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

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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

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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

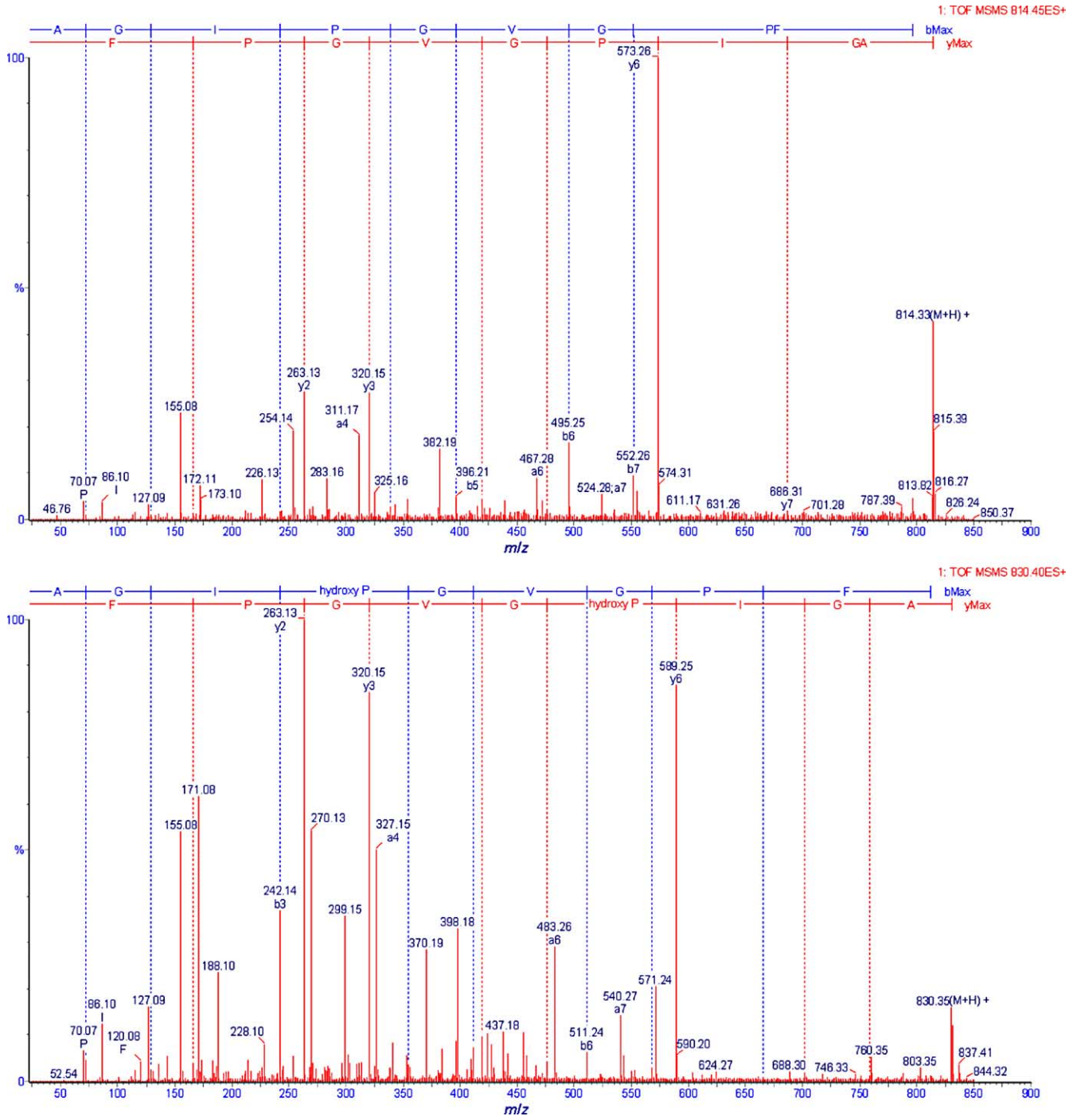

Fig. 2. Nano-ESI-qTOF fragment ion spectra of the non-hydroxylated peptide AGIPGVGPF, M_r 813.44 (top) and its hydroxylated counterpart AGI**p**GVGPF, M_r 829.43 (bottom). The b and y-series ions are labeled and their respective amino acid residues are indicated.

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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