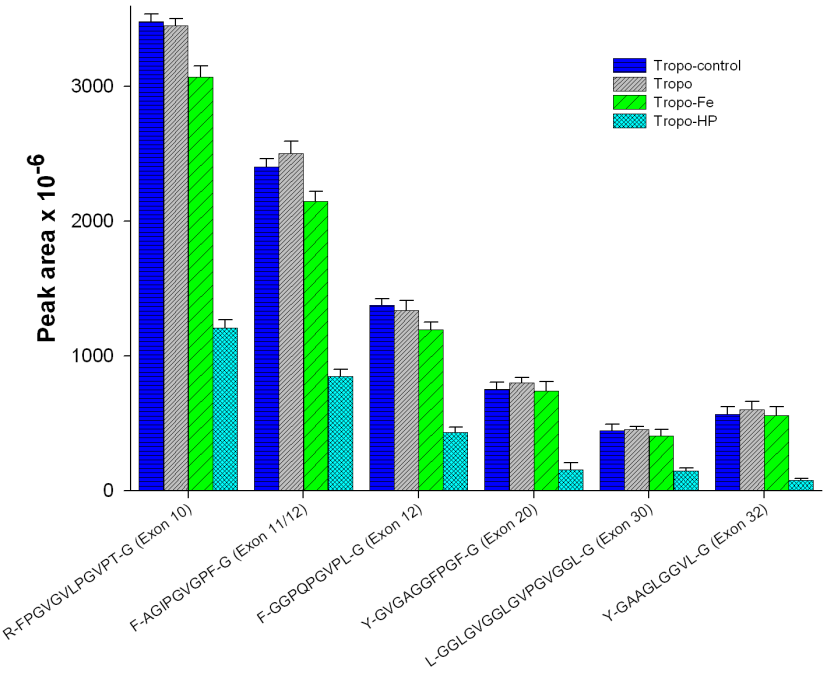



Figure 3.6: Influence of UVB (250 mJ/cm$^2$) on recombinant human tropoelastin peptides alone (Tropo) or in the presence of ferrous sulfate (Tropo-Fe) or HP (Tropo-HP). Peptides were generated by chymotrypsin digestion after treatment of the protein.

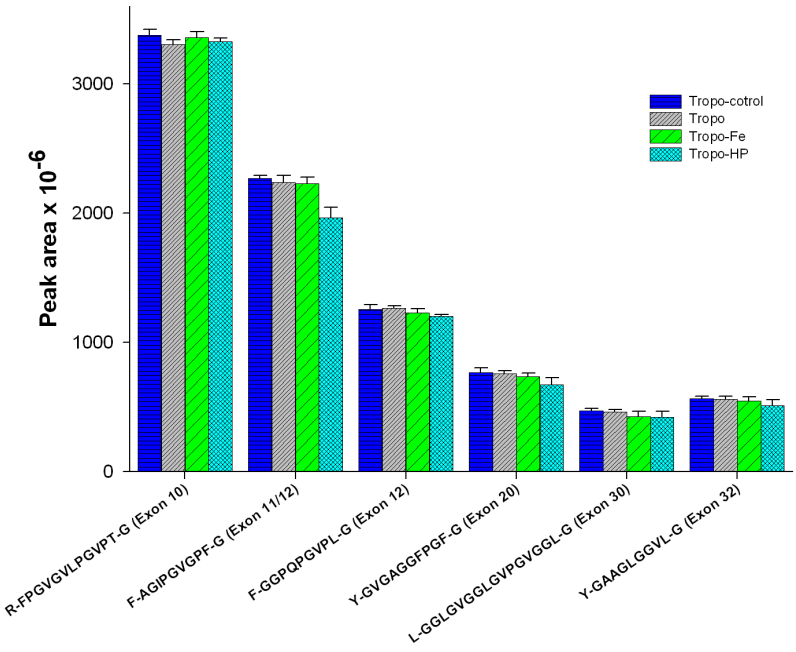



Figure 3.7: Recombinant human tropoelastin peptides exposed to UVB (250 mJ/cm$^2$) alone (Tropo) or in the presence of ferrous sulfate (Tropo-Fe) or HP (Tropo-HP). Treatment was done after peptides were generated by chymotrypsin digestion.

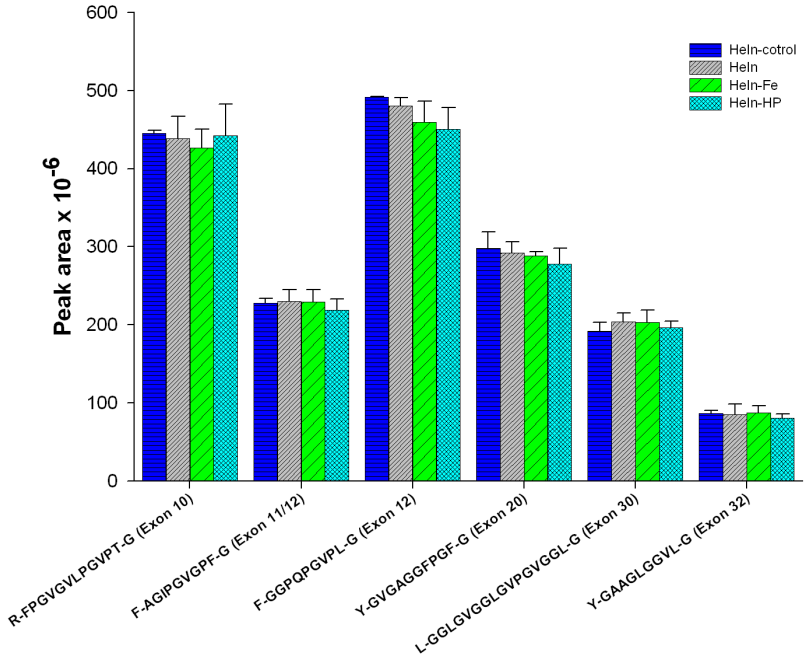



Figure 3.8: Effect of UVB (250 mJ/cm$^2$) on human skin elastin peptides alone (Heln) or in the presence of ferrous sulfate (Heln-Fe) or HP (Heln-HP). Digestion of the protein was performed before exposure to UVB.

### 3.7. Conclusive remark

In addition to providing an important framework for future efforts aimed at characterizing structural changes on elastin and other elastic fiber components in response to photoaging, this work established a comprehensive database of biomarkers for qualitative and quantitative characterization of elastin. Exposure of the skin to UV radiation results in a cascade of molecular events that affect a number of proteins and other cellular components. Although stepwise approaches focusing on the analysis of single molecules could serve well in obtaining specific information, comprehensive molecular approaches comprising the whole skin proteome would contribute to a better understanding of how skin responds to UV radiation. Therefore, proteomic initiatives to study changes in the profile of global protein expressions in skin due to photoaging are recommended for future work.

## 4. SUMMARY

The objective of this work was to characterize human skin elastin peptides and to evaluate the influence of radiation on the molecular structure of elastin using mass spectrometry approach. In the first step of this study, the effect of radiation generated from artificial sources was studied on two elastin cross-linked amino acids, DES and IDE, in the absence or presence of $Fe^{2+}$ and $H_2O_2$, which generate hydroxyl radicals by Fenton reaction.

Since biochemical analysis of the intact elastin is hardly possible due to its extreme insolubility both in water and in organic solvents, an effort was exerted to develop a method that enables characterization of its primary structure as well as that of its precursor, tropoelastin. Thus, tandem MS, using ESI-ion trap, nano-ESI-qTOF and/or MALDI-TOF MS, were performed on the peptides obtained by digestion of the proteins with elastase, thermitase, pepsin, or chymotrypsin. Database matching and/or *de novo* sequencing were then employed to identify the sequences of peptides from the fragment mass spectra. Finally, the effect of UVB on recombinant human tropoelastin and its peptides as well as peptides of human skin elastin was evaluated in the presence or absence of hematoporphyrin or ferrous sulfate. The results observed by the mentioned experiments are summarized below.

**1. Effect of radiation on cross-linked amino acids (DES and IDE)**

- No alteration in the stability of the amino acids was observed from samples treated with all forms of radiation in the absence of $Fe^{2+}$ and $H_2O_2$.
- Incubation of the amino acids with $Fe^{2+}$ and $H_2O_2$ or irradiation of the same sample with
    - A lamp that emits a sun-like spectrum (300 W),
    - UVA (305-420 nm) - 1.25-30 J/cm$^2$, or
    - UVB (280-350 nm) - 0.125-3 J/cm$^2$ produced a drastic decrease in the amount of the elastin cross-links and resulted in the formation of degradation products with mol. mass of 496 and 481.
- Structural elucidation of the degradation products suggested that oxidation of the amino acids catalysed by hydroxyl radicals, which is generated synergistically from radiation and Fenton reaction between $Fe^{2+}$ and $H_2O_2$, could have taken place.

- Irradiation of the amino acids with IR (520 W) in the presence of $Fe^{2+}$ and $H_2O_2$ did not affect their amount or produce any detectable degradation product.

**2. Characterization of human skin elastin peptides**

- Several peptides were identified from the elastase, thermitase and peptic digests of elastin, making the sequence coverage 58.8, 44.2 and 48.8%, respectively.
- The sequence coverage obtained by combining the results of the three enzymes was 74.1%.
- As has been reported before, the absence of amino acids expressed by exon 26A in the elastin sample studied was unambiguously confirmed by the absence of amino acids identified from this region and the presence of peptides, which share amino acids expressed by exons before and after exon 26A.
- Contrary to what has been published earlier, the identification of some peptides, which share amino acids expressed from exon 22 indicate that this exon exists in the human skin elastin mRNA.
- About 40% of the proline residues in the sequenced regions of the protein have a potential for hydroxylation by the enzyme prolyl hydroxylase.
- The hydroxyproline/proline ratios determined from the relative LC-MS chromatogram peak areas of the hydroxylated and non-hydroxylated peptide pairs differ region-wise, indicating regional variation in the extent of proline hydroxylation.

**3. Characterization of recombinant tropoelastin peptides**

- A total of 100 and 49 peptides with sequence coverage of 86.7 and 84.7% were identified from the peptic and chymotrypsin digests, respectively.
- An overall sequence coverage of 94.4% was achieved from the digests of the two enzymes.

**4. Cleavage sites**

- The cleavage sites of the enzymes used in this study were determined based on the cumulative data obtained from human skin elastin and recombinant tropoelastin.
- Elastase, thermitase, and pepsin principally cleaved at the C-terminal of three of the top four dominant amino acids, (i.e., Gly, Ala, and Val) as well as Leu.

- A similar degree of cleavage was observed at the C-terminal of Ile by Elastase, and at the C-terminal of Phe by thermitase and pepsin.
- Chymotrypsin cleaved predominantly at the C-terminal of Tyr, Phe, and Leu and, occasionally, at Ala, Ile, Thr, and Gly.

**5. The influence of UVB on recombinant human tropoelastin, its peptides and peptides of human skin elastin**

- The MALDI mass spectra of the tropoelastin showed two peaks at 30237.91 ($z$ = 2) and 60571.17 ($z$ = 1).
- No significant difference was observed between the intensity of the peaks from untreated tropoelastin and that exposed to UVB (250 mJ/cm$^2$) in the presence of ferrous sulfate.
- Both peaks almost disappeared after treatment of the protein with UVB in the presence of HP, indicating a high degree of degradation.
- The degradation of tropoelastin by this treatment was further confirmed by analyzing tropoelastin peptides using LC-MS.
- Treatment of the already produced peptides of the tropoelastin or the human skin elastin under similar conditions did not show any detectable qualitative or quantitative change.

## ZUSAMMENFASSUNG

Die Zielstellung dieser Arbeit war, menschliche Hautelastinpeptide zu charakterisieren und den Einfluss von Strahlung auf die molekulare Struktur des Elastins mittels Massenspektrometrie zu bestimmen. Im ersten Schritt wurde der Effekt von künstlicher Strahlung auf zwei, für die Quervernetzung des Elastins verantwortliche Aminosäuren, DES und IDE, untersucht. Dies erfolgte sowohl mit als auch ohne Zugabe von $Fe^{2+}$ und $H_2O_2$, die über eine Fenton Reaktion Hydroxylradikale erzeugen.

Da die biochemische Charakterisierung des intakten Elastins aufgrund seiner Unlöslichkeit in Wasser und organischen Lösungsmitteln kaum möglich ist, wurde eine Methode zur Charakterisierung der Primärstruktur von Elastin sowie von Tropoelastin entwickelt. So wurden Tandem MS mit ESI-Ionfalle, nano-ESI-qTOF und/oder MALDI-TOF MS an Peptiden, die durch Verdauung der Proteine mit Elastase, Thermitase, Pepsin bzw. Chymotrypsin erhalten wurden, durchgeführt. Über Datenbanksuche und/oder *de novo* Sequenzierung konnte die Sequenz der Peptide aus den Fragmentmassenspektren bestimmt werden. Schließlich wurde der Einfluss von UVB-Strahlung auf rekombinantes menschliches Tropoelastin und dessen Peptide sowie Peptide des menschlichen Hautelastins in Abwesenheit oder in Anwesenheit von Haematoporphyrin bzw. Eisensulfat ausgewertet. Die Ergebnisse der Arbeit lassen sich wie folgt zusammenfassen:

**1. Einfluss von UV-Strahlung auf quervernetzte Aminosäuren (DES und IDE)**

- Bei den Proben, die in unterschiedlicher Weise ohne die Zugabe von $Fe^{2+}$ und von $H_2O_2$ bestrahlt wurden, trat keine Änderung in der Stabilität der Aminosäuren auf.
- Inkubation der Aminosäuren mit $Fe^{2+}$ und $H_2O_2$ oder Bestrahlung der gleichen Probe mit
    - einer OSRAM-Vitalux 300 W-Lampe, die das Spektrum des Sonnenlichtes gut simuliert,
    - UVA Strahlung (305-420 nm) - 1,25-30 J/cm$^2$ oder
    - UVB Strahlung (280-350 nm) - 0,125-3 J/cm$^2$

  führte zu einer drastischen Abnahme der Anzahl an Elastinquervernetzungen und ergab die Bildung von Abbauprodukten mit einer molaren Masse von 496 und 481.
- Die Strukturaufklärung der Abbauprodukte zeigte, dass eine durch Hydroxylradikale katalysierte Oxidation der Aminosäuren stattgefunden haben könnte. Die

Hydroxylradikale entstehen synergistisch durch Bestrahlung und durch die Fenton Reaktion zwischen $Fe^{2+}$ und $H_2O_2$.

- Die Behandlung der Aminosäuren mit IR-Strahlung (520 W) in Anwesenheit von $Fe^{2+}$ und $H_2O_2$ beeinflusste weder ihre Menge noch führte sie zur Bildung von Abbauprodukten.

**2. Charakterisierung der menschlichen Hautelastinpeptide**

- Zur Charakterisierung des humanen Elastins wurden mehrere Peptide nach einem Verdau mit Elastase, Thermitase und Pepsin identifiziert. Die Sequenzabdeckung lag bei 58,8, 44,2 bzw. 48,8%.
- Durch Kombination der Resultate der drei Enzyme wurde eine Sequenzabdeckung von 74,1% erreicht.
- Die bereits aus der Literatur bekannte Abwesenheit von Aminosäuren, die in menschlichen Hautelastin durch Exon 26A codiert werden, konnte eindeutig bestätigt werden. Aminosäuren dieser Region des Proteins fehlten, wohingegen einige Peptide, deren Aminosäuren von Exons vor und nach Exon 26A exprimiert werden, vorhanden waren.
- Im Gegensatz zu einem früher publizierten Bericht ergab die Identifizierung einiger Peptide, die von Exon 22 codierte Aminosäuren enthalten, dass dieses Exon in der mRNA des humanen Hautelastins vorhanden ist.
- Ca. 40% der aus den sequenzierten Teilen des Proteins stammenden Prolinmoleküle könnten durch Prolylhydroxylase hydoxyliert werden.
- Aus den LC-MS Chromatogrammen wurden die relativen Peakflächen der hydroxylierten und nicht-hydroxylierten Peptidpaare entnommen. Die sich daraus ergebenden Hydroxyprolin-Prolin-Verhältnisse unterschieden sich je nach Proteinregion, was auf ein lokale Variation des Ausmaßes der Prolin-Hydroxylierung hindeutet.

**3. Charakterisierung der rekombinanten menschlichen Tropoelastinpeptide**

- Nach Verdau mit Pepsin bzw. Chymotrypsin betrug die Anzahl der identifizierten Peptide 100 bzw. 49, was einer Sequenzabdeckung von 86,7 bzw. 84,7% entspricht.
- Die gesamte Sequenzabdeckung von 94,4% wurde durch Kombination der Verdaue beider Enzyme erzielt.

