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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,