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Study of human lung elastin degradation by different elastases using high-performance liquid chromatography/mass spectrometry

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Abstract

Elastin is a structural insoluble protein which gives elasticity to tissues and organs. Although its hydrophobic and highly cross-linked nature makes it a very durable polymer, degradation of elastin in relation with several pathological conditions, such as pulmonary emphysema, has been documented. Since different enzymes may be involved in elastolysis, it is of interest to determine which enzyme is responsible for the degradative effects observed in a certain disease. The aim of this work was to study elastin degradation by proteases from different families (serine, cysteine, and metalloproteases) using liquid chromatography coupled to mass spectrometry to characterize the elastin-derived peptides. Incubation of insoluble human elastin with different elastases revealed that, indeed, each protease degrades elastin in a preferential way giving rise to specific peptide patterns. This opens the possibility of using a given set of peptides as biomarkers for disease-related elastolysis.

Keywords: Elastin; Elastases; Lung tissue; Elastolysis; HPLC; MS

Elastin is an insoluble fibrous protein of high tensile strength, which is part of the lung extracellular matrix, supporting its structural framework and providing its stretchable characteristics.

Elastin is secreted as a 72-kDa soluble polypeptide known as tropoelastin [1]. Tropoelastin occurs as multiple, distinct isoforms due to extensive alternative splicing of a single-gene transcript [2]. The polypeptide structural units are covalently cross-linked via two special amino acids (desmosine and isodesmosine) formed by enzymatic oxidative deamination of lysine residues by lysyl oxidase constituting a three-dimensional network [3]. The formation of these cross-links is essential for the unique properties of the mature elastin protein [4].

Elastin possesses an unusual chemical composition responsible for its characteristic physical properties. It consists of an alternation of highly hydrophobic and more hydrophilic residues. The hydrophobic domains contain numerous overlapping repetitive sequences of aliphatic residues (P, A, V, L, I) interrupted by glycyl residues. The fundamental building blocks of these peculiar elastin sequences are GX, PX, GGX, and PGX, where X = G, A, V, L or I. The lysine-containing regions correspond to the potential cross-linking domains [5]. Some of the prolyl residues may be hydroxylated [6]. Despite its extreme hydrophobicity, elastin is highly hydrated. It swells in water and is not elastic in the absence of water [7].

The mature elastin molecule is a remarkably stable and insoluble protein, with a metabolic turnover approaching the life duration of the animal under normal circumstances [8]. However, there are several disorders such as incomplete cross-linking or excessive proteolysis, which can result in pathologic conditions such as aneurysms and impaired pulmonary function [9,10]. Elastin degradation plays an important role in pathogenesis of some cardiovascular [11] and pulmonary [12] diseases and an enhanced elastolytic activity in malignancies has been observed [13–15].

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Although elastin is quite resistant to enzymatic proteolysis (it is not hydrolyzed by trypsin, chymotrypsin, or pepsin), it is acted upon by special proteases called elastases. Elastases may belong to the class of serine proteases, cysteine proteases, or metalloproteases. The elastolytic activity varies from one elastase to another and is usually not correlated with the catalytic efficiency of these proteases.

Uncontrolled elastases play pathologic roles in several diseases such as pulmonary emphysema, cystic fibrosis, infections, inflammation, and atherosclerosis [16–19]. Although repair of the damaged elastic fibers can occur, the physiologic function of the tissue may never return to normal, especially for the lung, where the unique alveolar architecture is very difficult if not impossible to reconstruct [20].

In our research we are interested in the pathophysiological mechanisms leading to the development of pulmonary emphysema in chronic obstructive pulmonary disease (COPD)[1]. In emphysema there is permanent destruction of the alveoli, the tiny elastic air sacs of the lung. Irreversible degradation of lung elastin is known to be a major factor in the development of emphysema. The loss of elastin also causes collapse or narrowing of the smallest air passages (bronchioles), which in turn limits airflow out of the lung.

Next to the deleterious effect of proteolytic activity on lung function, it has recently been proposed that peptides resulting from the proteolytic degradation of elastin can induce biological effects such as regulating the activity of matrix metalloproteases (MMPs) [21] and functioning as chemoattractants for neutrophils [22], thus perpetuating chronic inflammation.

Several studies have been performed to monitor the degradation of this protein in biological fluids in relation to emphysema development, for example, measuring urinary concentrations of desmosine and isodesmosine derived from mature elastin [23–25]. However, the results are not unequivocal. While some researchers found a correlation between desmosine in bronchoalveolar lavage fluid (BALF) and/or urine with disease progression, others did not [26]. Moreover, desmosine is strongly retained by the kidneys, and urinary desmosine is therefore doubtfully useful as a biomarker to assess elastolytic activity in the lung [27].

