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Proteinase 3 hydrolysis of peptides derived from human elastin exon 24

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Summary. In normal and pathological tissues, elastin-derived peptides proceed of elastin degradation by polymorphonuclear leukocyte proteases: elastase, cathepsin G and proteinase 3. They were demonstrated to have a chemotactic activity, to promote cell proliferation and protease release, …. To be biologically active, their structures, which reflect elastase specificity, must adopt a β-turn conformation which accommodate to the cell surface-located elastin binding protein. In this study, we establish that human elastin exon 24-derived peptides containing at least two repeated VGVAPG sequences are hydrolyzed by the proteinase 3 (Pr3). As shown by mass spectrometry analyses, the demonstrated cleavage sites are in agreement with previously reported Pr3 substrate specificity and its lengthy substrate binding site. The characterization of the Pr3-generated products indicate that they contain at least one GXXPG sequence known to stimulate cellular effects after binding to the elastin receptor.

Keywords: Elastin peptides – Exon 24 – Proteinase 3 – Hydrolysis – GVAPGV

Abbreviations: Boc, Butyloxycarbonyle; ESI, Electrospray ionisation; Fmoc, 9-Fluoromethoxycarbonyle; HNE, Human neutrophil elastase; HOBt, 1-Hydroxybenzotriazole; MMP, Matrix metalloproteinase; PMNL, Polymorphonuclear leukocytes; Pr3, Proteinase 3

1 Introduction

Leukocyte proteinase 3 (myeloblastin; EC 3.4.21.76) is a neutral serine endopeptidase stored in the azurophilic granules of polymorphonuclear leukocytes (PMNL) (Baggiolini et al., 1978; Kao et al., 1988).

Its amino acid composition is similar to those of human neutrophil elastase (HNE) and cathepsin G, except a lower amount of arginine. A 60% identity to HNE and cathepsin G may be observed in the *N*-terminal sequence (Rao et al., 1991a). It may hydrolyze various proteins, including structural proteins such as elastin, fibronectin, laminin, vitronectin and type IV collagen (Rao et al., 1991b).

Proteinase 3 (Pr3) and HNE have similar P_1 specificities, Ala and Val leading to the highest k_{cat}/K_M values (Koehl et al., 2003) when catalytic activities are compared, using acyl-tetrapeptides-*p*-nitroanilides. Whatever the substrate used, activities are generally much lower for Pr3 than for HNE (Früh et al., 1996).

Analysis of degradation products from oxidized insulin (Rao et al., 1991a) confirmed a P_1 and P'_1 specificity for small (aliphatic or non charged) amino acids (A, V, L, S). On the other hand, hydrolysis of various synthetic fluorogenic peptides with intramolecularly quenched fluorescence demonstrated that various amino acids may be in the P'_1 position, including Lys (Koehl et al., 2003), Met and Cys (Korkmaz et al., 2002). Recently, Korkmaz et al. took advantage of small differences in S'_2 subsites of both Pr3 and HNE to prepare specific substrates for each of these proteases (Korkmaz et al., 2004). Nevertheless, it is likely that there is an important adaptability of substrates in the active site, if lateral chains are not too bulky. Elongation of the substrate strongly increases the catalytic efficiency of Pr3 (Koehl et al., 2003). The most important structural and enzymatic properties of Pr3 have been recently reported (Rao and Hoidal, 2004).

Direct measurements of Pr3-catalyzed elastin hydrolysis were realized (Kao et al., 1988; Ying and Simon, 2002) but no data exist concerning the cleavage sites and the hydrolyzed and solubilized peptides. Indeed, elastin-derived peptides (also called matrikines) were demonstrated to play