**4. Spaltungsstellen**

- Die Angriffspunkte der in dieser Arbeit eingesetzten Enzyme wurden mit Hilfe kumulativer Daten aus menschlichem Hautelastin und rekombinantem Tropoelastin ermittelt.
- Elastase, Thermitase und Pepsin greifen hauptsächlich am C-Terminus von drei der vier dominierenden Aminosäuren (Gly, Ala und Val) sowie an Leu an.
- Ein ähnliches Ausmaß der Spaltung wurde am C-Terminus von Ile durch Elastase und am C-Terminus von Phe durch Thermitase and Pepsin beobachtet.
- Chymotrypsin greift überwiegend am C-Terminus von Tyr, Phe und Leu sowie in selteneren Fällen an dem von Ala, Ile, Thr und Gly an.

**5. Der Einfluss von UVB-Strahlung auf rekombinantes menschliches Tropoelastin, dessen Peptide und Peptide des menschlichen Hautelastins**

- Die MALDI-Massenspektren des Tropoelastins zeigten jewils einen Peak bei 30237,91 (z = 2) und bei 60571,17 (z = 1).
- Zwischen der Peakintensität von unbehandeltem bzw. mit UVB (250 $mJ/cm^2$) und Eisensulfat behandeltem Tropoelastin konnte kein wesentlicher Unterschied beobachtet werden.
- Beide Peaks verschwanden nahezu nach Bestrahlung des Proteins mit UVB-Licht in Anwesenheit von HP, was auf einen weitgehenden Abbau des Tropoelastins hinweist.
- Der Abbau von Tropoelastin durch diese Behandlung wurde weiterhin durch die Charakterizierung der Tropoelastinpeptide mittels LC-MS bestätigt.
- Die Behandlung der bereits produzierten Peptide des Tropoelastins bzw. des menschlichen Hautelastins unter ähnlichen Bedingungen zeigte weder eine qualitative noch eine quantitative Veränderung.

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## 6. APPENDIX (LIST OF PUBLICATIONS)

6.1. **Getie, M.**; Raith, K.; Neubert, H.H.R., LC/ESI-MS analysis of two elastin cross-links, desmosine and isodesmosine, and their radiation-induced degradation products, *Biochim Biophys Acta, 1624 (2003) 81-87*.

6.2. **Getie, M.**; Schmelzer, C; Neubert, H.H.R., Characterization of peptides resulting from digestion of human skin elastin with elastase, *Proteins: Structure, Function, and Bioinformatics, 61 (2005a) 649-657*.

6.3. Schmelzer, C; **Getie, M.**; Neubert, H.H.R., Mass spectrometric characterization of human skin elastin peptides produced by proteolytic digestion with pepsin and thermitase, *J Chromatogr A, 1083 (2005) 120-126*.

6.4. **Getie, M.**; Schmelzer, C.; Weiss, A.; Neubert, H.H.R., Application of complementary mass spectrometric techniques to achieve a complete sequence coverage of recombinant human tropoelastin, *Rapid Commun Mass Spectrom, 19 (2005b) 2989-2993*.

### POSTER PRESENTATIONS (from this work)

- **Getie, M.**; Schmelzer, C; Neubert, H.H.R., *Determination of the positions of hydroxylated prolines in human skin elastin using mass spectrometry*, the Gordon Research Conference on Elastin and Elastic Fibers, 31$^{st}$ July- 5$^{th}$ August 2005, Waterville Valley, NH, USA.
- Schmelzer, C; **Getie, M.**; Raith, K.; Neubert, H.H.R., *Identification of peptides from enzymatic digestion of human skin elastin by nanoelectrospray tandem mass spectrometry*. The 53rd American Society for Mass Spectrometry Conference on Mass Spectrometry, 5$^{th}$ - 9$^{th}$ June 2005, San Antonio, Texas, USA.
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# CURRICULUM VITAE

## I. Personal Data

| | |
|---|---|
| Name: | Melkamu Getie Kebtie |
| Date of Birth: | 02 October 1972 |
| Place of Birth: | Gundewoin, Ethiopia |
| Sex: | Male |
| Nationality: | Ethiopian |
| Marital Status: | Married |

## II. Educational Background

***Pre-university***

- 1980-1987 Gundewoin Elementary & Junior School, Ethiopia
- 1987-1991 Motta Senior Secondary School, Ethiopia

***University***

- 1991- 1996 School of Pharmacy, Addis Ababa University, Ethiopia (Bachelor of Pharmacy)
- 1998-2000 School of Pharmacy, Addis Ababa University, Ethiopia (Masters of Science in Pharmaceutics)
- 2002-present PhD Candidate, Institute of Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Halle/Saale, Germany

## III. Professional Experience

- 1996-1997 Graduate Assistant, School of Pharmacy, Addis Ababa University, Ethiopia
- 1997-1999 Assistant Lecturer, School of Pharmacy, Addis Ababa University, Ethiopia
- 1999-2001 Lecturer, School of Pharmacy, Addis Ababa University, Ethiopia

## IV. Oral and poster presentations

- **Getie, M.**; Schmelzer, C; Neubert, H.H.R., *Determination of the positions of hydroxylated prolines in human skin elastin using mass spectrometry*, the Gordon Research Conference on Elastin and Elastic Fibers, 31st July- 5th August 2005, Waterville Valley, NH, USA.
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**VI. Membership**

- Member, Ethiopian Pharmaceutical Association, since 1996

## 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.

Melkamu Getie
Halle/Saale

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# LC/ESI-MS analysis of two elastin cross-links, desmosine and isodesmosine, and their radiation-induced degradation products

Melkamu Getie, Klaus Raith, Reinhard H.H. Neubert*

*Institute of Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck Str. 4, D-06120, Halle(Saale), Germany*

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**Abstract**

In this work, the effect of Fenton reaction on two elastin cross-linked amino acids, desmosine (DES) and isodesmosine (IDE), in the absence or presence of different wavelength radiations generated from artificial sources has been evaluated using LC/ESI-MS. Irradiation as well as incubation of DES or IDE solutions in the presence of $Fe^{2+}$ and $H_2O_2$ resulted in products with *m/z* 497.1 and 481.1 for $[M+H]^+$. A strongly dose-dependent degradation of both amino acids was observed upon exposure to UVB at doses ranging from 0 to 3 J/cm$^2$ and a moderate dose-dependent degradation upon exposure to UVA at doses 10 times higher than that of UVB. A significant time-dependent degradation of DES and IDE was also observed upon exposure of these amino acids to a lamp emitting visible light similar to sunlight. Exposure of both amino acids to IR radiation (520 W) for 8 h did not cause significant degradation.


*Keywords:* Desmosine; Isodesmosine; Skin; Elastin; LC/MS; Radiation

## 1. Introduction

Due to its function as a barrier between the organism and its environment, the skin is directly exposed to exogenous oxidative stress such as solar and artificial radiation, chemical oxidants and air pollutants, which are potent inducers of reactive oxygen species (ROS) [1,2]. Ultraviolet (UV) radiation represents the most deleterious part of solar light to cells and has been associated with the incidence of skin cancer [3]. In addition, fractions of UVB (290–320 nm) and UVA (320–400 nm) radiations that reach the earth surface are of paramount importance in causing several other skin health complications such as wrinkling, sagging, and pigmentary alterations; collectively referred to as photoaging [4,5]. The major histopathologic alteration in the dermis of photoaged skin underlying wrinkling, sagging, and yellow discolouration is the accumulation of large amount of abnormal elastin, termed solar elastosis, replacing the normally collagen-rich dermis [6].

Elastin is a highly hydrophobic fibrous protein that comprises the main part of elastic fibres, which are responsible for the elastic properties of several vertebrate tissues such as skin, lung and large blood vessels. It is secreted from cells as a soluble precursor protein, tropoelastin. Then, the tropoelastin molecules are highly cross-linked into a rubber-like network in the extracellular matrix [7,8]. The principal step in the biosynthesis of elastin is well characterized; first, the lysine residues of the tropoelastin react with lysyl oxidase to form α-amino adipic acid δ-semialdehyde (allysine). Then, allysine molecules react with lysine and/or another allysine to form polyfunctional cross-links such as desmosine (DES), isodesmosine (IDE), lysinonorleucine, merodesmosine and cyclopentenone [7–10]. Since they are specifically present in elastin, DES and IDE are used as markers to study the role of elastic fibre in the pathology of skin diseases. They were first isolated from elastin hydrolysate by Thomas et al. [11].

Biochemical studies report a decrease of elastin content and particularly of its cross-links in the tissue with aging, which is proposed to be due to oxidative stress [12–15]. The reduction of cross-links appears to be of key importance, because the elasticity and strength of elastin are maintained by the existence of these cross-links. The mechanism and causes are still not well defined. However, it is assumed that ROS are causal factors in aging [16,17]. More recently, Umeda et al. [18] reported that hydroxyl radicals derived from Fenton reaction between transition

* Corresponding author. Tel.: +49-345-5525000; fax: +49-345-5527292.
*E-mail address:* neubert@pharmazie.uni-halle.de (R.H.H. Neubert).

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metal ions, such as $Fe^{2+}$ and $Cu^{2+}$, and $H_2O_2$ play a role in the generation of oxodesmosine (OXD) from DES.

Transition metal ions such as $Fe^{2+}$ are known to be cofactors and electron suppliers, which mediate the oxidation of biological components such as lipids, DNA and proteins [19]. $Fe^{2+}$ is a nearly ubiquitous transition metal ion and is an essential trace element in the human diet. Upon exposure to sunlight, the human, as well as animal, skin accumulates iron in the epidermis and dermis [20]. It is also well known that hydrogen peroxide is constantly generated under normal physiological conditions, and that the rate of its generation increases with age [21]. For example, it is always generated as part of the biochemical process involving the transformation of tropoelastin to elastin. Since it is documented that the reaction between $Fe^{2+}$ and $H_2O_2$ produces the highly reactive hydroxyl radical, which is widely believed to be the main agent of oxidative damage [22,23], the event seems to be a self-catalysing danger for skin.

A number of articles are available regarding the analysis of DES and IDE including electrophoresis [24,25], liquid chromatography [14,26–30], radioimmunoassay [31] and enzyme immunoassay [32]. However, these methods demonstrated no or only poor separation of the cross-links and require large amount of sample and long analysis time. Therefore, a rapid, sensitive and specific LC/MS assay is highly desirable, particularly since mass spectrometry enables a specific quantitation even without baseline separation.

This study was therefore undertaken to evaluate the effect of the Fenton reaction on the two elastin cross-linked amino acids in the absence or presence of different wavelength irradiation generated from artificial sources, and to quantify the cross-links as well as their oxidation products using LC/MS.

## 2. Experimental

### 2.1. Materials

Methanol and formic acid were both of HPLC grade and were obtained from J.T. Baker (Deventer, The Netherlands) and from Merck (Darmstadt, Germany), respectively. Desmosine and isodesmosine isolated from bovine neck ligament were purchased from ICN Biomedicals Inc. (Eschwege, Germany). Morphine, Purpald® and Ferrous sulf ate heptahydrate were from Sigma (Munich, Germany). Hydrogen peroxide was from Solvay (Rheinberg, Germany).

### 2.2. Oxidation by Fenton reaction

Solutions of DES or IDE (0.1 mg/ml) in distilled water were incubated with $FeSO_4$ (10 μM) and $H_2O_2$ (1.5 mM) in a total volume of 2 ml at 37 °C for 8 h. Samples (50 μl) were taken at 0, 2, 4, 6 and 8 h. The samples were then diluted in methanol and analysed by LC/MS using morphine (0.5 μg/ml in methanol) as an internal standard.

### 2.3. Sample preparation and irradiation

Solutions of DES or IDE (0.1 mg/ml) containing $FeSO_4$ (10 μM) and $H_2O_2$ (1.5 mM) were prepared in distilled water. Prior to irradiation, the samples were transferred to 55-mm glass dishes and a uniform optical path length of 2 mm was ensured. Then, they were exposed to a Vitalux lamp of 300 W (OSRAM, Hamburg, Germany), which produces a sun-like spectrum; to different doses of UVA (1.25 to 30 J/cm$^2$) or UVB (0.125 to 3 J/cm$^2$) by a special irradiation equipment that emits specific wavelength ranges of UVA (305–420 nm) or UVB (280–350 nm) radiations (Veith import export, Westerau, Germany); or to an IR instrument (Hydrosun, Müllheim, Germany) that emits 520 W of radiation from 1 m above the sample. In case of the Vitalux lamp, the distance between the lamp and the sample was set to be 50 cm. In all cases, 50 μl of sample was taken at different time intervals or different doses of radiation and analysed by LC/MS using morphine (0.5 μg/ml in methanol) as an internal standard.

### 2.4. Mass spectrometric analysis

The LC/MS analysis was performed using an HPLC pump SpectraSystem P4000 equipped with an autosampler AS 3000 and a membrane degasser coupled via an electrospray interface to an ion trap mass spectrometer Finnigan LCQ (ThermoFinnigan, San Jose, CA, USA). Ionisation was carried out in the positive ion mode applying an electrospray voltage of 4.5 kV and a heated capillary temperature of 200 °C. The mobile phase was 0.2% formic acid in a mixture of double distilled water–methanol (60:40, v/v), which was eluted through a Nucleosil® C8 column (CC 125/2, 120-3, Macherey-Nagel, Düren, Germany) at a flow rate of 0.2 ml/min. Tandem MS and $MS^n$ experiments were performed on trapped ions using argon collision gas. Exact mass determination was done using a hybrid quadruple orthogonal acceleration-Time-of-Flight mass spectrometer (Q-TOF 2™, Micromass, Manchester, UK) equipped with an electrospray interface.

### 2.5. Test for aldehydes

The presence of aldehyde(s) in the incubated as well as irradiated solutions of DES or IDE was tested using the method previously described by Dickinson and Jacobsen [33]. A drop of an incubated or irradiated solution containing DES or IDE (0.1 mg/ml), $FeSO_4$ (10 μM) and $H_2O_2$ (1.5 mM) or only $FeSO_4$ (10 μM) and $H_2O_2$ (1.5 mM) was added to test tubes containing 20 mg of Purpald® (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) dissolved in 2 ml of 1 N NaOH. Then, presence of colour change was observed.

### 2.6. Data analysis

All analytical data represent the average values of sextupled measurements. The standard deviation was less than 5% throughout.

## 3. Results and discussion

### 3.1. Qualitative analysis

The positive ion ESI mass spectrometry analysis on a solution of DES or IDE irradiated with the OSRAM lamp for 6 h in the presence of $Fe^{2+}$ and $H_2O_2$ resulted in a monoisotopic ion peak at $m/z = 526.1$, which is consistent with an elemental composition of $C_{24}H_{40}N_5O_8$ that contains a quaternary ammonium ion. In addition, two peaks appeared at $m/z = 497.1$ and 481.1 for both DES and IDE. Not only irradiation but also incubation of DES or IDE solutions in the presence of $Fe^{2+}$ and $H_2O_2$ gave rise to peaks corresponding to $m/z = 497.1$ and 481.1 (spectra not shown).

In their study on the oxidative metabolism of pyridinium cross-links in elastin, Umeda et al. reported that OXD having a molecular weight of 495 was formed from DES whereas no formation of the corresponding isooxodesmosine (IOXD) (having an identical molecular weight of 495) from IDE was observed. Their experiments featured the Fenton reaction using distilled water as reaction medium [18]. In contrast, our results demonstrated that Fenton reaction alone or accompanied with irradiation with a sun-like spectrum, UVA or UVB produced compounds of molecular weight 496, instead of 495, both from DES as well as IDE, the effect being quite prominent upon exposure to radiation. Our MS experiments proved that these oxidation products, which have been designated as OXD, and its isomer IOXD have one mass more than what has been reported. Electrospray ionisation (ESI)-TOF MS experiments of irradiated or incubated solutions of DES and IDE showed protonated molecular ion peaks at 497.2621 consistent with elemental compositions of $C_{23}H_{37}N_4O_8$, with standard errors of 1.86 ppm each. Supplemented by tandem MS experiments using ESI-ion trap, it was possible to postulate the structures of the oxidation products that provided peaks at $m/z = 497.1$ and 481.1. Fig. 1 shows the postulated scheme of oxidation of DES. The oxidation products are arbitrarily designated as $DESU_{497}$ and $DESU_{481}$, for oxidation products of DES that gave peaks at $m/z = 497.1$ and 481.1.