There are several hypotheses concerning the enzymes that are involved in the degradation of elastin. For example, it is known from clinical studies that patients with a hereditary deficiency of alpha-1-antitrypsin develop early and more severe lung emphysema, pointing toward neutrophil elastase as one of the major players in this disease. Whether this correlation also holds for patients with acquired emphysema such as smokers developing COPD is presently not well established [28]. The role of other proteolytic enzymes found in the lungs of patients with chronic inflammation is unclear. Preliminary data in the literature indicate that both MMPs and cysteine proteases of the cathepsin family may play important roles [29–32]. However, many of the published data are based on in vitro or animal studies and a correlation with the human situation has in most cases not been established. Therefore in COPD patients it would be important to assess the situation with regard to the proteases involved in tissue destruction and emphysema development for further development of diagnostic and therapeutic approaches.

A way to gain insight into these subjects would be by characterizing the peptides resulting from elastin degradation under action of proteases involved in different pathological conditions.

The aim of this work was to identify these peptides using modern analytical techniques based on liquid chromatography coupled to mass spectrometry (LC/MS) and to elucidate the elastolytic preferences of the different proteolytic enzymes.

It must be considered that elastin-derived peptides constitute an analytical challenge due to their hydrophobicity and the low abundance of positively charged amino acids which limits their efficient ionization. Even more, the repetitive nature of the amino acid sequences in this biopolymer makes it in some cases difficult to assign the identified peptides to unique positions in elastin.

Materials and methods

Materials and reagents

Human lung elastin and human neutrophil elastase (HNE) were purchased from Calbiochem (La Jolla, CA, USA). Pancreatic elastase, anti-rabbit IgG –alkaline phosphatase antibody, dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), Brij, and human insoluble lung elastin prepared by nondegradative extraction as described in Starcher and Galione [33] were from Sigma (Zwijndrecht, The Netherlands). Rabbit polyclonal antibody to elastin from several species was obtained from Elastin Product Co. (Owensville, MO, USA). Recombinant human MMP-12 (catalytic domain) and recombinant human MMP-9 were provided by Astra Zeneca, R&D (Lund & Mölndal, Sweden). Cathepsin-K was kindly donated by Dr. Dieter Brömme (University of British Columbia, Vancouver, Canada). Ammonium bicarbonate, ethylenediaminetetraacetic acid (EDTA), sodium acetate, sodium chloride, formic acid, and acetonitrile were from Merck (Darmstadt, Germany). Tris–hydrochloride was from Duchefa Biochemie B.V. (Haarlem, The Netherlands).

Human lung tissue was obtained using protocols approved by the medical ethics committee and provided by the Pulmonary Research Unit of the University Medical Centre Groningen (Groningen, The Netherlands).

Acrylamide/bis solution (30%) (37.5:1), TEMED, ammonium persulfate, precision plus protein standards were from Bio-Rad Laboratories BV (Veenendal, the Netherlands). Ultra-pure water (conductivity: 18.2 MΩ) was obtained from a Maxima System (Elga Labwater, Ede, The Netherlands).

[1] *Abbreviations used:* COPD, chronic obstructive pulmonary disease; MMPs, matrix metalloproteases; HNE, human neutrophil elastase; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; ESI, electrospray ionization; PVDF, polyvinylidene fluoride; TIC, total ion current; BALF, bronchoalveolar lavage fluid.

Instruments

All incubations were performed in a Thermomixer (Eppendorf, Hamburg, Germany). Centrifugations took place at 4 °C in a microliter centrifuge (Mikro 20; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany).

High-performance liquid chromatography/mass spectrometry

The HPLC part of the analytical system consisted of an Agilent Series 1100 LC system (Waldbronn, Germany) comprising a degasser, a binary pump, a temperature-controlled autosampler, and a thermostated column compartment. Chromatographic separation of the peptides generated took place in a reverse-phase C18 column (Zorbax; 0.5 mm I.D. × 150 mm length).

Mobile phase A consisted of 0.1% formic acid in ultrapure water. Mobile phase B was 0.1% formic acid in acetonitrile. The separation was performed with a 0.5% increase of B/min (0 to 60 % B in 120 min). Flow rate employed was 10 µl/min. The column was maintained at 28 °C. The analytes were detected by a diode array detector at 214 nm and subsequently with an Agilent SL ion-trap mass spectrometer equipped with an electrospray ionization (ESI) source operated in the positive mode. MS data were acquired over a scan range of 100–1500 amu and 5500 *m/z* per s scan rate.

For the MS/MS analysis, the fragmentation cutoff was set to 27% of the *m/z* of the precursor ion.

Preparation of elastin digests

Human lung elastin was dispersed in phosphate-buffered saline solution, pH 7.1, at a concentration of 1 mg/ml. A small aliquot of this suspension was used to mix with each of the enzymes prepared in the appropriate buffers (see Result and discusion).