an important role in normal and pathological extracellular matrixes. They may induce monocyte and PMNL activation (Fülöp et al., 1986), apoptosis (Péterszegi et al., 1999), be chemotactic for fibroblasts (Senior et al., 1984) and stimulate their proliferation (Kamoun et al., 1995). Two recent reviews described their implication in tumor invasion (Maquart et al., 2004; Duca et al., 2004). Among these peptides, three hydrophobic ones were studied in detail. VGVAPG, which is repeated several times in elastin sequence, is, among many other effects, able to stimulate matrix metalloproteinases MMP-2 (Brassart et al., 1998; Ntayi et al., 2004) and MMP-1 (Brassart et al., 2001) expressions and to induce myofibrillogenesis (Karnik et al., 2003). PGAIPG, another repeated hexapeptide of human elastin, is chemotactic for fibroblasts (Grosso and Scott, 1993a), neutrophils and M27 tumor cells (Grosso et Scott, 1993b) and stimulate MMP-1 in cultured fibroblasts (Brassart et al., 2001). Contradictory results were obtained concerning the ability of VPGVG to stimulate cell proliferation (Wachi et al., 1995) and to decrease elastin production (Tajima et al., 1996; Tajima et al., 1997). It was proposed that these effects occur via an elastin receptor, the 67-kDa elastin binding protein.

In vivo, VGVAPG and other matrikines could result from elastin hydrolysis by various elastases acting individually or synergistically. In this paper, we study the possibility to obtain such peptides after Pr3-catalyzed splitting of peptides derived from the human elastin exon 24-encoded product, $LV(PGVGVA)_3PGVGLA(PGVGVA)_3$ PAIG.

2 Material and methods

2.1 Peptide synthesis

Peptides were synthesized using 9-fluoromethoxycarbonyl (Fmoc) chemistry, from a Fmoc-Val-resin (0.39 meq/g). Couplings were performed with a four molar excess of Fmoc-amino acid-pentafluorophenyl esters (Novabiochem, France), in presence of four molar excess of HOBt. Each Fmoc deprotection step involved a treatment with 20% piperidine/dimethylformamide for 2×10 minutes. Cleavage of the peptides from the resin was achieved by a 2×30 min treatment with trifluoroacetic acid/water (95:5, v/v). After solvent evaporation, peptides were precipitated and extensively washed with diethyl ether at 4°C. Purity of peptides was confirmed by HPLC and by electrospray mass spectrometry (ESI).

2.2 Peptide enzymatic hydrolysis

Peptide hydrolyses were performed in a 5 mM Tris buffer, pH 8.6, at room temperature, with human proteinase 3 (Elastin Products Co, USA). Peptide and enzyme concentrations were $2\,mg \cdot mL^{-1}$ and $0.51\,\mu M$ (specific activity: $50.2\,U \cdot mg^{-1}$, with Boc-Ala-Ala-Nva-SBzl as substrate), respectively.

2.3 Analysis of hydrolysis products

Hydrolysis products were separated by reverse phase HPLC on a C18 column (LiChrospher® 100; 4 mm × 25 cm) using a Waters system consisting of a binary pump (Waters 1525), a diode-array detector (Waters 2996) controlled by Millenium 2010 software. Peptides ($0.8\,mg \cdot mL^{-1}$ in TFA 0.025%) were eluted with a linear (0–100%, v/v) gradient of acetonitrile in 0.05% trifluoroacetic acid for 45 minutes. Eluted peaks were monitored at 214 nm.

Mass spectra were acquired on a ThermoFinnigan LCQ Advantage ion trap instrument, detecting positive (+) ions in the ESI mode. Samples (1 to $10\,mg \cdot mL^{-1}$ in methanol:dichloromethane:water, 45:40:15, v/v/v) were infused directly into the source ($5\,mL \cdot min^{-1}$) using a syringe pump. The following source parameters were applied: spray voltage 3.0–3.5 kV, nitrogen sheath gas flow 5–20 arbitrary units. The heated capillary was held at 200°C. MS^n spectra were obtained by applying a relative collision energy of 25 to 40% of the instrumental maximum.

3 Results

We synthesized five peptides from the repeating sequence of human elastin exon 24. In order to keep their *C*-terminal extremities blocked, we added a *C*-terminal valine, corresponding to the first amino acid of the next repeating sequence. The peptides were characterized by HPLC and ESI mass spectrometry (Table 1). All five have a purity higher than 98%.

No hydrolysis by Pr3 could be evidenced for peptides 1 and 2, after 1-day incubation. In the same conditions, peptides 3, 4 and 5 were hydrolyzed.