The $MS^2$ and $MS^3$ spectra of $DESU_{497}$ revealed subsequent losses of $NH_3$ followed by CO confirming the presence of three unsubstituted amino groups and three unsubstituted carboxyl groups (Fig. 2). The tandem MS fragmentation pattern of $IDEU_{497}$ was exactly the same as that of $DESU_{497}$. Interestingly, incubation or irradiation of the solutions of either DES or IDE yielded ions with $m/z$

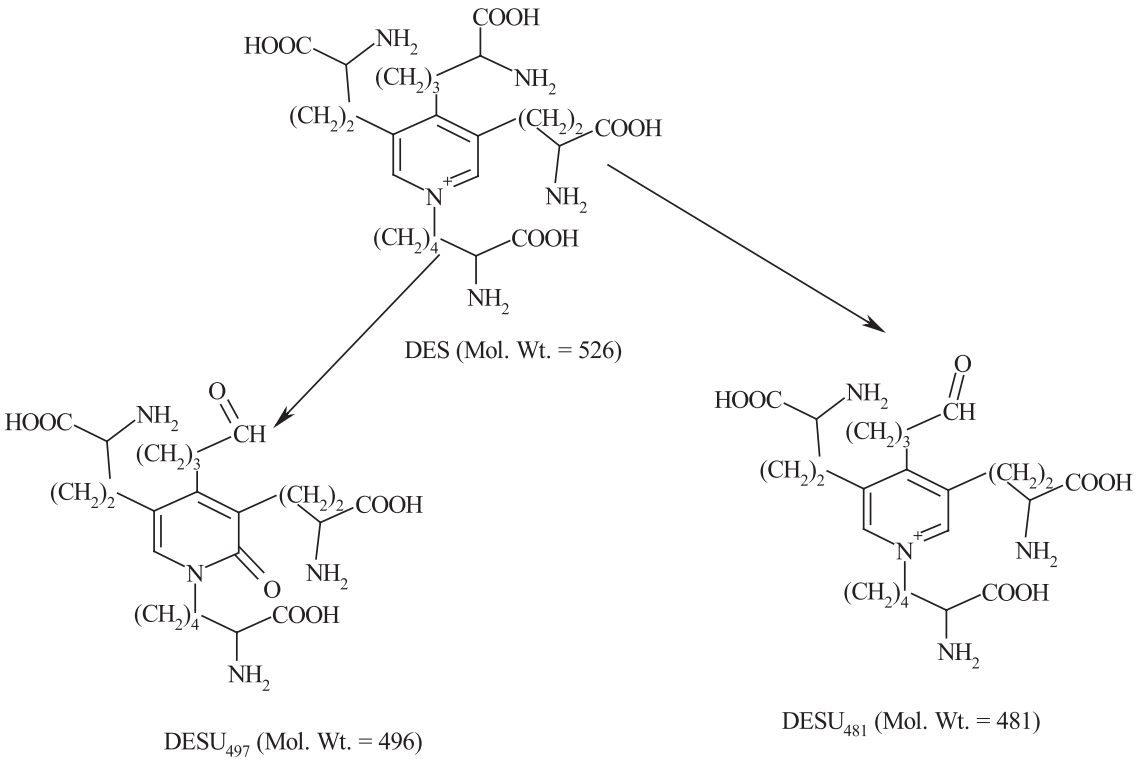


Fig. 1. Postulated scheme of oxidation of DES.

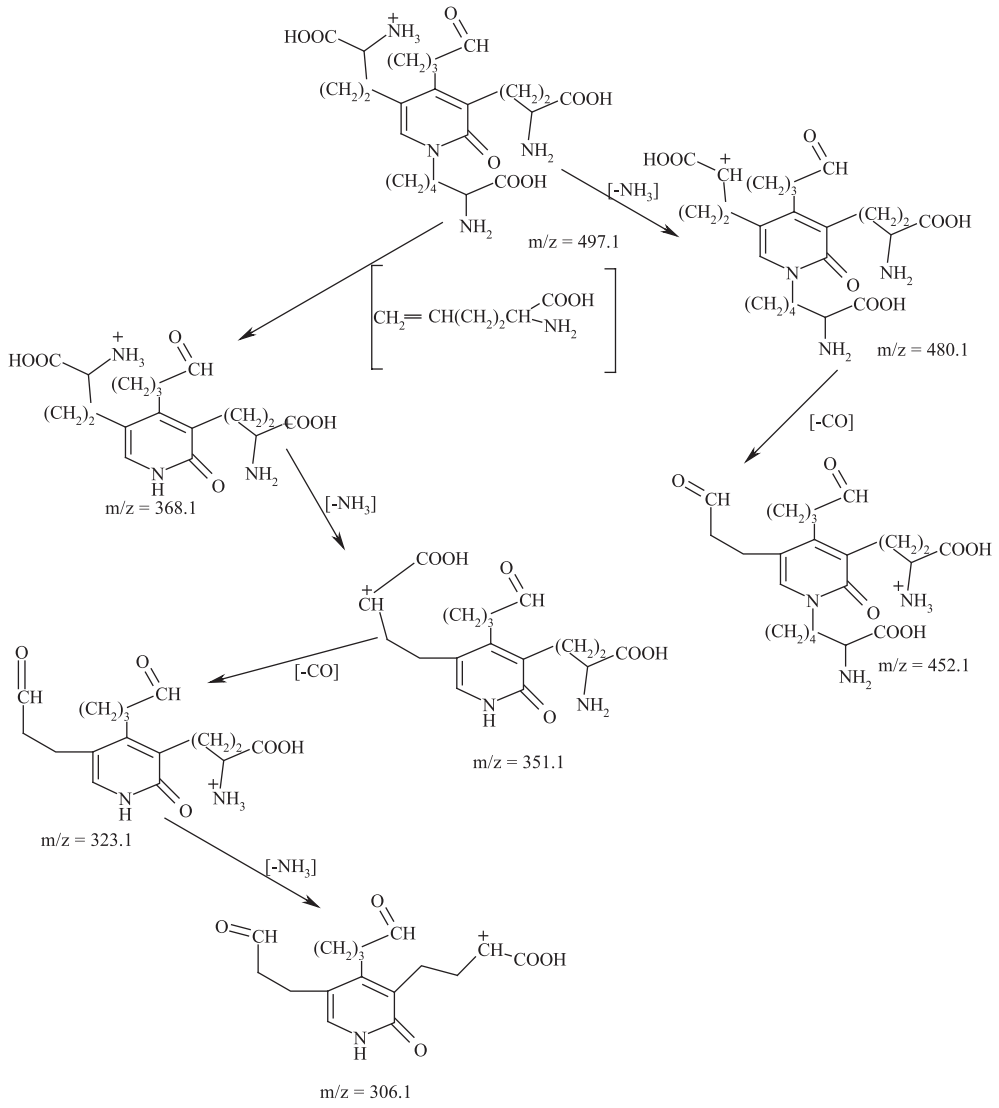


Fig. 2. Proposed MS/MS fragmentation scheme of $DESU_{497}$.

481.1, which were also found by MS/MS fragmentation of either of the amino acids. The tandem MS of the products obtained after incubation or irradiation and that obtained upon MS/MS fragmentation of both amino acids were exactly identical. This observation leads to the conclusion that $DESU_{481}$ and $IDEU_{481}$ were formed upon loss of a CO group preceded by deamination of their respective parent molecules.

The incubated and irradiated solutions of DES or IDE in the presence of $Fe^{2+}$ and $H_2O_2$ produced a deep purple colour within 2 min upon addition of Purpald®, a reagent that specifically reacts with aldehydes [33,34], whereas the solution that contained only $Fe^{2+}$and $H_2O_2$ demonstrated no colour change. This may be confirmatory for the presence of an aldehyde functional group on the chemical structures of $DESU_{481}$, $DESU_{497}$, $IDEU_{481}$ and $IDEU_{497}$. The structures of OXD and IOXD reported by Umeda et al. do not posses such a functional group.

### 3.2. Quantitative analysis

Quantitative determination of DES and IDE as well as their oxidative products was possible by LC/MS with a better sensitivity and shorter analysis time than obtainable by the methods that were employed so far, such as electrophoresis, thin-layer chromatography, liquid chromatography, radioimmunoassay and enzyme immunoassay [24–32]. Most of the authors that employed liquid chromatography, in an attempt to separate the elastin cross-links, used phosphate buffer and a surfactant such as sodium dodecyl sulfate in their mobile phase. Since both are incompatible to the MS detector, it was mandatory to search for a mobile phase composition avoiding such detrimental additives. A number of combinations of distilled water and organic solvents (methanol and acetonitrile) were tested during method development. Finally, a mobile phase that contains 0.2% formic acid in a mixture of distilled water and

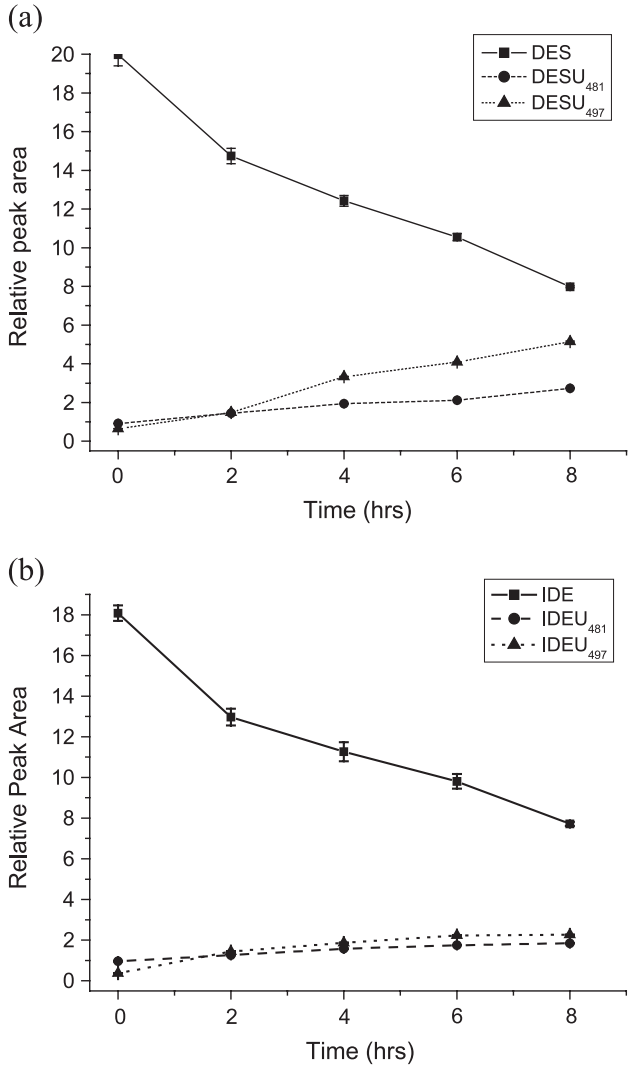


Fig. 3. The effect of irradiation of solutions of DES (a) and IDE (b) by the OSRAM lamp (300 W) in the presence of $Fe^{2+}$ (10 μM) and $H_2O_2$ (1.5 mM).

methanol (60:40, v/v) was selected. It was observed that the higher the percentage of methanol in the mobile phase, the better was the MS peak intensity. However, increasing the methanol concentration beyond 40% resulted in an increased tailing of the chromatographic peak. Although a C8 column was used in this work, there was no significant difference between this and a C18 column with respect to the resulting peak shape.

Morphine (0.5 μg/ml) was used as an internal standard for positive mode ESI-MS. The relative peak area versus concentration plots of DES and IDE resulted in linear curves of excellent correlations ($R = 0.9998$ and 0.9997, respectively) in a concentration range of 0.5 to 20 μg/ml. The limits of quantification for both DES and IDE were 0.1 μg/ml. In all cases, relative peak area represents peak area obtained from the substance analysed divided by peak area obtained from morphine.

It has been found that, following incubation of the solutions of DES and IDE, there was a slight time-dependent reduction in the concentration of the amino acids and a slight increase in the products having $m/z$ for $[M+H]^+$ of 497.1 and 481.1. Exposure of the solutions of the cross-links to IR radiation for 8 h does not alter their concentrations. On the other hand, upon exposure with OSRAM lamp, there was a higher time-dependent reduction in DES as well as IDE concentrations (down to 40% and 50%, respectively in 8-h time) and higher time-dependent increase in the amounts of $DESU_{497}$ and $IDEU_{497}$ (about eight times the initial concentration in 8 h). However, the amounts of $DESU_{481}$ and $IDEU_{481}$ were not significantly increased (Fig. 3). Whereas incubation did not seem to affect DES and IDE to a different extent, in the case of the OSRAM lamp, irradiation-induced degradation seems to be more focused on DES rather than IDE. The effect of irradiation

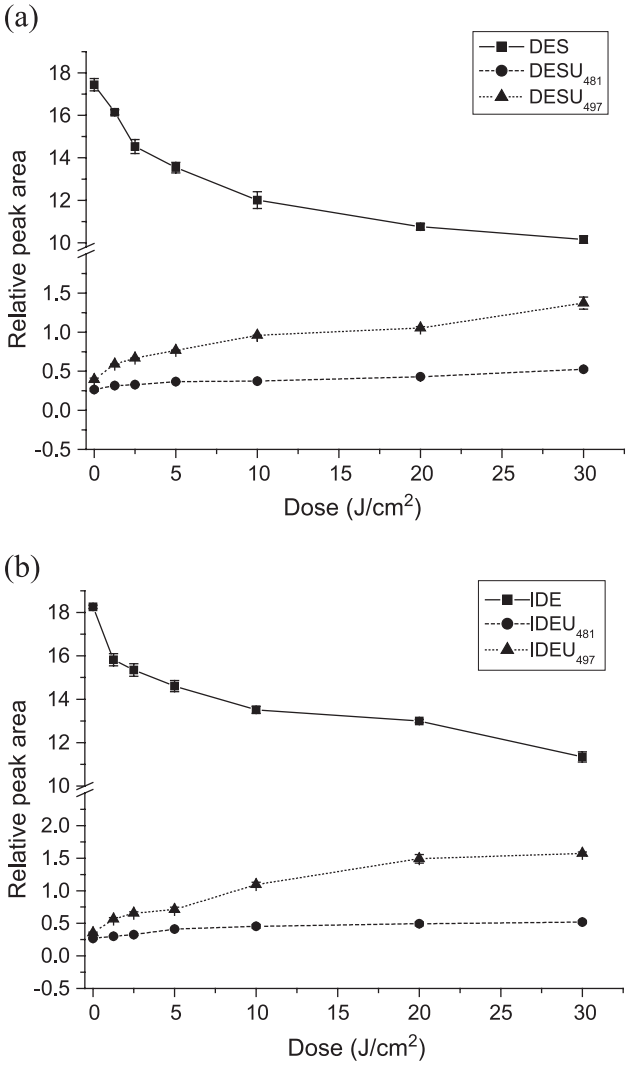


Fig. 4. The effect of irradiation of solutions of DES (a) and IDE (b) with different doses of UVA in the presence of $Fe^{2+}$ (10 μM) and $H_2O_2$ (1.5 mM).

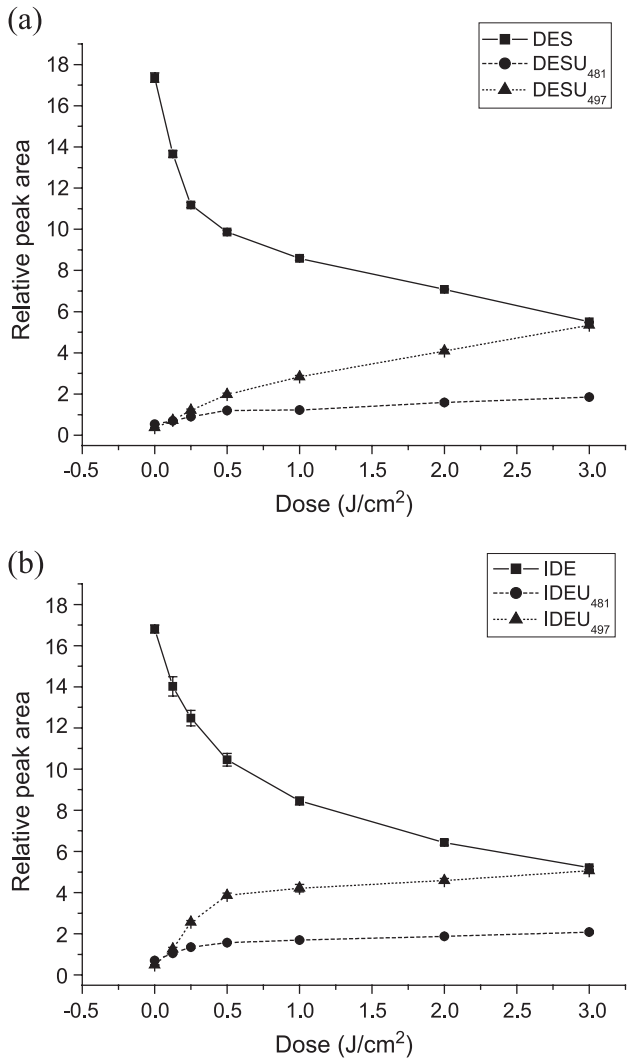


Fig. 5. The effect of irradiation of solutions of DES (a) and IDE (b) with different doses of UVB in the presence of $Fe^{2+}$ (10 μM) and $H_2O_2$ (1.5 mM).

was clearly greater than that of incubation for both amino acids.