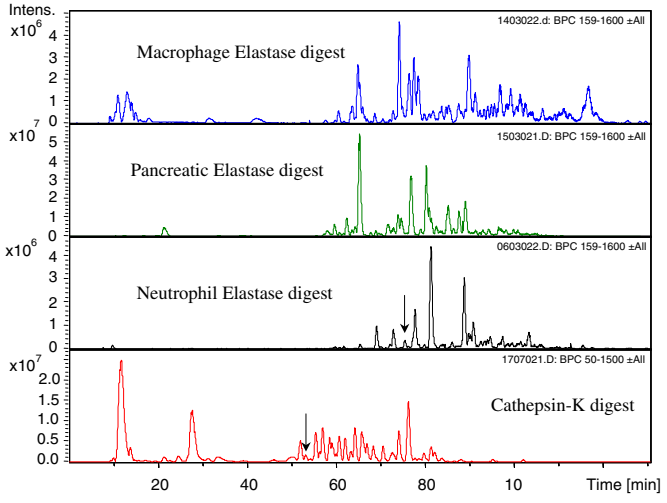

Fig. 1. Chromatographic profiles (HPLC-MS base peak chromatogram) obtained after digestion of human lung elastin with different proteases. Peptides were separated on a C18 column with an increasing gradient of acetonitrile in 0.1% aqueous formic acid (see text for further details). The peaks marked with an arrow correspond to the peptides sequenced by MS/MS in Fig. 2.

Influence of ionic strength on elastase activity

Human insoluble lung elastin (100 µg) was incubated in 50 mM NH_4HCO_3, pH 8.0, with HNE (1 µg) with different final concentrations of NaCl (0, 128, and 536 mM). The samples were incubated 18 h at 37 °C, 1000 rpm in a Thermomixer.

PMSF (1 mM) was added to stop the digestion. The remaining insoluble elastin was spun down (20 min at 13,000 rpm) and the supernatant was subsequently analyzed by LC MS.

HNE-mediated digestion of human lung tissue

NH_4HCO_3 (8 ml, 20 mM, pH 8.0) was added to 1.08 g human lung tissue. Homogenization was performed with an Ultra-Turrax TP18-10 instrument (Janke & Kunkel GmbH, Ika-Werk, Staufen, Germany).

Lung tissue homogenate (100 µl) was diluted 1:1 with 50 mM NH_4HCO_3, pH 8.0, containing different quantities of HNE (0, 0.5, and 5 µg), and NaCl was added to a final concentration of 127 mM (excluding the endogenous salts

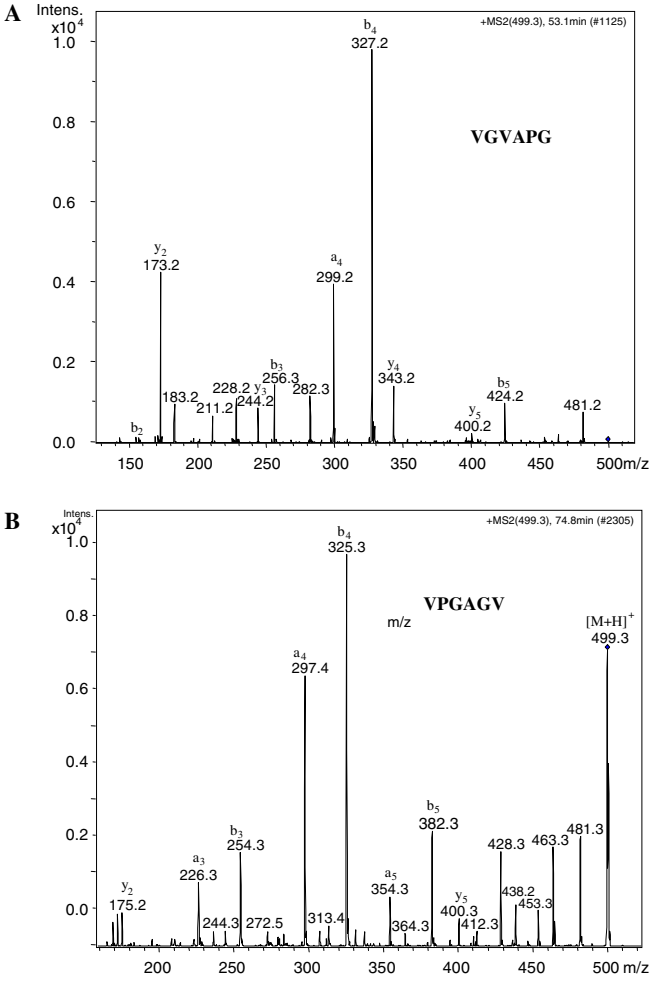

Fig. 2. MS/MS spectra corresponding to two elastin peptides obtained with different elastases (A) cathepsin K and (B) human neutrophil elastase having a parent ion with the same *m/z* value (499.3).

initially present in the piece of tissue). The samples were incubated 18 h at 37 °C, 1000 rpm in a Thermomixer. To stop the HNE-mediated digestion and avoid further tissue degradation due to endogenous serine and metalloproteases, 1 mM PMSF and 1 mM EDTA were added to each sample. The insoluble material was spun down (20 min at 13,000 rpm) to be separated from the supernatant, which was kept for further analyses by SDS–PAGE / Western blot and LC MS.