The HPLC profile obtained for peptide 3 hydrolysate is illustrated in Fig. 1, demonstrating the presence of two new peaks. The ESI mass spectrum indicated the presence of three peptides, at m/z 499.3, 598.3 and 1078.5, the last one corresponding to the intact peptide. Addition of the two other masses corresponding to that of peptide 3, there is only one cleavage site, but, due to the sequence, two possibilities exist: one leading to (VGVAPGV + GVAPGV) and the other to (VGVAPG + VGVAPGV). MS/MS spectrum of peak 499.3 unambiguously identifies the peptide GVAPGV (Fig. 2), demonstrating the following cleavage site:

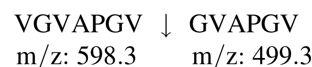


Table 1. Characterization of the synthesized peptides. Retention times (t_R) of peptides were determined by RP-HPLC on a C18 column with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid (flow $1\,mL \cdot min^{-1}$). Experimental masses were calculated from electrospray analyses. M_r (exp.) and M_r (calc.) are experimentally determined and calculated molecular masses, respectively

	Peptide	t_R (min)	M_r (exp.)	M_r (calc.)
1	VGVAPGV	13.6	597.2	597.7
2	VAPGVGVAPGV	14.1	921.4	922.1
3	$(VGVAPG)_2V$	15.7	1077.5	1078.2
4	$APG(VGVAPG)_2V$	15.8	1302.6	1303.5
5	$(VGVAPG)_3V$	16.5	1557.6	1558.8

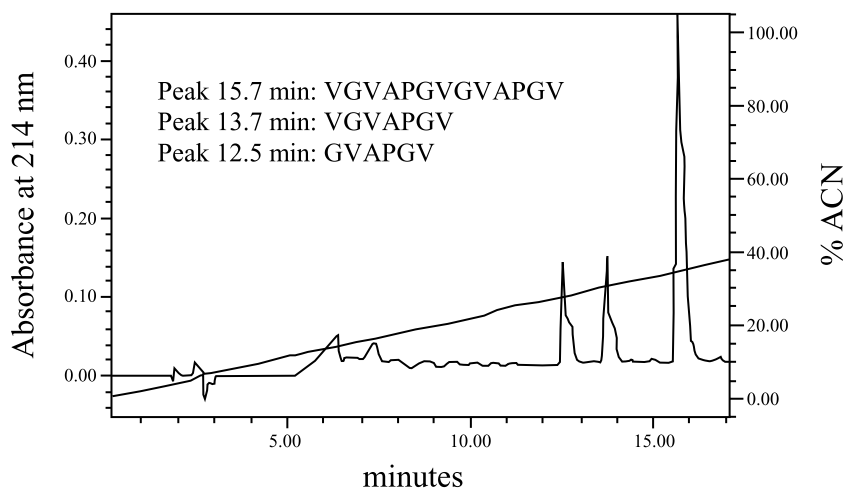


Fig. 1. Profile of chromatographic separation of $(VGVAPG)_2V$ (peptide 3) and its products, VGVAPGV and GVAPGV, obtained after 1 day-hydrolysis by Pr3. Peptide 3 ($2\,mg \cdot mL^{-1}$) was incubated with Pr3 ($0.51\,\mu M$) in 5 mM Tris buffer pH 8.6 at room temperature. Peptides were separated by RP-HPLC on a C18 column with a linear gradient of acetonitrile in 0.05% trifluoroacetic acid (flow $1\,mL \cdot min^{-1}$)