In this study, it has been observed that exposure to UVA radiation resulted in a significant dose-dependent decrease in the concentrations of DES and IDE and an increase in the concentrations of their degradation products (Fig. 4). An increase in the dose of UVB from 0 to 30 J/cm$^2$ results in a 42% reduction of the concentration of DES, but a 100% and 250% increase in the concentrations of its $DESU_{481}$ and $DESU_{497}$, respectively. Similarly, the concentration of IDE was reduced by 40% while the concentrations of its $IDEU_{481}$ and $IDEU_{497}$ were increased by 98% and 350%, respectively. Exposure to UVB radiation of doses 10 times less than that of UVA radiation resulted in a more drastic degradation of the cross-links (Fig. 5). Increasing the dose to 3 J/cm$^2$ reduced the concentrations of DES and IDE by 68% and 69%, respectively. On the other hand, the concentrations of $DESU_{497}$ and $IDEU_{497}$ were increased 13 and 10 times, respectively. Since approximately one third of UVA radiation and about 10% of UVB radiation incident on Caucasian skin penetrate to the dermis [34], it is essential to consider that prolonged exposure of the skin to solar radiation certainly could have a damaging effect to the elastin matrix of the skin.

## 4. Conclusion

The results of this study clearly indicated that solar radiation, accompanied with the ubiquitous transition metal ion ($Fe^{2+}$) and $H_2O_2$, has a drastic oxidative effect on two elastin cross-links, DES and IDE. Two oxidative products were detected from both amino acids that have molecular masses of 481 and 496. Their chemical structures are postulated from mass spectrometric and aldehyde specific analysis results. In addition, a sensitive and specific LC/MS analytical method that allows quantification of DES, IDE and their oxidative products was developed. The degradation of the cross-links is of paramount importance, because the elasticity and strength of elastic fibres are maintained by the existence of these cross-links in the elastin molecule. Answering the question whether the aforementioned degradation products are produced in vivo and if they produce any physiological alteration needs further study. In addition, for comprehensive understanding of the oxidative behaviour of the elastin molecule, further investigation on the effect of other oxidative agents that exist in the human skin on elastin and its components is required.

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# Characterization of Peptides Resulting from Digestion of Human Skin Elastin with Elastase

**M. Getie, C.E.H. Schmelzer, and R.H.H. Neubert***
*Institute of Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany*

***ABSTRACT*** **Several pathological disorders are associated with abnormalities in elastic fibers, which are mainly composed of elastin. Understanding the biochemical basis of such disorders requires information about the primary structure of elastin. Since the acquisition of structural information for elastin is hampered by its extreme insolubility in water or any organic solvent, in this study, human skin elastin was digested with elastase to produce water-soluble peptides. Tandem mass spectrometry (MS/MS) experiments were performed using conventional electrospray ionization (ESI) and nano-ESI techniques coupled with ion trap and quadrupole time-of-flight (qTOF) mass analyzers, respectively. The peptides were identified from the fragment spectra using database searching and/or *de novo* sequencing. The cleavage sites of the enzyme and, for the first time, the extent and location of proline hydroxylation in human skin elastin were determined. A total of 117 peptides were identified with sequence coverage of 58.8%. It has been observed that 25% of proline residues in the sequenced region are hydroxylated. Elastase cleaves predominantly at the C-terminals of the amino acids Gly, Val, Leu, Ala, and Ile, and to a lesser extent at Phe, Pro, Glu, and Arg. Our results confirm a previous report that human skin elastin lacks amino acid sequences expressed by exon 26A. Proteins 2005;61:649–657.**


**Key words: proline hydroxylation; peptide sequencing; liquid chromatography (LC); tandem mass spectrometry (MS/MS); electrospray ionization (ESI); nano-electospray; cleavage sites; *de novo* sequencing**

## INTRODUCTION

Elastin is an important connective tissue protein that provides elasticity to organs, such as skin, lung, blood vessels, and ligaments.[1] It is among the most hydrophobic proteins known. Although there is some species variation, elastin from higher vertebrates, including human beings, contains over 30% Gly, and approximately 75% of the entire sequence is made up of just four hydrophobic amino acids (Gly, Val, Ala, Pro). Due to the extreme insolubility of elastin in water or organic solvents, research into the primary structure of elastin was hampered until the discovery of the water-soluble precursor, tropoelastin, which was first isolated from copper-deficient animals. The amino acid sequences of tropoelastins from various sources have been determined using molecular biological techniques to isolate and sequence elastin genes. Human,[2,3] chicken,[4] bovine,[5,6] and rat[7] tropoelastin genes have all been sequenced and the amino acid sequences of the protein determined.

Studies on the primary structure of elastin are very useful in helping to understand the biochemical basis of several pathological conditions, including solar elastosis, emphysema, aneurysm, and atherosclerosis, in which the mechanical and elastic properties of tissues are altered. However, due to its extreme insolubility, such studies are mainly focused on complete hydrolysis of the protein in a strongly acidic environment and analysis of the resulting cross-linked amino acids, desmosine and its isomer isodesmosine, which are only found in elastin.[8–10] This approach has been challenged by several factors, including the incomplete separation of the amino acids with the analytical methods employed to date and the insufficient information that can be obtained about the entire elastin molecule only based on the cross-linked amino acids. Extraction of elastin from healthy and diseased tissue and comparison of the primary structures of the peptides resulting from enzymatic digestion of the protein could provide insight into the biochemical changes that occur due to the pathological conditions mentioned. Accomplishment of this task requires the identification of the resulting peptides by determining the primary sequences of these peptides.

Automated Edman degradation has been widely used for the determination of the primary structure of peptides. However, it is now being increasingly replaced by mass spectrometric methods[11,12] such as electrospray ionization (ESI)[13] or nano-ESI[14] combined with quadrupole time-of-flight (qTOF) or ion trap mass spectrometry and by matrix-assisted laser desorption/ionization (MALDI)-TOF[15,16] by virtue of the following advantages:

1. The ability to perform sequence analysis of a peptide within a mixture because it can be mass-selected by the instrument;
2. Fragmentation of a molecular ion is possible even in the

*Correspondence to: R.H.H. Neubert, Institute of Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck Str. 4, D-06120, Halle (Saale), Germany.

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presence of an N-terminal modification of the peptide, a considerable problem for the Edman degradation protocols; and
3. The sample amount necessary for analysis is usually less than 1 pmol to obtain a high-quality mass spectrum.[17]

Determination of the order of amino-acid residues in peptides resulting from enzymatic digestion or chemical hydrolysis of proteins requires tandem mass spectrometry (MS/MS). In this technique, a given parent (precursor) ion is selected and broken up, usually by collision with an inert gas. Then, the *m/z* (mass/charge) values for the resulting daughter (product) ions are measured. Under favorable conditions, this procedure may yield a series of specific ions that contains sufficient information to determine the peptide sequence. Even more information can be obtained in certain instruments (ion traps, for example) by breaking up the daughter ions themselves to yield a spectrum of granddaughter ions. Deducing peptide sequences from raw MS/MS data is slow and tedious when it is done manually. Instead, the most popular approach is to convert the resulting mass spectra into a mass list and to subject it to peptide sequencing either by matching with protein databases[18–21] or by *de novo* sequencing.[22–24] Even more comprehensive results could be obtained by coupling both methods when the protein of interest exists in databases.

Therefore, in this study, the amino-acid sequences of some of the peptides resulting from enzymatic digestion of human skin elastin with elastase were determined by tandem MS using ion-trap or qTOF analyzers, and the resulting mass spectra were sequenced by database matching or combination of *de novo* sequencing and database matching. As the effectiveness of the *de novo* sequencing depends on the availability of several fragment ions and the resolution of the analyzer used, this technique was employed for sequencing of the results from a nano-ESI–qTOF instrument.

## MATERIALS AND METHODS

### Materials

Human skin elastin (Product No. GH421) prepared by the method of Starcher et al.[25] and high-purity elastase isolated from elastase (Product No. E134), which was crystallized from porcine pancreas by the method of Brink et al.[26] were purchased from Elastin Products Company (Owensville, Missouri, USA). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was purchased from ICN Biomedicals (Aurora, Ohio, USA). High performance liquid chromatography (HPLC) grade acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands), and the water used was doubly distilled. Formic acid and trifluoroacetic acid (TFA), both analytical grade, were obtained from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland), respectively.

### Digestion of Human Skin Elastin with Elastase

Elastin was dispersed in 1 m*M* Tris buffer, pH 8.5 at a concentration of 1 mg/mL and digested with elastase for 24 h at 37°C. The enzyme–substrate ratio was 1:50.

### Liquid Chromatography (LC)–ESI–Ion Trap Mass Spectrometry (MS)

The elastin digest was analyzed using reversed-phase HPLC (RP-HPLC) coupled with electrospray interface to a Finnigan LCQ ion trap mass spectrometer (Thermo Electron, San José, CA, USA). The former comprised a Spectra System P 4000 HPLC pump equipped with an autosampler AS 3000. A quantity of 10 μL of each sample solution was loaded onto a Nucleosil 120-5 C18 column (125 × 2 mm i.d., Macherey Nagel, Düren, Germany), and peptides were eluted using a linear gradient: 5–60% acetonitrile in water, both containing 0.1% formic acid, over 60 min. The column was maintained at 30°C, and the flow rate was 0.2 mL/min. The mass spectrometer was operated in positive ion mode by applying an electrospray voltage of 4.5 kV and a heated capillary temperature of 220°C. The digest was initially analyzed in full scan mode, and the masses of all the peptides, the *m/z* values between 50 and 2000, were recorded. From this mass list, peptides of interest were selected manually based on the relative intensity of their chromatographic peaks for further tandem MS/MS experiments using collision-induced dissociation (CID). The mass isolation window for CID was set between 1 and 2 u depending on experimental conditions. Fragmentation was carried out varying the relative collision energy between 25 and 40% to achieve optimal results for $[M+H]^+$ ions.

### Nanoelectrospray-qTOF MS

Nano-ESI experiments were carried out using a qTOF mass spectrometer Q-TOF-2 (Waters/Micromass, Manchester, UK) equipped with a nanoelectrospray ZSpray source. The nano-ESI glass capillaries were obtained precoated from New Objective (Woburn, MA, USA) and DNU (Berlin, Germany). The instrument was calibrated everyday using a mixture of sodium iodide and caesium iodide. The peptide mixture was desalted by washing the sample bound to a ZipTip C18 unit (Millipore, Schwalbach, Germany) according to the manufacturer's instructions prior to elution with acetonitrile/water (1:1, v/v) containing 0.05% TFA. Two microliters of the sample solution were loaded into the capillary using Microloader pipette tips (Eppendorf, Hamburg, Germany).

The typical operating conditions for the qTOF mass spectrometer were as follows: capillary voltage, 900 V; sample cone voltage, 35–55 V; source temperature, 80°C. The instrument was operated in the positive ion mode. Full scans were performed over the *m/z* range 50 and 3500. Peptides of interest were selected manually for further MS/MS experiments using CID. The quadrupole mass filter before the TOF analyzer was set with low mass (LM) and high mass (HM) resolution settings of between 10 and 16 (arbitrary units), and the collision energy was varied between 20 and 70 eV according to the mass and charge state of the respective peptide.

### Database-Based and *De Novo* Sequencing

The fragment ion spectra of MS/MS obtained from LC-ESI were processed using Mascot Distiller (Matrix Science, London, UK), a software program that reduces MS raw data to high-quality peak lists for database searching. In the case of nano-ESI, the MS and MS/MS spectra were analyzed by MassLynx (version 3.4, Waters/Micromass). Then, the MS/MS spectra were processed by MaxEnt3, an add-on of MassLynx, and converted into SEQUEST files, which were suitable for further analysis. The Maximum Entropy 3 algorithm deconvolutes charge state and isotopic information in a continuum spectrum to generate a centroid spectrum containing only monoisotopic, singly charged peaks. Both the ESI and nano-ESI fragment ion peak lists generated as described were analyzed by searching sequence databases with Mascot (version 1.8, Matrix Science, London, UK).[18] For database searching, the MS protein sequence database (MSDB) and a database of human tropoelastin we developed considering its splice variants with the seven exons shown to be subject to alternative splicing: exons 22, 23, 24, 24A, 26A, 32 and 33[2,27,28] were used. The searches were taxonomically restricted to *Homo sapiens*, and the enzyme was set to "none." A varied hydroxylation of proline was considered.

Auto *de novo* sequencing with combined database searching was performed on the nano-ESI data using the software PEAKS Studio (version 2.4, Bioinformatics Solutions, Waterloo, Ontario, Canada) with a parent and fragment mass error tolerance of 0.08 u. The enzyme entry was set to "unknown," and a varied hydroxylation of proline was considered. Besides MSDB, the same homemade protein database as implemented in Mascot was also used for PEAKS.

## RESULTS AND DISCUSSION

In MS/MS sequencing of peptides resulting from enzymatic digestion of proteins, it has been common practice to use site-specific proteases such as trypsin. However, not all proteins are digested equally well by trypsin. For example, the enzyme does not digest highly hydrophobic and aggregated proteins, such as elastin, adequately. Therefore, in this study, we used a non-specific enzyme, elastase, which effectively digests elastin.

Owing to the complexity of possible fragmentation patterns and to ambiguities in mass signal interpretation, peptide sequencing with MS/MS is not always a straightforward task. To this end, the combined use of two or more methods, namely ESI, nano-ESI, and MALDI, may be necessary. ESI has the advantage over MALDI that it can easily be interfaced to chromatographic separation techniques. Unlike MALDI, ESI is as suitable for low molecular mass samples as it is for high molecular mass samples. This was an important advantage for our samples as we had several peptides with molecular masses of less than 500 u. With MALDI, such species may be difficult to detect as they are often hidden amongst the matrix-associated background ions. In addition, the poor selectivity of the timed ion selector in MALDI post-source decay (PSD) is an important limitation for the peptide identification in complex mixtures. When the resolution is insufficient, more than one peptide is subjected to PSD analysis, resulting in overlapping fragment patterns, which cannot be processed with the software tools currently available. A significant advance in the ESI technique was the introduction of nano-ESI, which utilizes a very low solvent flow rate. The signal-to-noise ratio of the technique is enhanced compared with standard ESI and a small aliquot of the sample (e.g., 1 $\mu$L of $10^{-6}M$) will spray for approximately 30 min., thus enabling a peptide mass map and several MS/MS sequence tag analyses to be performed on a single minimal sample introduction.[29] Moreover, advantages, such as a lower susceptibility to salt contamination and a more uniform response to chemically very different analytes such as peptides and oligosaccharides,[29] and high sensitivities down to the low attomol range[30] have been reported. Therefore, in this work, we chose to use ESI-ion trap, coupled with liquid chromatography, and nano-ESI–qTOF mass spectrometers for sequencing the elastin peptides.

The MS/MS raw data were processed with Mascot Distiller to create high-quality peak lists before database searching. The software tool detects peaks by attempting to fit an ideal isotopic distribution to the experimental data. This ideal distribution is predicted from the elemental composition expected for a peptide of average amino acid composition at that point on the mass scale. The advantage of this approach over conventional peak detection is that the charge state is automatically determined and the peak list contains only monoisotopic peaks, even when the signal-to-noise ratio is poor or the isotopic distribution is not fully resolved. It also reduces the time needed to create each single peak list from several minutes to some seconds.[31]

The fundamental approach of the Mascot search engine is to calculate the probability that the observed match between the experimental dataset and each sequence database entry is a chance event.[18] The probability is arithmetically converted to score such that the best match would be the one with the lowest probability or the highest score. The user is then provided with a list of peptides, the protein in which the peptide is found, and the corresponding scores. When the protein is unknown, the identity of the peptide will strictly depend on the value of the score, and sometimes *de novo* sequencing and supplementary information may be necessary for unequivocal identification. In the present study, since the protein of interest is known, peptides, which are found in elastin and have high Mascot scores, were automatically taken into consideration. The MS/MS data that were matched to elastin peptides with relatively lower scores, but not to any other elastin peptide or to a peptide from other protein with a higher score, were further subjected to automatic *de novo* sequencing. The peptides were then considered only if their identities were confirmed by the latter approach.