Table 1
Peptides obtained after digestion of human lung elastin with different elastases at a ratio 1/1000 (w/w) (enzyme/elastin)

m/z	*aa sequence*
Cathepsin-*K*	
317.2 (+)	(G)**AGLG**(A) aa 73-76, 77-80 or (G)**AGLG**(G) aa 110-113, (A)**AGLG**(G) aa 686-689 or (L)**GALG**(G) aa 49-52, 613-616 or (G)**GALG**(P) aa 54-57
331.2 (+)	(G)**VGVG**(G) aa 389-392, 409-412 or (G)**VGVG**(V) aa 561-564
428.2 (+)	(G)**PGVVG**(V) aa 331-335
428.2 (+)	(V)**VGVPG**(A) aa 334-338 or (G)**VGVPG**(A) aa 344-348 or (G)**VGVPG**(L) aa 563-567
435.2 (+)	(G)**VYPG**(G) aa 149-152
485.2 (+)	(G)**VPGVGG**(L) aa 115-120 or (G)**VPGVGG**(V) aa 561-564
499.2 (+)	(G)**VGVAPG**(V) aa 489-494, 495-500, 507-512, 513-518 or (G)**VGVAPG**(I) aa 519-524
581.3 (+)	(G)**IPVVPG**(A) aa 351-356
Pancreatic elastase	
442.2 (+)	(V)**PGLGV**(G) aa 566-570, 575-579
499.3 (+)	(G)**LGGVPG**(V) aa 112-117 or (G)**GLGVPG**(V) aa 660-665
513.3 (+)	(P)**GALVPG**(G) aa 89-94 or (A)**AGLVPG**(G) aa 320-325 ,477-482 or (G)**VGLAPG**(V) aa 501-506
598.3 (+)	(A)**GVLPGVG**(G) aa 272-278 or (A)**GLVPGVG**(V) aa 478-484
655.3 (+)	(G)**VGVAPGVG**(V) aa 483-490, 489-496, 507-514, 513-520 or (G)**VGVAPGVG**(L) aa 495-502 or (V)**GVPGLGVG**(A) aa 564-571 or (A)**GVPGLGVG**(A) aa 573-580
726.3 (+)	(V)**GVPGLGVGA**(G) aa 564-572 or (A)**GVPGLGVGA**(G) aa 573-581
Neutrophil elastase	
428.3 (+)	(F)**PGVGV**(L) aa 161-165, (V)**PGVGV**(P) aa 342-346, (R)**PGVGV**(G) aa 387-391, (V)**PGVGV**(A) aa 481-485, (A)**PGVGV**(A) aa 493-497,505-509, 511-515,517-521
442.3 (+)	(V)**PGLGV**(G) aa 556-570,575-579 (I)**PGLGV**(G) aa 557-561
444.2 (+)	(G)**LGVGV**(G) aa 559-563
449.3 (+)	(L)**GYPI**(G) aa 205-208
499.3 (+)	(G)**VPGAGV**(P) aa 336-341
572.4 (+)	(A)**GLGGLGV**(G) aa 652-658
632.3 (+)	(A)**GLGAFPA**(V) aa 74-84
653.4 (+2)	(V)**GVPGLGVGAGVPGLGV**(G) aa 564-579
754.4 (+)	(I)**PGLGVGVGV**(P) aa 557-565
Macrophage elastase	
487.3 (+)	(G)**LGALGG**(G) aa 48-53 or (G)**LGALGG**(V) aa 612-617
558.4 (+)	(G)**AGLGALG**(G) aa 46-52
629.3 (+)	(G)**LAGAGLGA**(G) aa 70-77
691.9 (+2)	(G)**PFGGPQPGVPLGYP**(I) aa 194-207

SDS–PAGE / Western blot with digested human lung tissue

The samples obtained after HNE-mediated digestion of human lung homogenate were mixed with an equal volume of loading sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 0.02% (w/v) bromophenol blue, 200 mM DTT in 62.5 mM Tris, pH 6.8) and then incubated in a boiling water bath for 5 min prior to loading on the polyacrylamide gel.

A 12.5% polyacrylamide gel was cast and the electrophoresis was performed with a constant voltage of 200 V using a Mini-Protean 3 Electrophoresis Cell (Bio-Rad Laboratories BV). The Western blot transfer from the gel to a PVDF membrane was done in 1 h with a 350-mA current in a Mini Trans-Blot Cell (Bio-Rad Laboratories BV) using as transfer buffer 25 mM Tris, 192 mM glycine, pH 8.3, 20% (v/v) methanol.

Results and discussion

To characterize the peptide bond specificity of different proteases, enzymes from three different protease families (human neutrophil elastase and pancreatic elastase (serine proteases), macrophage elastase (MMP-12) and gelatinase B (MMP-9) (metalloprotease), and cathepsin-K (cysteine protease)) were chosen to evaluate whether they generate different patterns of peptides. This selection was based on several literature references from research groups [19,29–32] indicating their implication in emphysema. Pancreatic elastase, although it is not involved in emphysema, was included to set up the analytical methodology.