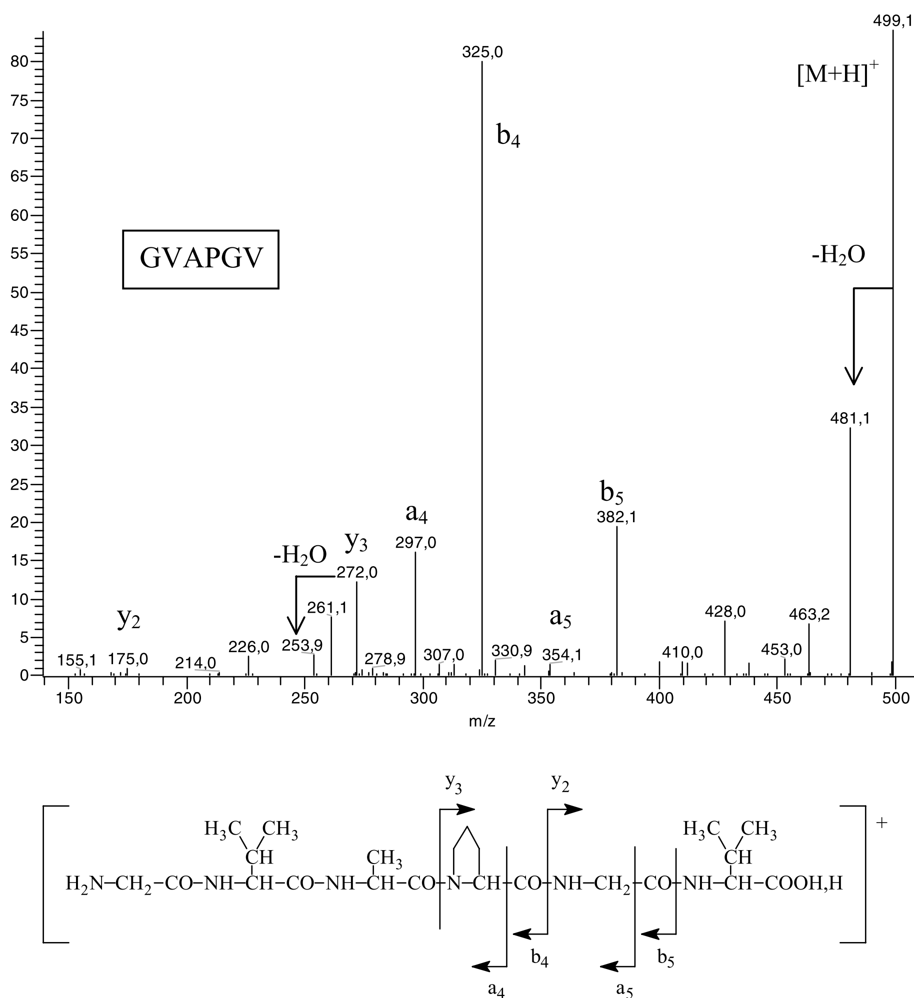
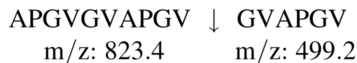


Fig. 2. MS/MS identification of the Pr3-catalyzed cleavage site of $(VGVAPG)_2V$ (peptide 3) MS/MS spectrum of peak 499.3 was obtained by ESI analysis. It indicates fragments a_4, a_5, b_4, b_5, y_2 and y_3 which may be attributed to GVAPGV

A similar result was obtained with peptide 4. Apart from the intact peptide, two other peaks were generated after Pr3 hydrolysis, corresponding to m/z 499.2 and 823.4. MS/MS analysis also confirmed a cleavage between V and G:



The hydrolysis pattern of peptide 5 is a little bit more complex. After a 24-hour incubation with Pr3, four new peaks with similar areas could be evidenced, corresponding to the m/z values of 499.3, 598.4, 979.4 and 1078. Considering the complementarity of $(1078 + 499.3)$ and of $(598.4 + 979.4)$, we may suggest that two independent cleavage sites occur:

Whereas m/z of 1078 corresponds to peptide 3, MS/ MS spectrum of the fragment at m/z 979.4 confirms our hypothesis (Fig. 3). The four peaks at 637.3, 708.3, 752.3 and 823.7 may be unambiguously assessed to b_8, b_9, y_9, y_{10} of the peptide GVAPGVGVAPGV, respectively, according to Biemann's nomenclature (Biemann, 1990).

This result also indicates that, at least initially, these peptides are independently generated by Pr3, as peak areas increase in parallel with time during the first two days. Nevertheless, after extensive hydrolysis (up to five days), secondary cleavage of the longest peptides could be evidenced, leading to the kinetic pattern described in Fig. 4.