A complete list of peptides obtained after digestion of human skin elastin with elastase is given in Table I. The exact positions of the peptides on the complete human tropoelastin sequence (Fig.1), which comprises the 786

**TABLE I. List of Peptides Obtained from Elastase Digestion of Human Skin Elastin After Analysis with LC-ESI and/or Nano-ESI Mass Spectrometry†**

| Residue no. | Sequence identified[d] | $M_r$[e] | LC/ESI-MS | Nano-ESI-MS |
|---|---|---|---|---|
| 34–47 | I<pGGVPGGVFYPGAG>L | 1246.60 | □ | |
| 42–48 | V<FYPGAGL>G | 723.36 | □ | |
| 74–84 | A<GLGAGLGAFPA>V | 929.50 | □ | |
| 78–84 | A<GLGAFPA>V | 631.33 | □ | ■ |
| 85–100 | A<VTFPGALVPGGVADAA>A | 1440.76 | □ | |
| 87–94 | A<VTFPGALVPG>G | 956.53 | □ | |
| 90–99 | G<ALVPGGVADA>A | 868.47 | □ | |
| 92–101 | L<VPGGVADAAA>A | 826.42 | □ | |
| 93–100 | V<PGGVADAA>A | 656.31 | □ | |
| 95–105 | G<GVADAAAAYKA>A | 1006.51 | □ | |
| 113–121 | L<GGVPGVGGL>G | 711.39 | □ | |
| 150–156 | V<YPGGVLP>G | 701.37 | | ■ |
| 158–171 | G<ARFPGVGVLPGVPT>G | 1365.78 | □ | |
| 159–171 | A<RFPGVGVLPGVPT>G | 1294.74 | □ | |
| 160–168 | R<FPGVGVLPG>V | 841.47 | □ | |
| 160–171 | R<FPGVGVLPGVPT>G | 1138.64 | | ■ |
| 161–170 | F<PGVGVLpGVp>T | 922.51 | | ■ |
| 165–171 | G<VLPGVPT>A | 681.41 | □ | |
| 165–173 | G<VLPGVPTGA>G | 809.46 | □ | |
| 166–170 | V<LPGVP>T | 481.29 | | ■ |
| 186–195 | A<FAGIpGVGPF>G | 976.50 | □ | |
| 187–195 | F<AGIPGVGPF>G | 813.44 | □ | |
| 187–195 | F<AGIpGVGPF>G | 829.43 | □ | ■ |
| 187–204 | F<AGIpGVGPFGGPQPGVPL>G | 1631.87 | | ■ |
| 188–195 | A<GIpGVGPF>G | 758.40 | □ | ■ |
| 189–194 | G<IpGVGP>F | 554.31 | | ■ |
| 190–195 | I<PGVGPF>G | 572.30 | □ | |
| 190–195 | I<pGVGPF>G | 588.29 | □ | |
| 196–204 | F<GGPQPGVPL>G | 820.44 | □ | ■ |
| 272–287 | A<GVLPGVGGAGVPGVPG>A | 1288.71 | □ | |
| 272–287 | A<GVLPGVGGAGVpGVPG>A | 1304.71 | □ | |
| 281–286 | A<GVPGVP>G | 524.30 | □ | ■ |
| 281–298 | A<GVPGVPGAIPGIGGIAGV>G | 1486.85 | □ | |
| 283–288 | V<PGVpGA>I | 512.26 | □ | |
| 288–295 | G<AIPGIGGI>A | 696.42 | | ■ |
| 288–296 | G<AIPGIGGIA>G | 767.45 | □ | |
| 288–298 | G<AIPGIGGIAGV>G | 923.54 | □ | |
| 288–302 | G<AIPGIGGIAGVGTPA>A | 1249.70 | □ | |
| 290–298 | I<PGIGGIAGV>G | 739.42 | □ | |
| 314–320 | A<AKYGAAA>G | 650.34 | □ | |
| 320–334 | A<AGLVPGGPGFGPGVV>G | 1279.69 | □ | ■ |
| 321–333 | A<GLVPGGPGFGPGV>V | 1109.59 | □ | |
| 321–334 | A<GLVPGGPGFGPGVV>G | 1208.66 | □ | ■ |
| 321–334 | A<GLVPGGpGFGPGVV>G | 1224.65 | □ | |
| 322–334 | G<LVPGGPGFGPGVV>G | 1151.63 | | ■ |
| 324–334 | V<PGGPGFGPGVV>G | 939.48 | | ■ |
| 330–343 | F<GPGVVGVPGAGVPG>V | 1118.61 | □ | |
| 335–349 | V<GVPGAGVPGVGVPGA>G | 1189.65 | □ | |
| 335–353 | V<GVPGAGVPGVGVPGAGIPV>V | 1555.87 | □ | |
| 335–356 | V<GVPGAGVPGVGVPGAGIPVVPG>A | 1809.01 | □ | ■ |
| 335–356 | V<GVPGAGVpGVGVPGAGIPVVPG>A | 1825.01 | □ | ■ |
| 342–350 | V<pGVGVpGAG>I | 741.37 | | ■ |
| 342–355 | V<PGVGVPGAGIPVVp>G | 1230.70 | | ■ |
| 351–356 | G<IPVVPG>A | 580.36 | | ■ |
| 351–359 | G<IpVVpGAGI>P | 853.49 | □ | |
| 354–372 | V<VpGAGIpGAAVPGVVSPEA>A | 1675.88 | | ■ |
| 363–371 | A<AVPGVVSPE>A | 853.45 | □ | |
| 386–390 | A<RPGVG>V | 484.28 | □ | |
| 402–409 | A<GGFPGFGV>G | 736.35 | □ | |
| 402–411 | A<GGFPGFGVGV>G | 892.44 | □ | |
| 402–418 | A<GGFPGFGVGVGGIPGVA>G | 1459.75 | □ | |

**TABLE I. (Continued)**

| Residue no. | Sequence identified[d] | $M_r$[e] | LC/ESI-MS | Nano-ESI-MS |
|---|---|---|---|---|
| 419–429 | A<GVpSVGGVpGV>G | 955.50 | | |
| 421–428 | V<PSVGGVPG>V | 668.35 | □ | |
| 425–440 | G<GVPGVGGVPGVGISPE>A | 1376.73 | □ | |
| 431–440 | G<GVPGVGISPE>A | 910.48 | □ | |
| 504–508 | L<NLAGL>V | 486.28 | □ | |
| 506–525 | L<AGLVPGVGVAPGVGVAPGVG>V | 1628.93 | □ | |
| 507–517 | A<GLVPGVGVAPG>V | 921.53 | □ | |
| 507–523 | A<GLVPGVGVAPGVGVAPG>V | 1401.80 | □ | |
| 517–536 | P<GVGVAPGVGVAPGVGLAPGV>G | 1628.93 | □ | |
| 526–541 | G<VAPGVGLAPGVGVAPG>V | 1316.75 | □ | |
| 544–556 | G<VAPGVGVAPGIGP>G | 1089.62 | | ■ |
| 544–559 | G<VAPGVGVAPGIGPGGV>A | 1302.73 | □ | |
| 549–555 | V<GVAPGIG>P | 569.32 | □ | |
| 550–560 | G<VAPGIGPGGVA>A | 893.50 | □ | |
| 551–559 | V<APGIGPGGV>A | 723.39 | □ | |
| 554–559 | G<IGPGGV>A | 498.28 | □ | |
| 555–564 | I<GpGGVAAAAK>S | 813.43 | □ | |
| 582–592 | L<GAGIPGLGVGV>G | 895.51 | □ | |
| 582–592 | L<GAGIpGLGVGV>G | 911.51 | □ | |
| 584–592 | A<GIPGLGVGV>G | 767.45 | □ | |
| 584–592 | A<GIpGLGVGV>G | 783.45 | □ | |
| 584–601 | A<GIPGLGVGVGVPGLGVGA>G | 1474.85 | □ | |
| 589–597 | L<GVGVGVPGL>G | 753.44 | □ | |
| 592–600 | V<GVPGLGVG>A | 654.37 | □ | |
| 598–612 | L<GVGAGVPGLGVGAGV>P | 1165.65 | □ | |
| 600–610 | V<GAGVPGLGVGA>G | 853.47 | □ | |
| 663–669 | K<YGAAVPG>V | 633.31 | | ■ |
| 672–689 | L<GGLGALGGVGIPGGVVGA>G | 1406.79 | □ | |
| 672–689 | L<GGLGALGGVGIpGGVVGA>G | 1422.78 | □ | |
| 678–687 | L<GGVGIPGGVV>G | 810.46 | □ | |
| 678–689 | L<GGVGIPGGVVGA>G | 938.52 | □ | |
| 678–689 | L<GGVGIpGGVVGA>G | 954.51 | □ | |
| 706–711 | A<QFGLVG>A | 619.33 | □ | |
| 706–712 | A<QFGLVGA>A | 690.37 | □ | |
| 713–723 | A<AGLGGLGVGGL>G | 869.50 | □ | |
| 713–729 | A<AGLGGLGVGGLGVPGVG>G | 1335.75 | □ | |
| 714–723 | A<GLGGLGVGGL>G | 798.46 | □ | |
| 722–738 | G<GLGVPGVGGLGGIPPAA>A | 1387.78 | □ | |
| 725–738 | G<VPGVGGLGGIPPAA>A | 1160.55 | | ■ |
| 749–762 | A<GLGGVLGGAGQFPL>G | 1241.68 | □ | |
| 771–779 | G<FGLSPIFPG>G | 933.50 | □ | |
| 772–779 | F<GLSPIFPG>G | 786.43 | □ | |
| 772–781 | F<GLSPIFPGGA>C | 914.49 | □ | |
| a | A<GVPGFGAVPG>A | 856.44 | □ | |
| | V<GAGVPGFGAVPG>A | 984.50 | □ | |
| b | A<GVPGVGGVPGVGGVPGVG>I | 1416.77 | | ■ |
| | A<GVPGVGGVPGVGGVPGVGI>S | 1529.86 | □ | |
| | A<GVPGVGGVPGVGGVPGVGISPE>A | 1842.98 | □ | ■ |
| c | PGLGV | 441.26 | □ | |
| | PGVGGA | 456.23 | □ | |
| | PGLGVGA | 569.32 | □ | |
| | GVPGLGVGA | 725.41 | □ | |
| | GVpGLGVGA | 741.40 | □ | |
| | VAPGVGVAPG | 822.46 | □ | |
| | VGVAPGVGVAPG | 978.55 | □ | |
| | VAPGVGVAPGVGVAPG | 1302.73 | □ | |

†Residue numbers are based on the tropoelastin sequence in PIR database with the accession number A32707.
(□) Identification by LC/ESI tandem MS fragment spectra
(■) Identification by nano-ESI tandem MS fragment spectra
**p** refers to hydroxylated proline.
[a]Peptides found in tropoelastin sequence lacking exon 26A.[28]
[b]Peptides found in tropoelastin sequence after Ota et al.[37]
[c]Peptides with several possible positions in the sequence.
[d]Sequences of peptides identified with adjacent amino acids.
[e]All masses are monoisotopic.


Fig. 1. Amino-acid sequence of human tropoelastin found in the PIR database with the accession number A32707. The region from which peptides were identified is designated in bold and underlined letters. **p** refers to proline residues with potential hydroxylation. The peptide sequences are the result of tandem MS experiments, ESI-ion trap and nano-ESI–qTOF, on human skin elastin digest followed by *de novo* and/or database-based sequencing of the fragment spectra.

amino acids derived from the mRNA of fetal human aorta[2] and an additional six-amino-acid peptide (residue 501–506) obtained from human skin fibroblast,[28] are also indicated in terms of residue numbers. This sequence is found in the protein information resource (PIR) database with the accession number A32707.

In general, 117 peptides were identified from the combined data of the ESI-ion trap and the nano-ESI–qTOF instruments, and the sequence coverage was found to be 58.8%. Ten of the peptides were identified as the result of analysis by both methods, while 17 peptides were identified by nano-ESI–qTOF alone. It has previously been reported that human skin elastin does not contain amino-acid sequences expressed by two exons, exon 22 (residues 453–481) and exon 26A (residues 618–650).[28] In addition, the amino-terminal signal sequence of 26 amino acids (residues 1–26) does not belong to the extracellular tropoelastin molecule.[32] The fact that no single peptide was detected in all of these locations by our experiments supported these previous reports (see Fig. 1). In addition, the presence of the two peptides, GVPGFGAVPG ($M_r$ 856.44) and GAGVPGFGAVPG ($M_r$ 984.50) in the digest demonstrated the excision of exon 26A (see Table I). Therefore, these parts of the sequence were not considered in the calculation of the sequence coverage. It can be seen from Figure 1 that almost no peptide was identified in regions consisting of stretches of lysine separated by two or three alanine residues such as AAAKAAKAA. This is due to the fact that the lysine molecules in tropoelastin are modified to form polyfunctional cross-links such as desmosine, isodesmosine, lysinonorleucine, merodesmosine, and cyclopentenone.[33–36] To date, sequencing peptides containing such modifications has been impractical both by database searching and by automated *de novo* sequencing. Alanine was detected in the sample, indicating that some or most of the alanine residues in these regions could have been cleaved out. Consequently, the actual sequence coverage could be higher than reported in this article.

The presence of the six-amino-acid exon (residues 501–506) in human skin elastin[28] was also confirmed by the presence of peptides NLAGL (residues 504–508), AGLVPGVGVAPGVGVAPG (residues 506–523), and AGLVPGVGVAPGVGVAPGVG (residues 506–525). We have identified peptides GVPGVGGVPGVGGVPGVGISPE ($M_r$ 1842.98), GVPGVGGVPGVGGVPGVGI ($M_r$ 1529.86), and GVPGVGGVPGVGGVPGVG ($M_r$ 1416.77) that were found only in a sequence of a new human tropoelastin variant recently submitted by Ota et al. to the European Molecular Biology Laboratory (EMBL).[37]

Tropoelastin is known to undergo very little post-translational modification. Hydroxylation of proline (Pro) residues is reported to occur in some animals to a varying degree with 0–33% of the total Pro being hydroxylated by the enzyme prolyl hydroxylase.[38–41] It has also been reported that cross-linking and the formation of elastin from tropoelastin is reduced by overhydroxylation of Pro.[42] The presence of such modifications in human elastin has not yet been investigated. Therefore, in this work, an attempt was made to determine the extent and the positions of hydroxylation in the human skin elastin sample. The combined use of the Mascot search engine and *de novo* sequencing enabled the unequivocal assignment of hydroxylation sites. Twenty-five percent of the total Pro residues in the identified tropoelastin sequence were hydroxylated. The prolines where hydroxylation was observed are designated as **p** in Table I and in Figure 1. The presence of peptides, which contain non-hydroxylated Pro(s) and hydroxylated Pro(s), such as PGVGPF and **p**GVGPF (residues 190–195); GLVPGGPGFGPGVV and GLVPGG**p**GFGPGVV (residues 321–334); GVPGAGVPGVGVPGAGIPVVPG and GVPGAGV**p**GVGVPGAGIPVVPG (residues 335–356); GAGIPGLGVGV and GAGI**p**GLGVGV (residues 582–592); and GGVGIPGGVVGA and GGVGI**p**GGVVGA (residues 678–689) in elastase digest indicated the possibility of partial hydroxylation in the precursor tropoelastin molecule or mature elastin. Figure 2 shows exemplary nano-ESI–qTOF fragment ion spectra of the hydroxylated and non-hydroxylated forms of the two peptides at residues 335–356 on which the b and y-series ions are indicated. Despite their higher masses, the peptides containing hydroxylated Pro were consistently observed 1 to 2 min ahead of their non-hydroxylated counterparts in the LC-MS chromatograms. This is in a good agreement with the general understanding that, under the chromatographic conditions employed in this experiment, if all other variables remain constant, the more hydrophilic peptides are eluted earlier than the less hydrophilic peptides.