Human lung elastin (500 μg) was incubated with each of the above-mentioned proteases at a ratio 1/1000, w/w, (enzyme/elastin), during 18 h at 37 °C.

Digestion buffer was ammonium bicarbonate, 50 mM, pH 8.0, for the serine proteases and MMP-12. For cathepsin-K, a mixture of 2 mM EDTA and 2 mM DTT in 20 mM sodium acetate, pH 5.0, was used [34] and for MMP-9, 12.5 mM Tris, pH 7.5, 1.5 mM $CaCl_2$, 33 mM NaCl, 0.015% Brij was used.

After digestion, visual examination of the solutions revealed that only a small part of insoluble elastin had been solubilized, therefore the resultant digest was centrifuged for 5 min at 4000 rpm and 10 μl of the supernatant was injected in the HPLC-UV-ESI-MS system.

Fig. 1 shows the base peak chromatogram obtained in each case. It is clearly observed that each protease generates a different chromatographic profile, confirming that each enzyme has its particular elastolytic activity. Cathepsin-K generates preferentially smaller peptides than the other enzymes.

The main peaks detected in each profile were submitted to MS/MS analysis for peptide identification. Although chromatographic peaks with the same parent masses were found in some cases, the MS/MS spectra confirmed that they corresponded to different peptide sequences (see Fig. 2). In Table 1 the elastin peptides identified after digestion with each protease are listed. It is interesting to observe that digestion of elastin with MMP-9 under these conditions did not result in detectable amounts of small peptides when analyzed by LC/MS.

The influence of the contact time between the enzyme and the elastin substrate was studied. Longer incubation times resulted in higher concentrations of peptides produced but no new peptides were generated, indicating the completeness of the reaction under these conditions (data not shown).

As expected, incubation of elastin with increasing amounts of proteolytic enzyme resulted in the appearance of new peptides in the chromatogram. In Fig. 3 the chromatograms obtained after digestion with two different ratios of HNE (1/1000 and 1/500 enzyme/protein (w/w)) illustrate this observation.

The digestion of elastin with different proteases was performed again utilizing a higher ratio of protease/elastin (1/200) to compare the identity of the resulting peptides with those obtained with less enzyme (Table 2). Comparing Tables 1 and 2 reveals that cathepsin K produces smaller peptides than the other enzymes with a marked preference to cleave after glycine. Macrophage elastase (MMP-12) on the other hand produces longer peptides with a stronger preference to cleave after alanine than any of the other enzymes. The two serine proteases (HNE and pancreatic elastase) behave similarly to each other with a marked trend to cleave after valine and glycine. This is in partial agreement with the results obtained by Mecham et al. [35], who studied the elastolytic preferences of MMP12 and HNE by sequencing the newly generated N termini after digestion of insoluble elastin. They reported a marked preference of HNE for glycine and alanine and less for valine and they found in the case of

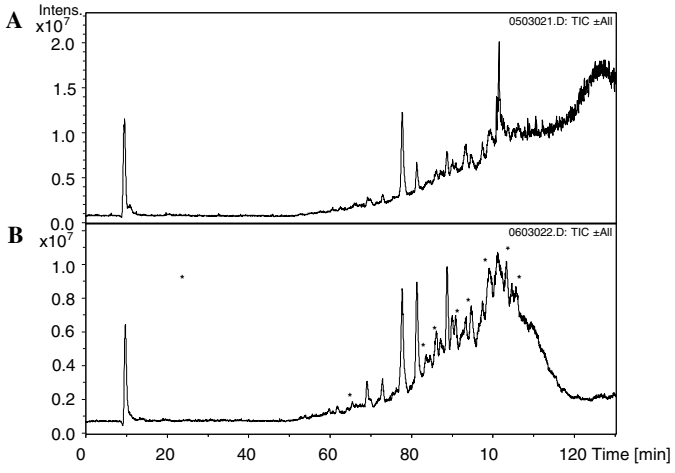

Fig. 3. Digestion of human elastin with different ratios of HNE. (A) 1/1000 enzyme/protein; (B) 1/500 enzyme/protein (w/w). As expected, an increase in the amount of elastase generates more peptides (marked with ∗).