Then, we studied the kinetics of hydrolysis of peptides 4 and 5. For peptide 4, at substrate concentrations of 0.75 mM, 1.14 mM and 1.53 mM, a first-order kinetics was evidenced, indicating that $[S] < K_M$, and the calculated first-order constant was $(6.2 \pm 1.6) \cdot 10^{-3}\,h^{-1}$. For peptide 5, as two cleavage sites were demonstrated, we measured both the decrease of substrate peak area and the increase of product peak areas. In both cases, at substrate concentrations of 0.58 mM, 0.94 mM and 1.30 mM, first-order kinetics were also demonstrated, at least within the first two days of hydrolysis. Considering the substrate, the first-order constant of hydrolysis was $(12.6 \pm 2.0) \cdot 10^{-3}\,h^{-1}$. For VGVAPGVGVAPGV formation, the k_1 constant was $(4.9 \pm 0.8) \cdot 10^{-3}\,h^{-1}$ and for GVAPGVGVAPGV, the k_2 constant was $(6.1 \pm 0.9) \cdot 10^{-3}\,h^{-1}$. These values agree well with the model proposed on Fig. 4 as, when the

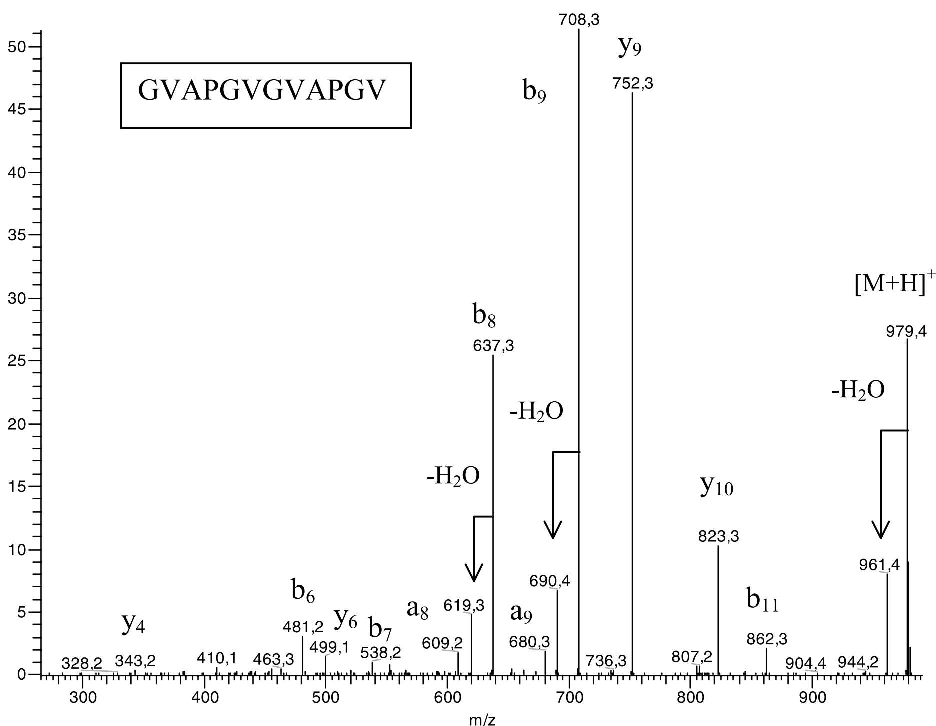


Fig. 3. MS/MS identification of a second Pr3-catalyzed cleavage site of $(VGVAPG)_3V$ (peptide 5). MS/MS spectrum of peak 979.4 was obtained by ESI analysis. It indicates fragments which derived from GVAPGVGVAPGV

VGVAPGVGVAPGVGVAPGV
k_1 k_2
VGVAPGVGVAPGV + GVAPGV VGVAPGV + GVAPGVGVAPGV
k_3 k_4
VGVAPGV + GVAPGV 2 GVAPGV

Fig. 4. Kinetic pattern of hydrolysis of VGVAPGVGVAPGVGVAPGV (peptide 5) by Pr3 showing two independent cleavage sites

substrate hydrolysis may be considered as the result of two independent cleavages, the apparent first-order constant $k = k_1 + k_2$, which is the case in our study. Plotting the data of product formation *vs* time for longer hydrolysis allowed us to confirm the further splitting of VGVAPGVGVAPGV and GVAPGVGVAPGV, but k_3 and k_4 constants could not be precisely estimated.

