The specificity of macrophage elastase on the insulin B-chain

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The specificity of macrophage elastase obtained from mouse peritoneal exudative macrophages was determined in the hydrolysis of the oxidized insulin B-chain. This elastase hydrolysed two bonds, namely Ala–Leu and Tyr–Leu. The rate of hydrolysis of the latter was two to three times greater than that of the former. The hexapeptide Glu–Ala–Leu–Tyr–Leu–Val, obtained by cleavage of the insulin B-chain, was not hydrolysed by macrophage elastase. When EDTA was present, proteolysis of the B-chain was not observed. The macrophage elastase is therefore different from the neutrophil elastase in specificity and mechanism.

Proteolysis of lung connective tissue is probably responsible for the pathogenesis of pulmonary emphysema (Johanson & Pierce, 1972; Kaplan *et al.*, 1973; Pump, 1974). Elastolytic proteinases have been implicated in this process (Blackwood *et al.*, 1973), and neutrophils and alveolar macrophages are generally regarded as the likeliest sources of such enzymes in the lung (Janoff, 1972; Rosenberg *et al.*, 1972; Rodriguez *et al.*, 1977). However, neutrophils and macrophages produce elastases that are distinctly different. The macrophage elastase is a metalloenzyme (Werb & Gordon, 1975; White *et al.*, 1980), whereas neutrophil elastase is a serine proteinase (Janoff and Zeligs, 1968). The relative contribution of each of these enzymes in emphysema is still uncertain. For example, whereas the neutrophil enzyme is present in considerably larger quantities than the macrophage elastase, the latter is more resistant to anti-proteinases (White *et al.*, 1980; Janoff *et al.*, 1979).

In view of the possible contribution of macrophage elastase to lung disease, it would be desirable to have further knowledge of the properties of this enzyme. Although considerable information is known concerning the specificity of the neutrophil elastase, no such data are available on the macrophage enzyme. The insulin B-chain is a reference substrate for comparison of proteinase specificities since it has been used with a variety of enzymes.

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; SDS, sodium dodecyl sulphate.

These include both neutrophil (Blow, 1977) and pancreatic (Narayanan & Anwar, 1969) elastase.

In specificity studies with the oxidized insulin B-chain as a substrate for the neutrophil elastase, Blow (1977) observed that several of the major cleavage sites occurred at bonds where the P₁ residue (using the notation of Schechter & Berger, 1967) was valine. This observation led to the development of highly specific inhibitors (Powers *et al.*, 1977) and substrates (Castillo *et al.*, 1979; Nakajima *et al.*, 1979) for neutrophil elastase.

We here report the use of the insulin B-chain to study the specificity of the macrophage elastase. As this elastase is available only in small quantities, we have separated the hydrolysis products of the B-chain by h.p.l.c., since this technique is adaptable to nanomolar amounts of peptides.

Materials and methods

Mouse macrophage elastase was purified from serum-free culture medium conditioned by thio-glycollate-elicited peritoneal macrophages. The purification procedure was described previously (White *et al.*, 1980). The enzyme preparation was further purified by ion-exchange chromatography on DEAE-Sephadex A-50 equilibrated at 4°C with 0.05 M-Tris/HCl, pH 8.35, containing 0.05 M-NaCl and 0.005 M-CaCl₂. In a final purification step, gel filtration was performed on Sephadex G-50 (Superfine grade) equilibrated with the same buffer.

Neutrophil elastase was prepared by the pro-

cedure of Feinstein & Janoff (1975). Elastase activity was measured on agar plates containing particulate SDS-treated elastin (Werb & Gordon, 1975; White *et al.*, 1977). Elastolytic activity of the macrophage enzyme, determined by lysis zones on the agar plates, was compared with the activity of neutrophil elastase run in adjacent wells of the same plate. The activity of macrophage elastase was then expressed in units of concentration of the neutrophil enzyme.

The oxidized insulin B-chain (Boehringer-Mannheim) was digested for 5 h at 37°C with 0.67 mM-substrate and 0.4 µg of elastolytic activity/µl. The reaction was conducted in 50 mM-Tris/maleate buffer, pH 7.6, containing 5.0 mM-CaCl₂. The components of the digestion solution were separated by h.p.l.c. on a column (4.6 mm × 250 mm) packed with Lichrosorb RP-18 resin (10 µm particle size; Merck) by using Laboratory Data Control Constametric II pumps and a Gradient Master. Linear gradients from 0 to 40% acetonitrile in 20 mM-sodium phosphate buffer, pH 5.6, were developed over a period of 57 min at a flow rate of 2.0 ml/min. The elution pattern of the chromatogram was determined at 210 nm on a Spectromonitor II and fractions were collected manually. Fractions were evaporated to dryness and were hydrolysed with 6.0 M-HCl in sealed evacuated tubes for 2 h at 150°C. Amino acid analyses were performed on a column (0.63 cm × 24 cm) packed with Aminex A7 resin. Glu-Ala-Leu-Tyr-Leu-Val was prepared by digestion of the insulin B-chain (135 nmol) with neutrophil elastase (6 µg) in 0.20 ml of 0.10 M-NH₄HCO₃ buffer, pH 8.5, for 2 h at 37°C. The peptide was isolated in a yield of 30% by h.p.l.c., under the conditions described above, except that 10 mM-ammonium acetate buffer, pH 5.6, was substituted for the phosphate buffer and peptides were detected at 220 nm. The peptide was eluted from the column in 22 min, concentrated and freeze-dried. The following amino acid composition for the peptide was obtained: Glu (1.1), Ala (1.0), Leu (1.9), Tyr (0.66), and Val (1.0).

