SPECIFICITY OF ELASTASES: DEGRADATION OF THE OXIDIZED β-CHAIN OF INSULIN BY PORCINE PANCREATIC ELASTASE II AND DOG LEUCOCYTE ELASTASE**

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Porcine elastase II (EC 3.4.21.-), a pancreatic proteinase with elastolytic activity, hydrolyses the oxidized β-chain of insulin with major cleavages occurring at Leu₁₇₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋行政审批

In the earlier publications we described the purification of two elastolytic proteinases: porcine pancreatic elastase II (Ardelt, 1974) and dog leucocyte elastase (Ardelt et al., 1976a). Both enzymes hydrolysed elastin and several other protein substrates as well as N-t-butyloxycarbonyl-L-alanine p-nitrophenyl ester. Elastase II was also very active towards N-acetyl-L-tyrosine ethyl ester whereas the leucocyte enzyme exhibited only negligible activity towards this compound.

Recently, Gertler et al. (1977) succeeded in removing the accompanying proteinases from the elastase II preparation, using a turkey ovomucoid-Sepharose column, and showed that the enzyme did not hydrolyse N-acetyl-tri-L-alanine p-nitroanilide, the specific substrate for elastase I, but still could degrade elastin and the typical chymotryptic substrate, N-acetyl-L-tyrosine ethyl ester. The authors also determined C- and N-terminal residues released during elastolysis, and proposed that elastase II splits the bonds between large hydrophobic and small aliphatic residues. At the same time, Blow (1977) found that human leucocyte elastase easily hydrolyses peptide bonds adjacent to valine and (more slowly) to alanine residues.

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This paper presents the results of studies on specificity of elastase II and dog leucocyte elastase with the use of oxidized β-chain of insulin, the commonly employed polypeptide substrate with known amino acid sequence.

MATERIALS AND METHODS

Elastase II and dog leucocyte elastase were prepared as described previously (Ardelt, 1974; Ardelt et al., 1976a). Oxidized β-chain of insulin was prepared from crystalline porcine insulin (Polfa, Tarchomin, Poland). The hormone was oxidized with performic acid (Craig et al., 1960), the β-chain was isolated by DEAE-Sephadex A-25 chromatography and converted to a hydrochloride form (Bang-Jensen et al., 1964).

Butyloxy carbonyl-tri-L-alanine chloromethyl ketone was prepared by Dr. K. Bańkowski (Institute of Fundamental Problems of Chemistry, Warsaw University, Poland). Toluene sulphonphyl-L-lysine chloromethyl ketone, toluene sulphonphyl-L- -phenylalanine chloromethyl ketone and Dowex 50WX-2-200 were purchased from Sigma Chem. Co. (St. Louis, Mo., U.S.A.).

Elastase II preparation was treated with the chloromethyl ketones as described previously (Ardelt et al., 1976a,b) except that the reaction proceeded at a 100-fold molar excess of the appropriate inactivator; and with all the ketones 50 mM-Tris/acetate buffer (pH 7.2) was used.

Digestions of the insulin β-chain. The substrate (1.65 mM) was digested at 30°C in 50 mM-ammonium carbonate buffer (pH 8.5) containing 0.02% sodium azide. Concentration of the enzyme varied from 17 to 670 nM for elastase II (Fig. 1) and was 850 nM for the leucocyte elastase. The incubation times were 15 min or 24 h in the case of the former, and 60 min or 24 h of the latter enzyme. The final volumes of the incubation mixture were: 0.3 ml for analytical, and 3.0 ml for preparative purposes. The reaction was stopped by lowering the pH to about 3.0 with 4 M-HCl. The samples were dried in a rotary evaporator and submitted to the appropriate fractionation procedure.

Separation of the peptides. Samples of the digests obtained with elastase II were dissolved in 2 M-pyridine/acetate buffer (pH 3.5) and submitted to high-voltage electrophoresis (70 V/cm) in a flat-bed apparatus (Camag HVE-System) using Whatman no. 1 or 3 MM paper. The separated peptides were eluted with water and dried in a rotary evaporator. Digests by the leucocyte elastase were separated on a Dowex 50W column (1 x 50 cm) according to Schroeder (1972) as described by Blow (1977). The isolated peptides were further purified by ascending paper chromatography (Whatman no. 1 or 3 MM paper) or by high-voltage reelectrophoresis under the aforementioned conditions. The following solvents were used alternatively in paper

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1 Abbreviations: Boc-Ala₂,CH₂Cl, butyloxy carbonyl-tri-L-alanine chloromethyl ketone; Tos-Lys-CH₂Cl, toluene sulphonphyl-L-lysine chloromethyl ketone; Tos-Phe-CH₂Cl, toluene sulphonphyl-L-phenylalanine chloromethyl ketone.