4 Discussion

Elastolysis by proteinase 3 was previously evidenced and compared with that of HNE and cathepsin G (Kao et al., 1988; Ying and Simon, 2002). Quite pH dependent, at neutral pH, elastolysis rates by Pr3 were lower than with HNE, the values ranging from 12% (Ying and Simon, 2002) to 50% (Kao et al., 1988). Nevertheless, considering that Pr3 is the most abundant serine proteinase in neutrophils, it may play a significant role in proteolytic tissue destruction, even if it has been observed that Pr3 is nearly completely inhibited by α_1-proteinase inhibitor in non pathological conditions (Rao et al., 1991b; Duranton and Bieth, 2003).

Recently, *in vitro* studies with synthetic substrates gave detailed information on Pr3 specificity and allowed the authors to design highly specific substrates (Korkmaz et al., 2004). Their results demonstrated the importance of a negatively charged residue in P'_2. The presence of an arginine at P'_3 does not change the k_{cat}/K_M constant for Pr3, but protects against HNE activity.

These results are quite puzzling, as they are not in agreement with the elastolytic activity of Pr3, insoluble elastin being quite exclusively constituted of apolar residues.

Our data demonstrate that Pr3 may hydrolyze some apolar sequences derived fom the human elastin exon 24, but their lengths have a significant importance. As seen in Fig. 5, the cleavage only takes place between Val and Gly. This corroborates the specificity of Pr3 for small aliphatic amino acids in P_1 and P'_1, previously reported by Koelh et al. (2003) and Rao et al. (1991a). In this model, the proline residue fills the S_3 enzyme subsite without preventing its activity which is in agreement with a broad specificity to P_3 residue as suggested by structural analyses (Fujinaga et al., 1996).

We may notice that, despite the presence of alanine in synthesized peptides, no splitting was evidenced on the carboxyl end of this amino acid, probably because a proline residue is coming immediately after alanine.

Furthermore, cleavage at Val-Gly bonds occurs only if there are more than five amino acids on the *N*-terminal side (P_i residues). Because all peptides contain at least two GV sequences, one could postulate that a second hydrolysis site might be observed at GV ↓ A position allowing a greater interaction between enzyme and substrate both on P and P′ sides, especially for peptides 1 and 2. But, in this case, a proline residue would occupy the P'_2 position which is definitively unpropitious to enzymatic activity as mentioned earlier (Korkmaz et al., 2004).

An extended substrate binding site (larger than the $S_4 - S'_3$ mentioned by Fujinaga et al., 1996) may also explain why only peptides 3, 4 and 5 are hydrolyzed and why secondary cleavages may only be observed with peptide 5. This is also probably why it was observed (Rao et al., 1991b) that fibronectin is hydrolyzed in seven different fragments (two of which being between 200–220 kDa), HNE and cathepsin G leading to much lower masses.

Considering the peptides generated after hydrolysis, all contain at least one GXXPG sequence which was reported to have a folded β-turn conformation, a motif necessary for binding to the elastin receptor (Duca et al., 2004; Floquet et al., 2004). In a recent paper, it was mentioned that pro-MMP-1 (and pro-MMP-3) was stimulated by such peptides, the highest efficiency being observed with GVAPGV sequence (Brassart et al., 2001). In our work, we showed that this peptide may be generated by Pr3 from VGVAPG repeating sequences derived from elastin exon

Peptide 1 V GVAPGV
Peptide 2 VAPGV GVAPGV
Peptide 3 V GVAPGV | GVAPGV
Peptide 4 APGV GVAPGV | GVAPGV
Peptide 5 V GVAPGV | GVAPGV ¦ GVAPGV
VGVAPGV ¦ GVAPGV | GVAPGV

Fig. 5. Comparison of the cleavage sites of peptides 1 to 5. Primary cleavage sites are indicated by ⟶ and secondary cleavage sites by - ->

24 peptide. The biological effects of this peptide demonstrated with cell cultures should then be considered *in vivo*, when an imbalance between neutrophil serine proteinases and α_1-proteinase inhibitor occur, allowing then proteolysis of extracellular matrix proteins, and, in particular, elastolysis.

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