Results and discussion

Because of their availability in large numbers and their long period of viability in culture (White *et al.*, 1980), mouse peritoneal exudative macrophages were used as a source of macrophage elastase in the present studies. The specificity of the macrophage elastase in the hydrolysis of the oxidized insulin B-chain was determined by incubating the proteinase with the substrate at 37°C. The optimum incubation time for obtaining the maximum yield of initial cleavage products (5 h) was determined by monitoring the hydrolysis rate at timed intervals by h.p.l.c. Peaks A–D remained approximately in the same proportion as shown in Fig. 1, whereas peak E

Fig. 1. Peptide 'map' of the insulin B-chain after hydrolysis by macrophage elastase. Macrophage elastase was incubated with the B-chain for 5 h at 37°C and pH 7.6; the components were separated by h.p.l.c. under the conditions described in the Materials and methods section. See Table 1 for the composition of peptides indicated by peaks A–E (E is the undigested B-chain).

decreased. As a control, the macrophage elastase was also incubated with the B-chain in the presence of the metal chelator EDTA (10 mM), which has previously been shown to inhibit macrophage elastase (White *et al.*, 1980). As Fig. 2 shows, only two peaks were obtained in the presence of EDTA: the first corresponds to the peak obtained with buffer and EDTA alone and the second corresponds to the undigested B-chain.

The amino acid sequence of the digest peptides obtained from h.p.l.c. peaks A–D as well as undigested B-chain, peak E, (Fig. 1) and their yields are shown in Table 1. The amino acid sequence of the peptides was determined from their amino acid composition and the known amino acid sequence of the B-chain. Each major peak in Fig. 1 corresponds to a pure peptide. The initial peak, corresponding to the buffer peak in the EDTA control (Fig. 2), did not contain a significant amount of amino acids after hydrolysis.

Table 1. Composition of the macrophage-elastase digest of the insulin B-chain

The oxidized insulin B-chain (106 nmol) was incubated for 5 h with macrophage elastase in 156 μ l of 50 mM-Tris/maleate buffer, pH 7.6, containing 5.0 mM-CaCl₂ at 37°C. The components were separated by h.p.l.c. (Fig. 1) and the peptides were identified by their amino acid compositions. Peaks A-E correspond to the h.p.l.c. peaks shown in Fig. 1. Abbreviation used: Cys, cysteic acid.

H.p.l.c. peak	Residue no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	Yield (nmol) 8.7
A	...	Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala																														21.7
B		Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala																														19.1
C		Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr																														5.2
D		Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala																														34.0
E		Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

Fig. 2. Chromatography of EDTA-treated control Macrophage elastase was incubated with the insulin B-chain and the solution chromatographed under conditions identical with those described in Fig. 1, except that 10mM-EDTA was present during the incubation.

Analysis of the peptides obtained after digestion of the B-chain by macrophage elastase revealed two major cleavage sites, namely the Ala-Leu bond (residues 14-15) and the Tyr-Leu bond (residues 16-17). The two peptides resulting from the hydrolysis of the Ala-Leu bond of the B-chain, peptide A (residues 1-14) and peptide D (residues 15-30), were obtained in yields of 8.7 and 5.2 nmol respectively. Peptides B (residues 17-30) and C (residues 1-16), resulting from the hydrolysis of the Tyr-Leu bond, were obtained in yields of 21.7 and 19.1 nmol respectively. Thus the rate of hydrolysis of the Tyr-Leu bond is two to three times greater than that of the Ala-Leu bond. The remaining peak, E, corresponds in location on the chromatogram and in composition to the unhydrolysed B-chain. Recovery of the peptides and B-chain after digestion accounts for approx. 60% of the starting material.

Both cleavage sites of the insulin B-chain have in common a leucine residue in the P₁' position (Schechter & Berger, 1967), indicating that the macrophage elastase preferentially hydrolyses pep-

tide bonds in which leucine contributes the amino group. Since residues in the P_1 position of the two hydrolysis sites, tyrosine and alanine, are widely divergent, it appears that the specificity of the elastase is not dependent on the P_1 residue. However, histidine residue in the P_1 position may be an exception, since the His-Leu bond (residues 10–11) was not cleaved by the macrophage enzyme, although the amino acid sequence after this bond is similar to that of the P_1' – P_4' residues of the principal cleavage site.

In order to further characterize the activity of macrophage elastase on peptide substrates, Glu-Ala-Leu-Tyr-Leu-Val, a peptide corresponding to residues 13–18 of the B-chain and encompassing both cleavage sites of the macrophage enzyme, was prepared by digestion of the B-chain with purified neutrophil elastase. (The latter enzyme preferentially cleaves peptide bonds in which valine contributes the carboxylic acid group.) This peptide was then isolated and incubated with the macrophage elastase at the same molar concentration used previously with the B-chain substrate. After 5 h at 37°C, no hydrolysis products were detected by h.p.l.c. Absence of, or decreased, activity of macrophage elastase on the hexapeptide substrate indicated that the enzyme is a true endopeptidase and probably preferentially cleaves larger oligopeptide substrates. In addition to primary structure and size of the substrate, other factors may be important in determining the specificity of macrophage elastase in the hydrolysis of elastin. Such factors might include the unique secondary structure of elastin arising from the desmosine cross links.

In contrasting the specificity of mouse macrophage elastase with that of the human neutrophil enzyme, it is understood that the results may reflect species differences as well as differences in cell type.

Determination of the specificity of the macrophage elastase as described in the present paper should lead to rational design of specific inhibitors and substrates for this enzyme. Such agents may prove beneficial in determining the physiological role of this proteinase in the pathogenesis of emphysema and other connective-tissue disorders. The requirement of macrophage elastase for peptide substrates

longer than six residues (as shown above) should be considered in any attempt to design such inhibitors or substrates.

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