Analyses of tropoelastin cDNAs indicated that there is significant variation within a species in nucleotide sequence and size of both the isolated mRNA and

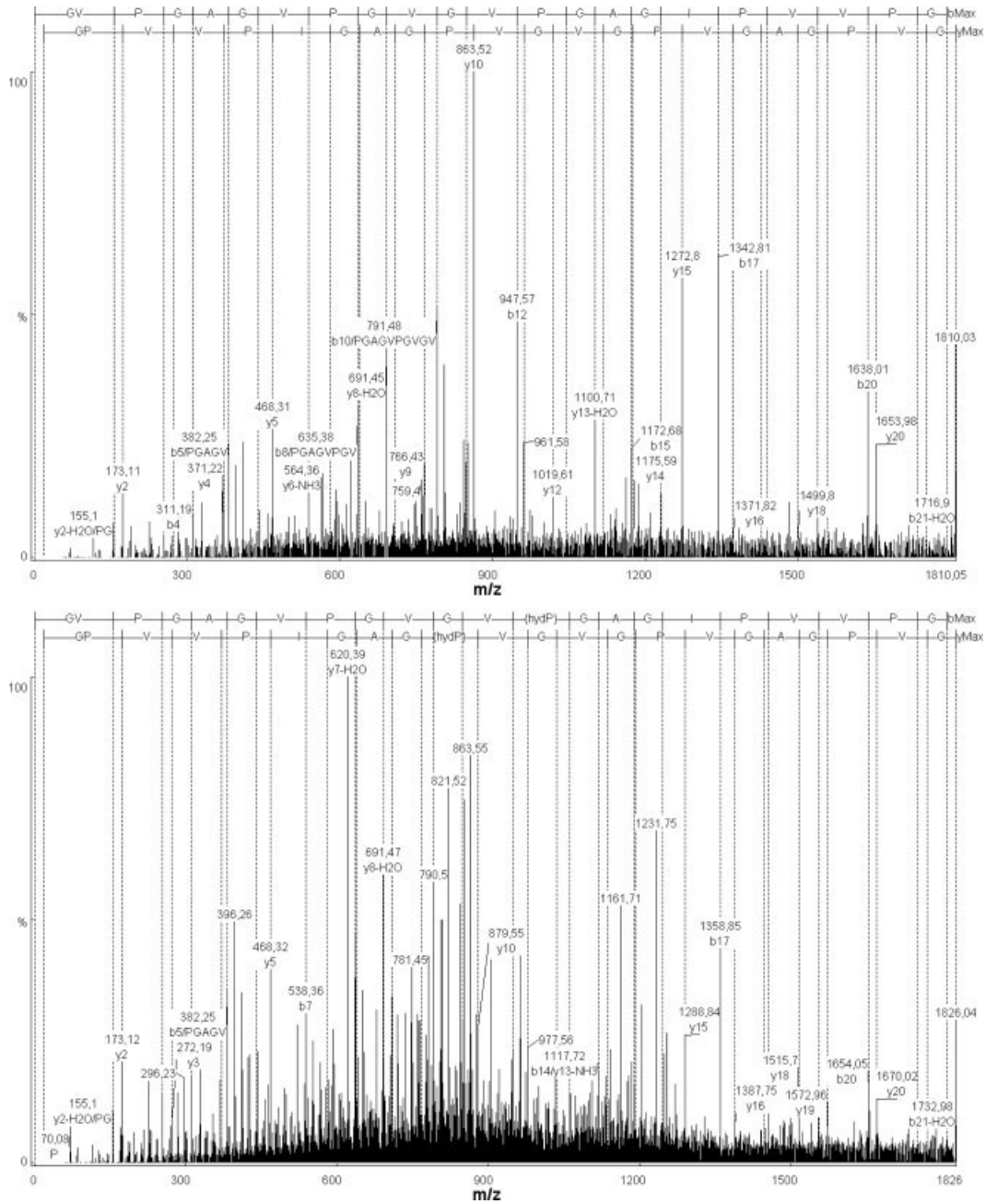


Fig. 2. Nano-ESI–qTOF fragment ion spectra of the non-hydroxylated peptide GVPGAGVPGVGVPGAGIPVVPG, $M_r$ 1809.01 (top) and its hydroxylated counterpart GVPGAGV**p**GVGVPGAGIPVVPG, $M_r$ 1825.01 (bottom). The hydroxylated proline is designated with the letter **p**. The b and y-series ions are labeled, and their respective amino acid residues are indicated.

cDNA.[7,28,43,44] cDNAs were found to differ only in the presence or absence of specific domains corresponding to entire exons or segments of exons. These variable cDNAs were shown to be the result of alternative splicing of tropoelastin mRNA. Several human tropoelastin splice variants have been identified, with seven exons shown to be subject to alternative splicing: exons 22, 23, 24, 24A, 26A, 32 and 33.[2,27,28] In an effort to investigate which of the splicing variants are present in our human skin elastin sample, we developed a database, which considers the aforementioned variants of elastin. As mentioned elsewhere in this article, two peptides were detected in the sample verifying that exon 26A is spliced out, which was observed in human aorta.[2]

Besides characterization of the primary structures of the peptides, our results have helped us to understand the sites of cleavage of the peptide bonds by the enzyme used. It has been reported that elastase cleaves predominantly at the C-terminals of the amino acids Gly, Val, Leu, Ala, and Ile, and to a lesser extent at Phe and Pro.[45] Our study revealed a similar result, except that C-terminal cleavage to two more amino acids, Glu and Arg, was observed.

## CONCLUSIONS

In this work, peptides resulting from digestion of human skin elastin with elastase were characterized with MS/MS using two independent mass spectrometric techniques: ESI-ion trap MS coupled to liquid chromatography and nano-ESI–qTOF MS. These methods were shown to be complementary, yielding results that help to identify the sequences of 117 peptides with sequence coverage of 58.8%, which neither of the two methods would have achieved alone. The use of the homemade database with a limited set of sequences of tropoelastin variants in the Mascot search engine resulted in a reduction in the number of false positive peptide sequences, especially in the case of smaller peptides. On the basis of the structure and location of the peptides identified, we were able to deduce the cleavage sites of the enzyme. The presence of proline hydroxylation on human skin elastin was demonstrated for the first time. We found that 25% of the total proline residues in the identified tropoelastin sequence are hydroxylated. Generally, this approach can be effectively employed to deduce the primary structure of elastin extracted from tissues exposed to elastin-related pathological disorders and to understand the biochemical basis of such disorders.

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# Mass spectrometric characterization of human skin elastin peptides produced by proteolytic digestion with pepsin and thermitase

Christian E.H. Schmelzer*, Melkamu Getie, Reinhard H.H. Neubert

*Institute of Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck-Str. 4, 06120 Halle (Saale), Germany*

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**Abstract**

This study investigated peptides resulting from the digestion of human skin elastin with pepsin and thermitase. Characterization of the peptides was performed using two complementary mass spectrometric techniques; LC/ESI-ion trap and nano-ESI-qTOF MS. 155 different peptides were identified using a combined database based and de novo sequencing approach resulting in a total sequence coverage of 65.4% calculated on the basis of the precursor tropoelastin (accession number A32707). A potential hydroxylation was found in 29% of the recovered prolines. Furthermore, the absence of amino acids expressed by exon 26A could be confirmed. However, contrary to earlier studies, amino acids expressed by exon 22 seem to exist.


*Keywords:* Skin elastin; Hydroxyproline; LC/MS; Tandem MS; Nanoelectrospray; De novo sequencing; Cleavage sites

## 1. Introduction

Elastin, a natural elastomer, is a primary component of elastic fibers that provide elasticity and resilience to elastic tissues such as skin, blood vessels, lung, and ligaments [1]. It is principally synthesized from its precursor, tropoelastin, during the development or growth of tissues, with tropoelastin expression occurring during mid- to late fetal or embryonic periods [2]. The principal step in the biosynthesis of elastin is well characterized. First, the lysine residues of the tropoelastin react with lysyl oxidase to form α-amino adipic acid δ-semialdehyde (allysine). Then, allysine molecules react with lysine and/or another allysine to form polyfunctional cross-links such as desmosine, isodesmosine, lysinonorleucine, merodesmosine, and cyclopentenone [3–6]. Although there is some species variation, elastin from higher vertebrates including human beings contains over 30% Gly and approximately 75% of the entire sequence is made up of just four hydrophobic amino acids: Gly, Ala, Pro, and Val [7]. The extensive cross-linking at Lys residues together with the high content of hydrophobic amino acids makes elastin one of the most hydrophobic proteins known.

Several pathological conditions are associated with abnormalities in elastin. With increasing age, changes such as wrinkling and sagging occur in sun exposed skin [8,9]. Diseases such as Williams syndrome [10], supravalvular aortic stenosis [11,12], emphysema [13], aneurysms [14], and atherosclerosis [15] are said to occur due to pathological modifications in elastin and elastic fibers. However, the exact mechanisms behind such disorders are unknown. Understanding the primary structure of elastin at molecular level would help to gain a better insight into the biochemical basis of the aforementioned pathological conditions.

Hydroxylation of proline residues is reported to occur in tropoelastin of some animals to a varying degree; between 0% and 33% of the total Pro being hydroxylated by the enzyme prolyl hydroxylase [16–19]. It has also been reported that

* Corresponding author. Tel.: +49 345 5525214; fax: +49 345 5527292.
*E-mail address:* schmelzer@pharmazie.uni-halle.de (C.E.H. Schmelzer).

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cross-linking and the formation of elastin from tropoelastin is reduced by overhydroxylation of Pro [20].

Elastin is virtually insoluble both in water and in any organic solvent. Consequently, studies on elastin are mainly restricted to complete hydrolysis of the protein in a strongly acidic environment and analysis of its characteristic cross-linked amino acids, desmosine and isodesmosine [21–24]. However, the difficulty for complete chromatographic separation of the amino acids and the scanty information available about the entire elastin molecule from the cross-linked amino acids limits this approach. Alternatively, analysis of peptides resulting from enzymatic digestion of elastin would show a better image of the entire protein. Besides, the latter approach has the advantage that the protein is not exposed to the destructive acidic environment and high temperature. Consequently, modifications, which occur on the elastin molecule, as the result of pathological conditions or due to physiological biotransformation, will have a better chance of preservation.

Tandem mass spectrometry (MS–MS) in conjunction with database searching [25] and/or de novo sequencing algorithms [26] has become an increasingly important tool in the determination of the primary structure of peptides and is well applicable also in the case of post-translational modifications [27,28]. An important step is the choice of a suitable enzyme. While the literature shows that very often, and particularly in the field of protein identification or proteomics, site-specific enzymes such as trypsin or chymotrypsin are used [29], when used separately, these proteases are suitable for the hydrolysis of elastin [30] only to a limited extent. To achieve effective and uniform degradation of elastin, the use of proteases, which cleave predominantly at hydrophobic amino acids, is preferable.

Therefore, in this work, the sequences of peptides resulting from enzymatic digestion of human skin elastin with the low-specificity acid protease pepsin and the serine protease thermitase [31] were determined by tandem MS using conventional electrospray ionization (ESI) coupled with reversed-phase HPLC and nanoelectrospray ionization (nano-ESI). The peptide sequences of the resulting mass spectra were identified by database matching and/or combination of de novo sequencing and database matching.

## 2. Experimental

### 2.1. Materials

Human skin elastin prepared using the method of Starcher and Galione [32] was purchased from Elastin Products Company (Owensville, Missouri, USA). Thermitase from *Thermoactinomyces vulgaris* was kindly offered by Dr. Ulrich Rothe (Institute of Physiological Chemistry, Martin Luther University Halle-Wittenberg, Germany). Pepsin derived from porcine stomach mucosa (471 U/mg), was obtained from Sigma (Taufkirchen, Germany). 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) was obtained from ICN Biomedicals (Aurora, OH, USA). Water was doubly distilled and acetonitrile of HPLC grade was obtained from J.T. Baker (Deventer, The Netherlands). Formic acid and trifluoroacetic acid (TFA), both of analytical grade, were obtained from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland), respectively.

### 2.2. Digestion of human skin elastin with thermitase

Elastin was dispersed in 1 mM Tris buffer, pH 8.5 at a concentration of 1 mg/mL and digested with thermitase for 24 h at 37 °C. The enzyme-substrate mass/mass ratio (m/m) was 1:50.

### 2.3. Digestion of human skin elastin with pepsin

Elastin was dispersed in water at a concentration of 1 mg/mL, adjusted to pH 2 with 1N HCl and digested with pepsin for 48 h at 37 °C. The enzyme–substrate ratio (m/m) was 1:20.

### 2.4. LC/ESI-ion trap mass spectrometry

The system used for reversed phase HPLC/ESI-MS consisted of a Spectra System P 4000 pump, equipped with an auto sampler AS 3000 and a controller SN 4000 (Thermo Electron, San José, CA, USA). The MS and tandem MS experiments were performed on an ion trap mass spectrometer Finnigan LCQ (Thermo Electron, San José, CA, USA) with electrospray interface. Ten microliters of each sample solution were loaded onto a Nucleosil 120-5 $C_{18}$ column (125 mm × 2 mm i.d., Macherey Nagel, Düren, Germany) and peptides were eluted using a linear gradient: 5–60% of acetonitrile in water, both containing 0.1% of formic acid, over 60 min. The column was maintained at 30 °C and the flow rate was 0.2 mL/min. The mass spectrometer was operated in positive ion mode by applying an electrospray voltage of 4.5 kV and the heated capillary temperature was 220 °C. The digests were initially analyzed in full scan mode and the masses of all the peptides, *m/z* between 50 and 2000, were recorded. From this mass list, peptides of interest were selected manually based on the relative intensity of their chromatographic peaks for further tandem MS experiments using collision-induced dissociation (CID). The mass isolation window for CID was set between 1 and 2 U depending on the experimental conditions. Fragmentation was carried out varying the relative collision energy between 25% and 40% to achieve optimal fragment spectra for $[M + H]^+$ ions.

### 2.5. Nanoelectrospray-qTOF mass spectrometry

Nano-ESI experiments were conducted on a quadrupole time-of-flight mass spectrometer Q-TOF-2 (Waters/Micromass, Manchester, UK) equipped with a nanoelectrospray ZSpray source. The nano-ESI glass capillaries were obtained precoated from New Objective (Woburn,

MA, USA) and DNU (Berlin, Germany). The TOF analyzer was calibrated every day using a mixture of sodium iodide and caesium iodide. The thermitase digest was desalted by washing the sample bound to a ZipTip $C_{18}$ unit (Millipore, Schwalbach, Germany) according to the manufacturer's instructions prior to elution with acetonitrile/water (1:1, v/v) containing 0.05% TFA. The peptic digest was used without further preparation. Two microliters of the sample solution were loaded into the capillary using Microloader pipette tips (Eppendorf, Hamburg, Germany).

The typical operating conditions for the qTOF mass spectrometer were as follows: capillary voltage, 900 V; sample cone voltage, 35–55 V; source temperature, 80 °C. The instrument was operated in the positive ion mode. Full scans were performed over the *m/z* range from 50 to 3500. Peptides of interest were selected manually for further tandem MS experiments using CID. The quadrupole mass filter before the TOF analyzer was set with low mass (LM) and high mass (HM) resolution settings of between 10 and 16 (arbitrary units) and the collision energy was varied between 18 and 70 eV according to the mass and charge state of the respective peptide.

### 2.6. Database based and de novo sequencing

The fragment ion spectra of tandem MS obtained from LC/ESI were processed using Mascot Distiller (Matrix Science, London, UK), a software program that reduces MS raw data to high quality peak lists for database searching. In the case of nano-ESI, the MS and MS–MS spectra were analyzed by MassLynx (version 3.4, Waters/Micromass). Then, the MS–MS spectra of each peptide were processed by the MassLynx add-on Maximum Entropy 3 (MaxEnt3), and converted into SEQUEST files, which were suitable for further analysis. The algorithm of MaxEnt3 deconvolutes charge state and isotopic information in a continuum spectrum to generate a centroid spectrum containing only monoisotopic, singly charged peaks. Both the ESI and nano-ESI fragment ion peak lists, generated as described, were analyzed by searching sequence databases with Mascot (version 1.8, Matrix Science, London, UK) [33]. For database searching, Mass Spectrometry protein sequence DataBase (MSDB), SWISS-PROT and a home-made database of human tropoelastin considering its splice variants with the seven exons shown to be subject to alternative splicing, namely exons 22, 23, 24, 24A, 26A, 32, and 33 [34–36] were used. The searches were taxonomically restricted to *Homo sapiens* and the enzyme was set to "none" because of the low specificity of the proteases used. A varied hydroxylation of proline was considered.

Auto de novo sequencing with combined database searching was mainly performed on the nano-ESI data using the software PEAKS Studio (version 2.4, Bioinformatics Solutions, Waterloo, ON, Canada) [37] with a parent and fragment mass error tolerance of 0.08 U. The enzyme entry was set to "unknown" and the varying degree of hydroxylation of proline was considered. Besides MSDB the same home-made protein database as implemented in Mascot was also used for PEAKS.