Table 2
Peptides obtained after digestion of human lung elastin with different elastases at a ratio 1/200 (w/w) (enzyme/elastin)

m/z	*aa sequence*
Cathepsin-*K*	
345.4 (+)	(G)**LGVG**(V) aa 582-585 or
	(G)**LGVG**(A) aa 591-594 or
	(G)**LGVG**(A) aa 600-603 or
	(G)**LGVG**(G) aa 712-715
442.4 (+)	(G)**VGLPG**(V) aa 144-148 or
	(G)**VGIPG**(G) aa 674-678
456.5 (+)	(A)**IPGIG**(G) aa 289-293 or
	(G)**IPGLG**(V) aa 579-583
473.4 (+)	(G)**ALGGVG**(I) aa 670-675
501.5 (+)	(G)**LGVGVG**(V) aa 582-587
541.5 (+)	(G)**VGVLPG**(V) aa 163-168
581.5 (+)	(G)**IPVVPG**(A) aa 351-356
584.4 (+)	(G)**AGIPGLG**(L) aa 577-583
709.5 (+)	(G)**AGIPVVPG**(A) aa 349-356
726.5 (+)	(G)**AGVPGLGVG**(A) aa 573-2-580
Pancreatic elastase	
328.5 (+)	(G)**VPL**(G) aa 273-275
416.5 (+)	(A)**GLGGL**(G) aa 708-712 or
	(G)**GLGGI**(P)aa 724-728
485.5 (+)	(S)**VGGVPG**(V) aa 423-428 or
	(G)**VGGVPG**(V) aa 429-434 or
	(V)**GVGVPG**(L) aa 585-590
515.4 (+)	(G)**LGGVLG**(G) aa 744-749 or
547.4 (+)	(G)**ARFPG**(V) aa 158-162
323.7 (+2)	(G)**ARFPGV**(G) aa 158-163
352.4 (+2)	(G)**ARFPGVG**(V) aa 158-164
401.7 (+2)	(G)**ARFPGVGV**(L) aa 158-165
456.7 (+2)	(G)**VGPFGGPQPG**(V) aa 192-201
Neutrophil elastase	
328.4 (+)	(G)**VPL**(G) aa 202-204
331.4 (+)	(S)**VGGV**(P) aa 423-426 or
	(G)**VGGV**(P) aa 429-432
345.4 (+)	(G)**VGGL**(G) aa 714-717 or 722-725
485.4 (+)	(G)**GVPGGV**(F) aa 36-41
499.4 (+)	(G)**VAPGVG**(V) aa 508-513,
	514-519,532-537,538-543 or
	(G)**VAPGVG**(L) aa 520-525
598.4 (+2)	(G)**GPQPGVP**(L) aa 197-203
323.7 (+2)	(G)**ARFPG**(G)aa 158-163
362.7 (+2)	(V)**APGIGPGGV**(A) aa 545-553
394.7 (+2)	(V)**AARPGFGL**(S) aa 760-767
557.3 (+2)	(A)**VTFPGALVPGGV**(A) aa 85-96
Macrophage elastase (MMP-12)	
416.4 (+)	(G)**IAGVG**(T) aa 295-299
449.4 (+)	(G)**YGLP**(Y) aa 217-220
321.2 (+2)	(G)**WGAGPAA**(A) aa 680-687
326.2 (+2)	(G)**GPQPGVP**(L) aa 197-203
655.4 (+)	(G)**LGVPGVGG**(L) aa 717-724
662.5 (+)	(P)**AVTFPGA**(L) aa 84-90
726.5 (+)	(A)**LGGVGIPGG**(V) aa 671-679
754.5 (+)	(G)**VGVGVPGLG**(V) aa 584-592
463.5 (+2)	(G)**WGAGPAAAAAA**(A) aa 680-691
476.1 (+2)	(G)**VFYPGAGLGA**(L) aa 41-50
506.5(+2)	(A)**LVPGGVADAAAA**(Y) aa 91-102

MMP-12 a higher preference for leucine/isoleucine followed by alanine and glycine.

Influence of ionic strength on the elastolytic process

Several reports [36–39] showed that activity of HNE was significantly enhanced by increased ionic strength, particularly over the range 200–750 mM NaCl. Our data indicate that, in the presence of HNE at a 1:100 (w/w) ratio, elastin is degraded at increasing rates with increasing salt concentration with a remarkable increase between 128 and 536 mM. Interestingly, despite the fact that some of the published literature [36,39] reported that no elastolysis occurred below 200 mM NaCl, we did find peptides when elastin was incubated with HNE, even with no addition of salt. This difference may be due to the fact that MS-based detection is much more sensitive than the techniques that were previously used. These results confirmed the observation done by Boudier et al. [36] that the activity of HNE is strongly enhanced by elevated ionic strength and we deduced that, for a relevant study of elastolysis, trying to emulate in vivo situations, a physiological salt concentration should be used.