## 3. Results and discussion

The samples obtained from the pepsin and thermitase digestion of human skin elastin were subjected to two independent mass spectrometric techniques in order to characterize the complex peptide pattern as comprehensively as possible. Conventional electrospray carried out on an ion trap mass spectrometer, following chromatographic separation, and nanoelectrospray on a quadrupole time-of-flight instrument were used in parallel.

Besides the standard approach of identifying peptide sequences from tandem MS data using database search engines, a powerful de novo sequencing software was applied for the re-evaluation of ambiguous results and the determination of the locations of hydroxylated prolines. The use of a home-made database with a restricted set of tropoelastin sequence variants in the Mascot search engine resulted in a reduction in the number of false positive peptide sequences, especially in the case of smaller peptides.

In general, 72 and 89 peptides with lengths of between 3 and 26 amino acids were identified from the pepsin and thermitase digests, respectively. Only 10 of the peptides were common to the digest of the two enzymes. Fig. 1 shows the peptides identified in the amino acid sequence of human tropoelastin for the respective enzymes used. The sequence, which is found in the protein information resource (PIR) database with the accession number A32707, comprises 786 amino acids derived from the mRNA of fetal human aorta [38] and an additional 6 amino acids peptide (residues 501–506) obtained from human skin fibroblast [36]. It is worth mentioning that the positions of 19 of the identified peptides could not be unequivocally determined due to their multiple occurrences in the primary structure of the precursor (data not shown).

One of the interesting features of protein characterization with mass spectrometry is its ability to determine post-translational modifications. The presence of hydroxyproline in elastin has been known for more than three decades. However, the function of this modification has not yet been fully described. Whereas proline hydroxylation plays a critical role in the synthesis and secretion of a related protein, procollagen, and for its complete maturation to insoluble collagen [39], its presence is not required for the synthesis and secretion of tropoelastin [40]. It has been reported that the existence of hydroxyproline in elastin may be a coincidental feature of the fact that the precursors of elastin and collagen are synthesized in the same region of the endoplasmic reticulum and the prolyl hydroxylase, which exists there, hydroxylates some of the proline residues occurring in the tropoelastin polypeptides [41]. On the other hand, reports suggest that cross-linking and the formation of elastin from


Fig. 1. Amino acid sequence of human tropoelastin found in the protein information resource (PIR) database with the accession number A32707. All unambiguously identified peptides from the digestion of human skin elastin with pepsin (left) or thermitase (right), respectively, are labeled with solid lines. Proline residues, which were found to be hydroxylated in at least one peptide, are labeled "**p**".

tropoelastin is reduced by overhydroxylation. For example, Urry et al. have shown that a synthetic polypentapeptide (Val-Pro-Gly-Val-Gly)$_n$, which is capable of coacervating at 37 °C similar in a mechanism to tropoelastin, requires a higher temperature to coacervate when some of the prolines are replaced by hydroxyprolines [20]. Amino acid analyses on elastin isolated from various sources indicate the hydroxyproline content of the protein can vary from 0% to 33% [16,17,19,42]. In this study, the extent of proline hydroxylation in the enzymatic digest of human skin elastin was determined.

Numerous prolines were found to exist in a hydroxylated and non-hydroxylated state in parallel, for example AAGLGAGIPGLGVG and AAGLGAGI**p**GLGVG (residues 578–591) in the peptic digest or AGIPGVGPF and AGI**p**GVGPF (residues 281–293) in the thermitase digest; "**p**" referring to the prolines in the sequences at which hydroxylation was observed. This indicates the possibility of partial hydroxylation of the precursor tropoelastin or matured elastin. Fig. 2 shows representative nano-ESI-qTOF fragment ion spectra of the hydroxylated and non-hydroxylated forms of the two peptides at residues 578–591 on which the b- and y-series ions are indicated. Therefore, although about 29% of the 73 recovered prolines from the digests of the two enzymes used (see Fig. 3) were found to be potential sites of hydroxylation, the actual percentage of the average hydroxylation of elastin should be smaller.

It has previously been reported that human skin elastin does not contain amino acid sequences expressed by two exons: exon 22 (residues 453–481) and exon 26A (residues 618–650) [36]. The absence of amino acids expressed by exon 26A, which was observed in human aorta [38], could be unambiguously confirmed by the presence of the two peptides, AV**p**GAL ($M_r$ 542.31) and GAVPGAL ($M_r$ 583.33), which share amino acids before and after exon 26A. Contrary to what has been published earlier by Fazio et al. [36], the

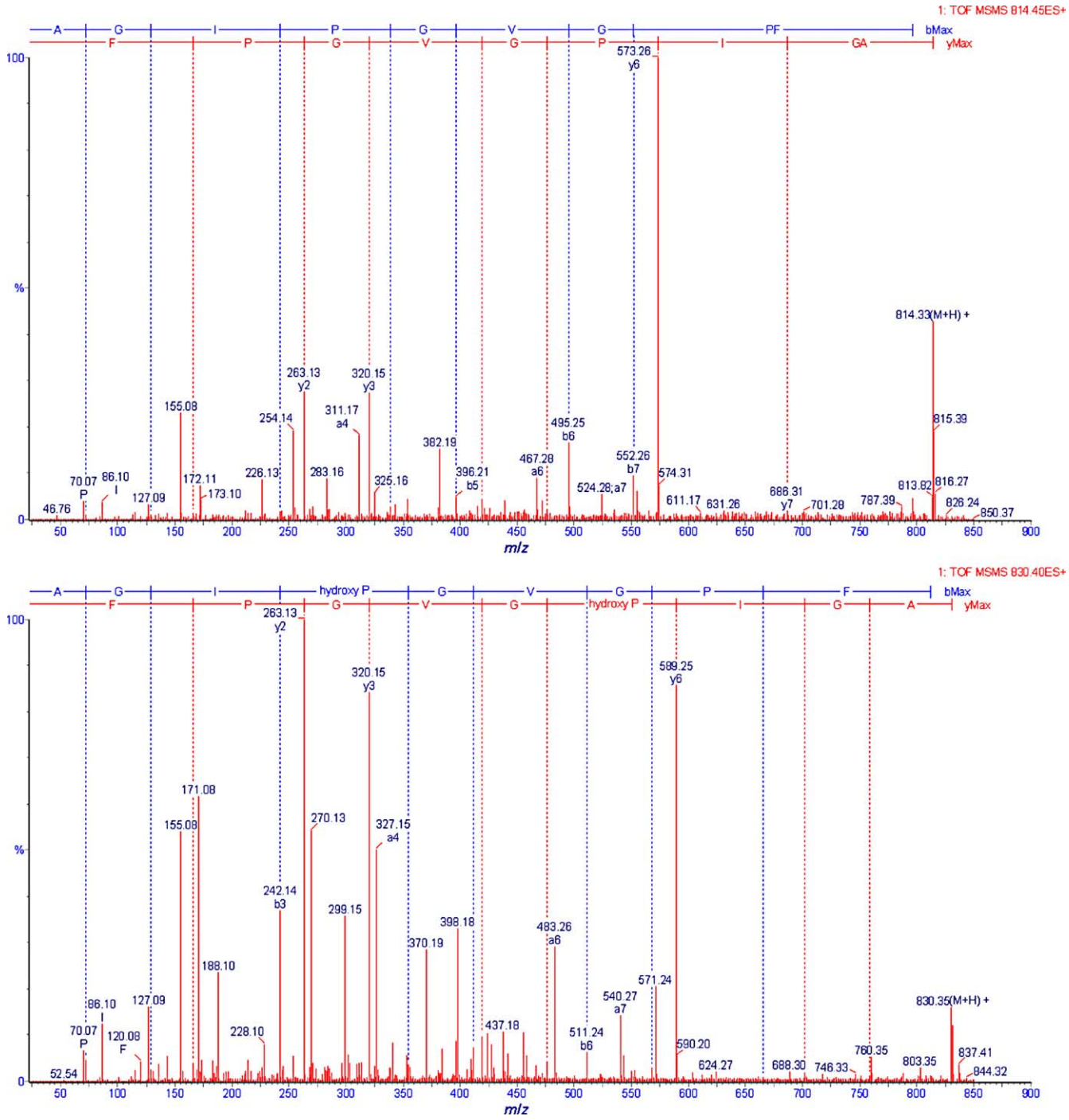


Fig. 2. Nano-ESI-qTOF fragment ion spectra of the non-hydroxylated peptide AGIPGVGPF, $M_r$ 813.44 (top) and its hydroxylated counterpart AGI**p**GVGPF, $M_r$ 829.43 (bottom). The b and y-series ions are labeled and their respective amino acid residues are indicated.

identification of three peptides (residues 461–482, 462–467, and 478–488), which share amino acids from exon 22 indicate that the region coded by this exon is not spliced out in the human skin elastin sample under investigation.

When determining the sequence coverage, the amino acids of exon 26A, the 26 amino acids of the signal sequence which do not belong to the extracellular tropoelastin molecule [43], and the 19 peptides with multiple occurrences were not considered. As regards the precursor tropoelastin, sequence coverages of 49% and 44% for the peptic and the thermitase digest, respectively, were found. Combining the results of both digests, the total sequence coverage was found to be 65.4%.

It can be seen from Fig. 3 that almost no peptide was identified in regions consisting of stretches of lysine separated by two or three alanine residues such as AAAKAAKAA. This


Fig. 3. Total sequence coverage from the results of both digests. Regions from which peptides are identified are designated in bold and underlined letters. Proline residues, which were found to be partially hydroxylated, are labeled "**p**".

is due to the fact that the lysine molecules in tropoelastin are modified to form polyfunctional cross-links. To date, sequencing peptides containing such modifications using database searching or conventional de novo sequencing has not been reported.

From the data obtained some remarks can be added here concerning the observed cleavage sites of the enzymes used. Previous reports have verified the cleavage sites for pepsin [44,45] and thermitase [46] on other proteins. In this work, it was observed that the two enzymes exhibited a high degree of similarity in terms of substrate specificity. Generally speaking, both proteases cleave predominately at the C-terminals of three of the top four dominant amino acids, i.e., Gly, Ala, and Val. In addition, there has been a similar degree of cleavage on the C-terminals of the relatively rare amino acids Leu and Phe. Table 1 summarizes the cleavage sites found from the hydrolysis of human skin elastin with the two respective enzymes.

Table 1
Identified cleavage sites of the proteases thermitase and pepsin used on human skin elastin (**bold**—frequent cleavage sites; *italics*—occasional cleavage sites)

| Enzyme | Cleavage sites (C-terminal) |
|---|---|
| Thermitase | **G**, **V**, **A**, **L**, **F**, *P*, *T*, *E*, *Q*, *K* |
| Pepsin | **G**, **V**, **A**, **L**, **F**, *Y*, *S*, *T*, *I*, *D* |

## 4. Conclusion

In this work, peptides derived from the digestion of insoluble human skin elastin with pepsin and thermitase were investigated. The proteases were found to be suitable for the hydrolysis of insoluble elastin that cannot be digested with site-specific enzymes. Characterization was performed with tandem MS using two complementary techniques: ESI-ion trap coupled with liquid chromatography and nano-ESI-qTOF mass spectrometry. Thus, 155 different peptides from the two digests were unambiguously identified using database searching and a combined de novo sequencing with database approach. This combination of different enzymes and complementary mass spectrometric and sequencing methods

made a high sequence coverage possible, which was found to be 65.4% with respect to the elastin precursor tropoelastin.

We have found that 29% of the proline residues recovered in the underlying tropoelastin sequence are partially hydroxylated. Furthermore, the data obtained confirm the absence of amino acids expressed by exon 26A, whereas amino acids expressed by exon 22 seem to exist in human skin elastin. In general, this approach can be effectively employed to derive the primary structure of elastin, extracted from biological tissue samples exposed to elastin-related pathological disorders, and to understand the biochemical basis of such disorders.

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# Letter to the Editor

*To the Editor-in-Chief*
*Sir,*

**Complementary mass spectrometric techniques to achieve complete sequence coverage of recombinant human tropoelastin**

Tropoelastin is the precursor of elastin, which is a primary component of elastic fibers that provide elasticity and resilience to elastic tissues such as skin, blood vessels, lung, and ligaments. Several pathological conditions, such as wrinkling and sagging in sun-exposed skin, Williams syndrome, supravalvular aortic stenosis, emphysema, aneurysms, and atherosclerosis, are associated with abnormalities in elastin. Analyses of tropoelastin cDNAs resulted in the identification of several human tropoelastin splice variants with seven exons shown to be subject to alternative splicing, namely exons 22, 23, 24, 24A, 26A, 32, and 33.[1,2] Comparison of peptides of matured elastin, extracted from normal or diseased tissue, with the peptides of tropoelastin generated and sequenced in a similar manner will help to understand primary structural changes resulting from pathological disorders, alternative splicing, or due to biotransformation of tropoelastin to elastin. In order to achieve this, it is essential to develop a method capable of fully characterizing the primary structures of tropoelastin and elastin.

Tandem mass spectrometry (MS/MS) accompanied by database searching and/or *de novo* sequencing is currently one of the most important techniques employed for this purpose. The identification of proteins with MS/MS is commonly based on the analysis of peptide mixtures produced by proteolytic digestion of the protein(s) under study. Complementary ionization techniques, when used together, lead to the identification of more peptides than can be identified by either technique alone.[3–5] The most commonly reported complementary ionization methods used in the mass spectrometric analysis of biomolecules are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). It has been suggested that the complementarity between ESI and MALDI, both coupled with a variety of mass analyzers, may be correlated to preferences of the two ionization techniques for certain classes of amino acids. MALDI is reported to preferentially ionize basic residues[6] or aromatic peptides,[5] while ESI might be better employed in the analysis of hydrophobic peptides.[7] A significant advance in the ESI technique was the introduction of nano-ESI, which utilizes a very low solvent flow rate and consequently a very small sample volume. It enables a peptide mass map and several MS/MS sequence tag analyses to be performed on a single minimal sample introduction without chromatographic separation.[8] More advantages over conventional ESI, such as a lower susceptibility to salt contamination and a more uniform response to chemically very different analytes such as peptides and oligosaccharides,[8] and high sensitivities down to the low-attomole range,[9] have been reported.

In this study, MS/MS experiments were performed on the peptides of recombinant human tropoelastin using ESI-ion trap, nano-ESI-quadrupole time-of-flight (qTOF) and MALDI-TOFMS in order to exploit their complementarity towards obtaining full sequence coverage of the protein. Then peptide identification was performed from the resulting fragment spectra by database searching and *de novo* sequencing.

Recombinant human tropoelastin lacking exon 26A (SHELΔ26A), synthesized as previously described by Martin *et al.*,[10] was digested either by pepsin in 0.01 M HCl (pH 2) for 24 h or by chymotrypsin in 1 mM Tris buffer (pH 8) for 6 h at 37°C. The enzyme/substrate ratio was 1:20 (m/m) for both enzymes.

The tropoelastin digests were analyzed using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with an ESI interface to a Finnigan LCQ ion trap mass spectrometer (Thermo Electron, San José, CA, USA). The former comprised a Spectra System P 4000 HPLC pump equipped with an autosampler AS 3000. Ten microliters of each sample solution were loaded onto a Nucleosil 120-5 $C_{18}$ column (125 × 2 mm i.d.; Macherey Nagel, Düren, Germany) and peptides were eluted using a linear gradient: 5–60% of acetonitrile in doubly distilled water, both containing 0.1% of formic acid, over 60 min. The column was maintained at 30°C and the flow rate was 0.2 mL/min. The mass spectrometer was operated in positive ion mode by applying an ESI voltage of 4.5 kV and the heated capillary temperature was set to 220°C. MS/MS experiments were performed using collision-induced dissociation (CID) varying the relative collision energy between 25 and 40%.

The LC system was disconnected from the MS system to collect the eluate from the column in fractions for further analysis using a MALDI post-source decay (PSD) instrument. MALDI experiments were carried out using a delayed extraction TOF mass spectrometer (Voyager-DE PRO; Applied Biosystems, Framingham, MA, USA) equipped with a pulsed nitrogen laser ($\lambda = 337$ nm, 3 ns pulse width, 20 Hz repetition rate). Solutions of α-cyano-4-hydroxycinnamic acid and sinapinic acid in acetonitrile/0.1% trifluoroacetic acid (TFA) (1:1, v/v) at concentrations of 10 and 20 mg/mL, respectively, were used as matrices. Measurements were performed operating in the positive-ion reflector mode at a total acceleration voltage of 20 kV, grid voltage set to 76%, 0.002% guide wire voltage and an extraction delay of between 100 and 350 ns. PSD experiments were carried out by varying the reflectron voltage from 10 to 15 steps, the voltage being reduced to ~75% of the previous step for each individual step. Using the timed ion selector (TIS), different precursor ions were selected from the peptide mixtures and subjected to fragmentation. The TIS resolution was specified with R = 80 (full width at half maximum). After acquisition, all spectra were stitched together by the data

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system. Each segment spectrum obtained was the mean of at least 1000 laser shots.