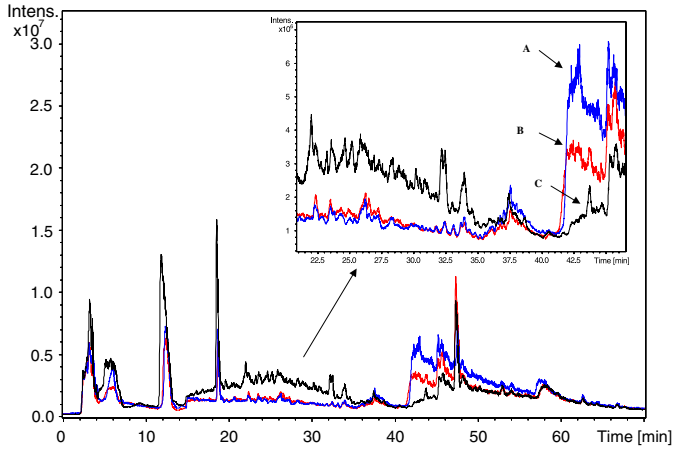

Fig. 4. Total ion current chromatograms corresponding to (A) lung tissue homogenate supernatant, (B) lung tissue digested with neutrophil elastase ratio 1/200 (w/w) enzyme/tissue, and (C) ratio enzyme/tissue 1/20 (w/w). The major chromatographic peaks eluting between 41 and 48 min diminish with increasing enzyme to tissue ratio while earlier eluting, smaller peaks appear between 20 and 35 min as observed in the enlarged inset. Some of these peaks were further identified by MS/MS as elastin-derived small peptides (see Figs. 5 and 6).

Digestion of lung tissue

Further experiments were performed to determine whether elastin could also be degraded into small peptides directly in lung tissue, where it is part of an intricate structural network together with collagens, glycoproteins, proteoglycans, and other extracellular matrix components.

A piece of lung tissue (1.08 g) was homogenized in 8 ml of ammonium bicarbonate buffer. Aliquots of 100 µl of homogenized lung tissue were transferred to a new tube and 0.5 and 5 µg of HNE were added. Considering the percentages of water and elastin in lung tissue [40] these amounts correspond roughly to 1:200 and 1:20 (w/w) enzyme/elastin ratios, respectively.

Samples incubated with different concentrations of HNE were separated by SDS–PAGE, and subsequently transferred to a PVDF membrane for Western blot analysis using a polyclonal antibody against elastin. We observed that the resolution of elastin peptides was limited in agreement with earlier reports showing that elastin-derived peptides are difficult to resolve by SDS–

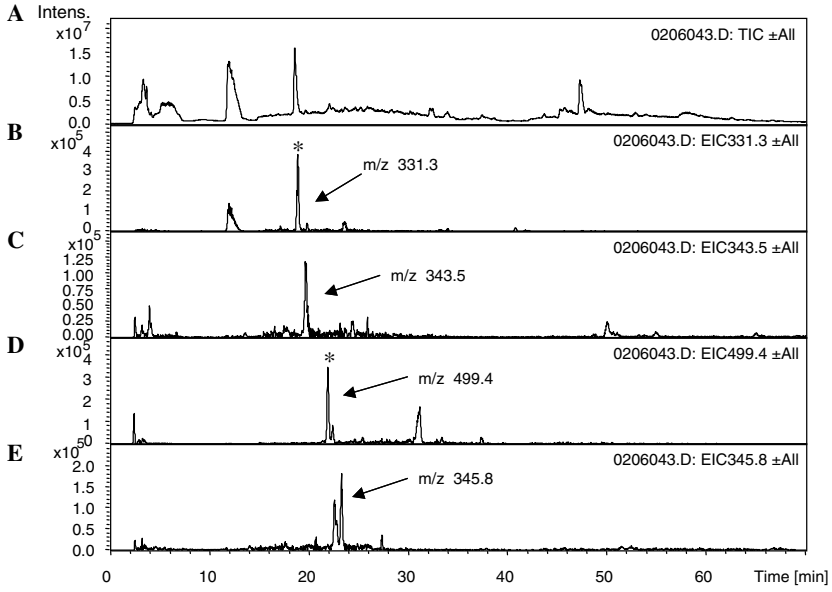

Fig. 5. LC/MS chromatogram corresponding to the digestion of lung tissue homogenate with neutrophil elastase at a ratio 1/20 enzyme/tissue (w/w). (A) TIC chromatogram; (B to E) extracted ion chromatograms corresponding to different *m/z* values corresponding to elastin-derived peptides. The peaks marked with * correspond to the peptides identified by MS/MS in Fig. 6.