Nano-ESI experiments were carried out using a quadrupole TOF mass spectrometer (Q-TOF-2; Waters/Micromass, Manchester, UK) equipped with a nano-ESI ZSpray source. The nano-ESI glass capillaries were obtained precoated from New Objective (Woburn, MA, USA) and DNU (Berlin, Germany). Two microliters of the sample solution were loaded into the capillary using Microloader pipette tips (Eppendorf, Hamburg, Germany). The typical operating conditions for the qTOF mass spectrometer were as follows: capillary voltage, 900 V; sample cone voltage, 35–55 V; source temperature, 80°C. The instrument was operated in the positive ion mode. For MS/MS experiments, the quadrupole mass filter before the TOF analyzer was set with low-mass (LM) and high-mass (HM) resolution values of between 10 and 16 (arbitrary units) and the collision energy was varied between 20 and 70 eV according to the mass and charge state of the respective peptide.

The fragment ion spectra from MS/MS experiments were processed using Mascot Distiller (Matrix Science, London, UK) or MassLynx (version 3.4, Waters/Micromass) to generate fragment ion peak lists that were analyzed employing a sequence database search engine (Mascot, version 1.8; Matrix Science, London, UK)[11] or using the PEAKS Studio software (version 2.4; Bioinformatics Solutions, Waterloo, ON, Canada), for automated *de novo* sequencing. For database searching, Mass Spectrometry Protein Sequence DataBase (MSDB) and SWISS-PROT were used.

Figure 1 shows the sequence of SHELΔ26A including the 26 amino acid signal sequence. The peptides identified after analysis using ESI, nano-ESI, and both methods are underlined in dashed, solid, and dotted lines, respectively. Overall, 100 peptides with lengths ranging from 4 to 54 amino acids were identified. Seven of these peptides: PGVG, PGAI/L, PGLGVG, VGTPAAA, VGTPAAAA, VAPGVGVA, and VAPGVGVAPGVG, had sequences that occur at more than one position in the tropoelastin sequence. The sequence coverage excluding the signal sequence and peptides, whose positions could not be unequivocally determined due to their multiple occurrences, was found to be 86.7%. The number of peptides identified by ESI and nano-ESI was 66 and 57, respectively, 23 of which were identified by both methods. Thus, the analysis of the peptide mixtures on both the ESI and the nano-ESI instruments increased the number of peptides identified by 52% ([100-66]/66) and 75% (from 57 to 100), respectively, over the number of peptides identified on either instrument alone. Despite the relatively fewer number of peptides identified by the nano-ESI instrument, the sequence coverage (72.1%) was far greater than that of peptides detected by ESI (48.4%). This can be attributed to the increase in the length of peptides detected by the former instrument.

Chymotrypsin produced fewer, but larger, peptides than could be obtained with pepsin. The use of the sequence-specific protease chymotrypsin, besides creating peptides in a preferred mass range for sequencing, will have the advantage that the peptides produced could generate information-rich and easily interpretable peptide-fragmentation spectra. The peptides produced by chymotrypsin digestion, detected by nano-ESI and MALDI-MS instruments and identified by *de novo* sequencing and database matching following MS/MS, are mapped on the SHELΔ26A sequence as shown in Fig. 2. The sequences of peptides obtained as the result of analysis with the nano-ESI and MALDI instruments are marked with solid and dashed-dotted lines, respectively. Peptide sequences marked with solid red lines were obtained from analysis using both instruments. A total of 49 peptides (20 by MALDI, 38 by nano-ESI, 9 of which by both methods) with the number of amino acids ranging from 6 to 59 were sequenced. Similar to the observation in the pepsin digest, the analysis of this sample using the MALDI and the nano-ESI instruments increased the number of peptides identified by 145% (from 20 to 49) and 29 (from 38 to 49)%, respectively, over the number of peptides identified by either instrument alone. This finding is consistent with previous experiments on other proteins.[5,12] Stapels and Barofsky,[5] e.g., reported that analyzing DNA-binding proteins on ESI and MALDI instruments increased the number of peptides identified on average by 49% over the number identified in a single analysis on either instrument alone. It was interesting to note that, despite their number being half the number of peptides identified from the pepsin digest, the overall sequence coverage of the peptides observed from the chymotrypsin digest (84.7%) was almost the same. Taking into consideration the peptides identified by nano-ESI analysis alone, it was necessary to sequence 57 peptides from the pepsin digest in order to achieve sequence coverage of 72.1%, whereas a relatively higher coverage (78.9%) was attained by only 38 peptides from the chymotrypsin digest. This is attributed to the residue specificity of the enzyme as compared to pepsin.

Another important observation made in this study was the determination of the cleavage sites of the enzymes used. We have observed that pepsin mostly cleaved at the C-terminals of Gly, Ala, and Val, which, together with Pro, form 75% of the entire sequence of tropoelastin. Pepsin cleaved at the C-terminals of Phe and Leu to a similar degree. It occasionally cleaved at the C-terminals of other amino acids: Ile, Asp, Tyr, Gln, Ser, and Thr, too. These results are in agreement with a previous report[13] that demonstrated the same cleavage sites. Chymotrypsin is known to hydrolyze peptide bonds specifically at the C-terminals of Tyr, Phe, Trp, and Leu.[14] Met, Ala, Asp, and Glu are also cleaved at a lower rate. Similarly, in this study, we observed that chymotrypsin cleaved predominantly at Tyr, Phe, and Leu residues and, rarely, at Ala, Ile, Thr, and Gly. Several peptides that were a result of cleavage at the C-terminals of Lys and Arg residues were found in the chymotrypsin digest. Since cleavage at these sites is characteristic of trypsin, contamination of the enzyme with traces of trypsin was anticipated and subsequently confirmed using MALDI-MS.

In conclusion, a comprehensive identification of the peptides produced by digestion of the recombinant tropoelastin with two enzymes, pepsin and chymotrypsin, with instruments of

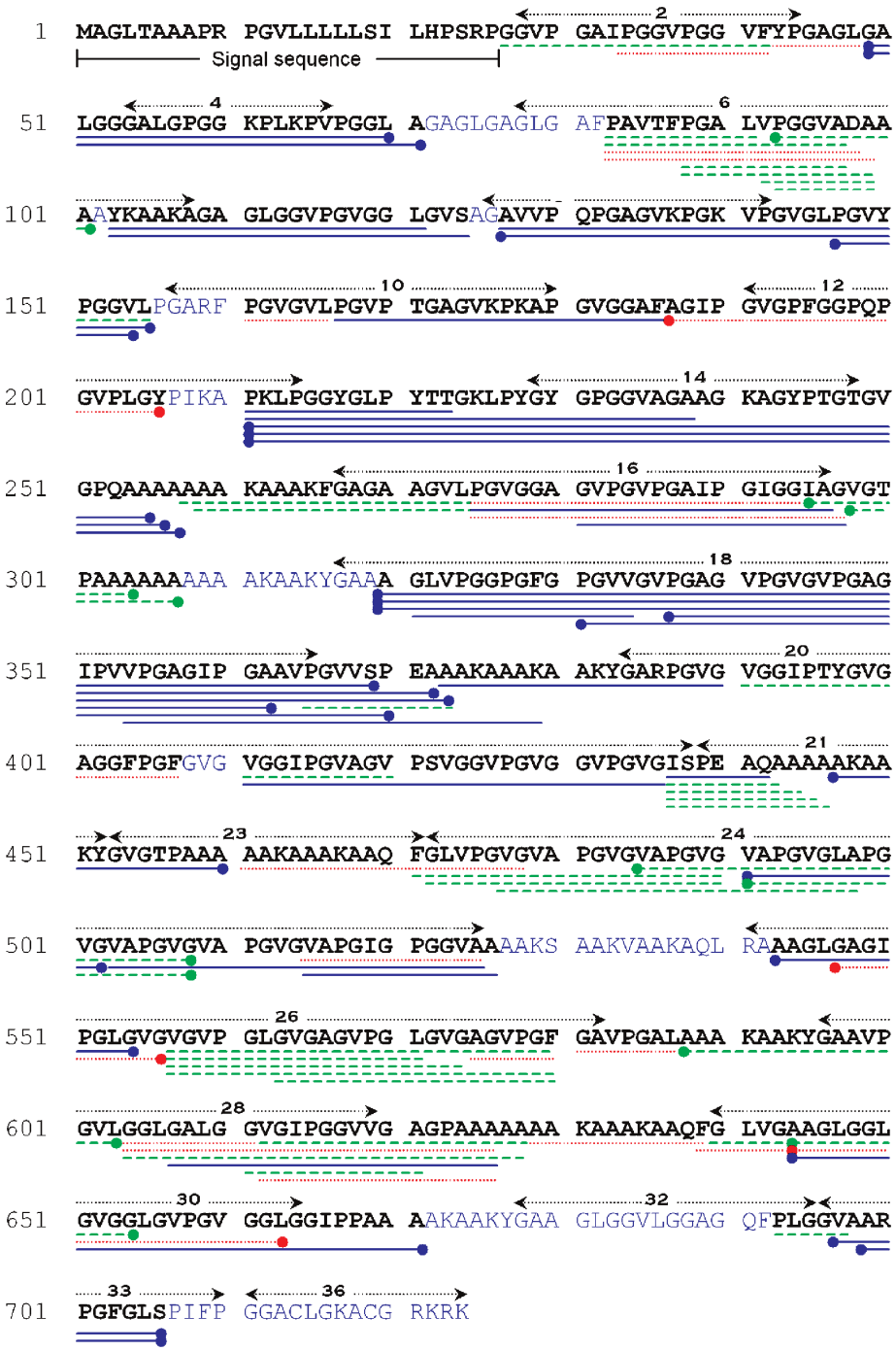


**Figure 1.** The sequence of recombinant human tropoelastin (SHELΔ26A) synthesized on the basis of the amino acid residues of the GenBank entry AAC98394. The stretches of protein sequence identified from the pepsin digest of the protein after analysis using the ESI (dashed), nano-ESI (solid) and both (dotted) instruments are underlined. Sequences extending to the next lines are underlined in oval arrow lines. Blue colored letters refer to the sequences of unidentified regions. Segments corresponding to some exons of tropoelastin are indicated with dotted lines and numbers; the regions adjacent to the marked exons refer to exons of the next higher or lower numbers.

three complementary ionization techniques of MS, namely ESI, nano-ESI, and MALDI, and *de novo* sequencing as well as database searching resulted in a sequence coverage of 94.4%. The sequence coverage could have been higher than reported if some detected ions with *m*/*z* corresponding to individual amino acids, such as Ala, Lys, Leu/Ile, Phe, Arg, and Tyr, and whose positions cannot be unequivocally determined in the protein sequence, had been considered. The presence of Ala and Lys in the samples is particularly important, because most of those

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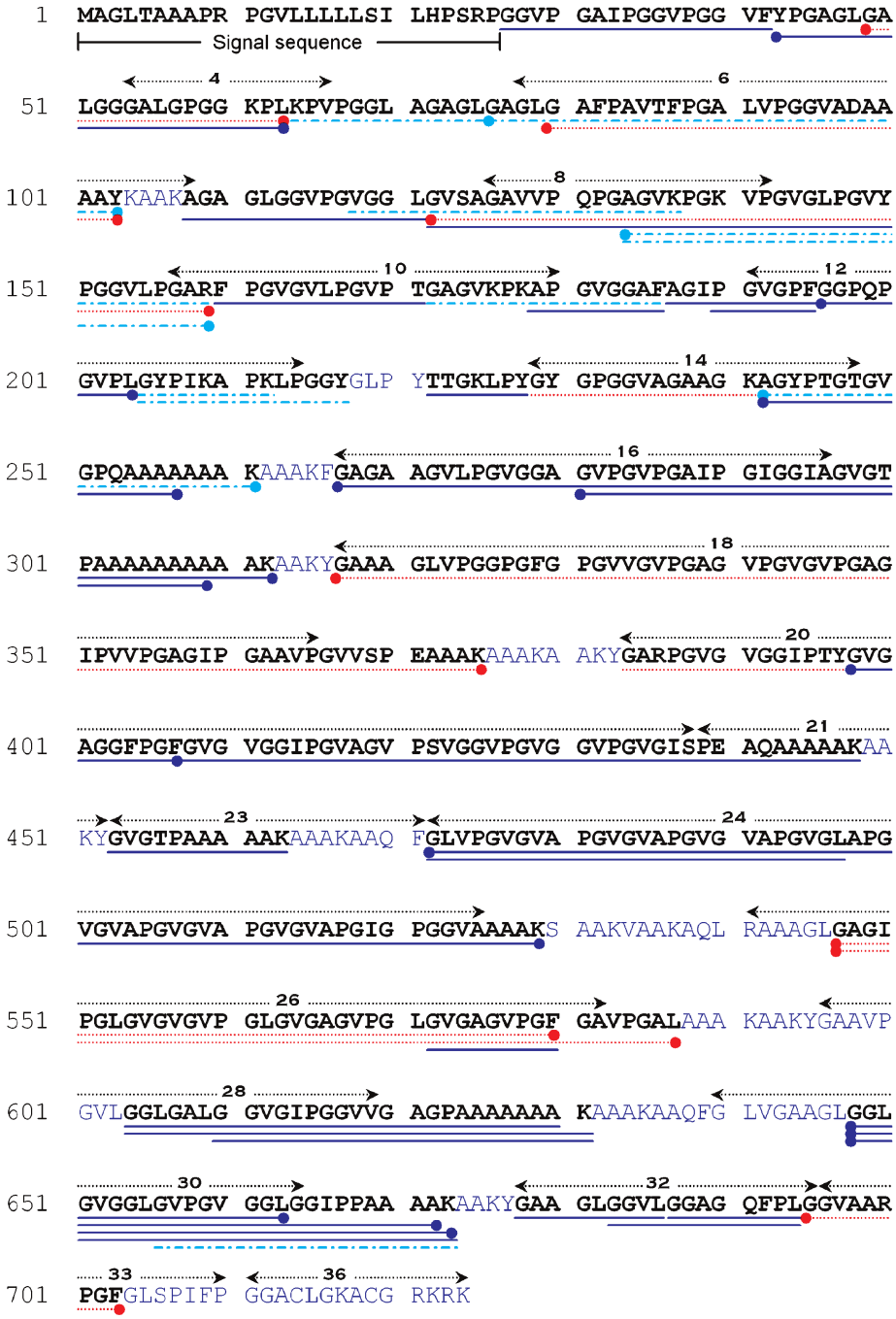


**Figure 2.** The sequence of synthetic human tropoelastin (Accession number: AAC98394); the peptide sequences underlined in dashed-dotted, solid, and dotted lines refer to peptides observed from the chymotrypsin digest of the protein after analysis using the MALDI, nano-ESI and both instruments, respectively. For the rest of the description, refer to Fig. 1.

stretches of protein sequence not detected by MS were rich in Ala and Lys. As has been demonstrated earlier, the complementarity of these methods is attributed to preferential ionization of certain residues by any one of the methods.

For studies involving the characterization of tropoelastin with significant sequence coverage, digestion of the protein with chymotrypsin and analysis of the peptides using MALDI and nano-ESI seems the best and most time-saving approach. However, matured elastin is not adequately digestible with chymotrypsin; thus digestion requires the use of low substrate-specific enzymes such as elastase, thermitase and pepsin. Therefore, characterization of elastin should employ digestion of the protein with one or more of these enzymes. Since large numbers of peptides will be obtained this way and most of the peptides will be of low mass, which are not suitable for analysis by MALDI-MS (due to matrix interference), the use of LC/ESI-MS or nano-ESI could be beneficial.

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*Melkamu Getie[1], Christian E. H. Schmelzer[1], Anthony S. Weiss[2] and Reinhard H. H. Neubert[1]**

[1]Institute of Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck Str. 4, D-06120, Halle (Saale), Germany

[2]School of Molecular and Microbial Biosciences, University of Sydney, NSW 2006, Australia

**Correspondence to: R. H. H. Neubert, Institute of Pharmaceutics and Biopharmaceutics, Martin Luther University Halle-Wittenberg, Wolfgang-Langenbeck Str. 4, D-06120, Halle (Saale), Germany.*

*E-mail: neubert@pharmazie.uni-halle.de*

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