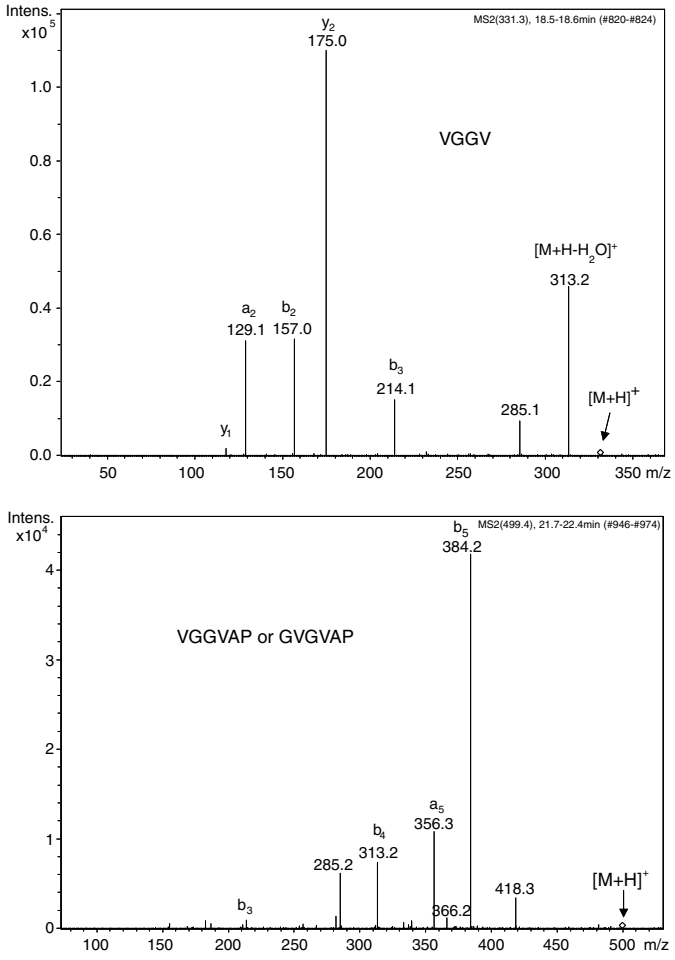

Fig. 6. MS/MS spectra corresponding to two of the peptides detected in human lung tissue digested with HNE (see Fig. 5; *m/z* 331.3, sequence VGGV, and *m/z* 499.4, sequence VGGVAP or GVGVAP). All peptides were identified taking into account their molecular weight and amino acid sequence deduced from their MS^2 spectra.

PAGE [39], resulting mainly in a smear containing some diffuse bands with higher protein density. Protein staining with Coomassie blue did not show any obvious differences between the samples treated with different concentrations of HNE. However, a clear increase in degraded elastin (appearance of bands with lower molecular weight) with increasing amounts of HNE was visible by Western blot (data not shown). It is clear that elastin-derived peptides are released from whole lung tissue upon digestion with HNE in a dose-dependent manner. Remarkably, a weak signal was observed even without addition of exogenous HNE. This low quantity of elastin-derived peptides may be attributed to endogenous elastolytic activity.

For a more detailed study of the released peptides, aliquots of the same lung tissue digests were analyzed by LC/MS. Fig. 4 shows in a comparative way the TIC chromatograms corresponding to lung tissue homogenate supernatant digested with HNE at different enzyme/tissue ratios. A simple visual inspection reveals that the major chromatographic peaks eluting between 41 and 48 min disappear after digestion and small peaks appear between 20 and 35 min. All peaks in the chromatograms were submitted to automatic MS/MS and the fragmentation patterns analyzed. Several of the small peaks found exclusively in the digested lung tissue samples were identified as elastin-derived small peptides based on their molecular weight and amino acid sequence deduced from their tandem MS spectra (Figs. 5 and 6): *m/z* 331 (VGGV), *m/z* 343 (GVAP), *m/z* 499 (VGGVAP or GVGVAP), and *m/z* 345 (VGGL). Some of these peptides (e.g., *m/z* 331 (VGGV) and *m/z* 345 (VGGL) were matched to those obtained after in vitro digestion of human lung elastin with neutrophil elastase (see Table 2). Based on the amino acid sequence and data base searching it cannot be excluded that some of these peptides could also correspond to collagen because they have exactly the same sequence.

Further work is needed to correlate these digestion patterns with in vivo proteolytic activity, for example screening of biological fluids during the development of pulmonary emphysema.

Conclusion

It has been known for a long time that degradation of elastin into elastin-derived peptides occurs in diseases involving destruction of extracellular matrix components, such as emphysema associated with COPD. Recently attention has also been paid to the degradation of elastin in vascular diseases involving pathology of the arteries such as atherosclerosis [41]. Several studies report an increase of circulating elastin-derived peptides measured using immunoassays in various diseases (e.g., specific manifestations of atherosclerosis and macular degeneration [42,43]). However, identification of these peptides has never been performed.

In our study we show that LC MS is a powerful technique to study in greater detail elastin degradation by proteases belonging to different families. The study focused on small peptides, which were sequenced by tandem MS in view of using them as well-characterized biomarkers for extracellular matrix degradation.

Elastin-derived peptides constitute a challenge from the analytical point of view. Their hydrophobic character together with the low abundance of positively charged amino acids renders their efficient ionization and fragmentation difficult. Moreover, the repetitive nature of the amino acid sequences in this biopolymer makes it difficult to assign the identified peptides to unique positions in elastin. Nevertheless, many of the identified peptides provide a fingerprint of the respective protease at work. Future work is needed to determine how far the results from in vitro degradation can be translated to the in vivo situation.

The approach will involve the screening of different biological fluids from patients suffering from emphysema and looking for the presence of any of these peptides. The first biofluid will be BALF due to its proximity with the lung.

However, due to its invasive nature, it should be replaced by others such as induced sputum or exhaled breath condensate once some biomarkers have been identified.

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