Amino acid analysis. The peptides were hydrolysed in 6 M-HCl in evacuated tubes, for 18 h at 105 - 110°C, and run on Beckman Model Unichrom or Beckman 119 B.L. analysers. In some cases, qualitative analysis was performed using paper chromatography in $n$-butanol/acetic acid/water (12:3:5, by vol.) or in phenol saturated with trisodium acetate (Bailey, 1967).

$N$-Terminal analysis. $N$-Terminal residues were identified by the dansyl chloride method (Gray, 1967) except that higher concentration of the label solution was used (5 mg/ml) and dansyl-amino acids were separated by means of thin-layer chromatography on silica gel plates (Folk & Cole, 1965).

RESULTS

Specificity of pancreatic elastase II

Elastase II preparation used in the present study was contaminated with traces of other pancreatic proteinases, mainly elastase I. Therefore, prior to digestions, samples of the preparation were treated alternatively with Tos-Lys-CH$_2$Cl, Tos-Phe-CH$_2$Cl or Boc-Ala$_2$-CH$_2$Cl, the respective synthetic inactivators of trypsin, chymotrypsin and elastase I. Figure 1 presents the results of high-voltage electrophoresis of the insulin $\beta$-chain degraded with Tos-Lys-CH$_2$Cl-treated elastase II.

![Figure 1](image)

Fig. 1. High-voltage electrophoresis of the insulin $\beta$-chain digested with porcine pancreatic elastase II treated with Tos-Lys-CH$_2$Cl. The electrophoresis was run in 2 M-pyridine/acetate buffer, pH 3.5, at 12 - 15°C, for 90 min, at 70 V/cm using Whatman no. 1 paper. Samples were applied as spots, and the separated peptides were visualized with 0.3% ninhydrin solution in acetone containing 1% acetic acid. In the exhaustive digest $e$ an additional, very faint peptide fast moving towards anode (not shown) was also detected.

The digestions were carried out for various periods of time, using various enzyme concentrations, as indicated on the Figure.

Only three "early" released peptides (numbered 5, 10 and 11) appeared in the digests obtained with elastase at concentrations up to 268 nm after 15 min, while
digestion with elastase at a concentration of 670 nm and for a longer time gave a more complex peptide mixture. The same electrophoretic pattern was obtained when elastase I treated with Boc-Ala₂₅-CH₂Cl was used as a substrate. On the basis of these results, two types of digests, $b$ ("short") and $e$ ("exhaustive") were chosen for further experiments.

Irrespective of the kind of pretreatment of elastase II (with Tos-Lys-CH₂Cl, Tos-Phe-CH₂Cl or Boc-Ala₂₅-CH₂Cl), spot II appeared to be a non-splittable peptide (Thr-Pro-Lys-Ala), representing four residues from the carboxyl end of the substrate (Craig et al., 1960). Spot 10 consisted of two peptides: Tyr₂₅-Thr-Pro-Lys-Ala₂₅, and (in smaller amount) Phe₂₅-Tyr-Thr-Pro-Lys-Ala₂₅. The composition of the peptide revealed as spots 4 and 5 was found to be dependent on the enzyme pretreatment. When the enzyme preparation was treated with Tos-Lys-CH₂Cl (trypsin blocked), two peptides: Leu₁₇-Tyr-Leu₁₇ and Phe₂₄-Phe-Tyr₂₆ were found. When the preparation of elastase II was treated with Boc-Ala₂₅-CH₂Cl (elastase I blocked) only one peptide was identified: Gly₂₃-Phe-Phe₂₅. It was, therefore, concluded that the former peptides arose as the result of action of both elastase II (Fig. 2, cleavages: Leu₁₇-Val₁₈ and Tyr₂₆-Thr₂₇) and the contaminating elastase I (cleavages: Ala₄-Leu₃ and Gly₂₃-Phe₂₄), whereas the Gly₂₃-Phe₂₄ peptide was cut off from the substrate by elastase II (cleavage: Phe₂₅-Tyr₂₆) and by trypsin (cleavage: Arg₈₂₂-Gly₂₂₃). When untreated or Tos-Phe-CH₂Cl-treated elastase II was used, spot 5 contained all three peptides described.

![Elastase II and Chymotrypsin C Diagram](image)

![Elastase II and Chymotrypsin A Diagram](image)

**Fig. 2.** Specificity of action of porcine pancreatic elastase II. Oxidized β-chain of insulin was used as a substrate. For comparison, specificities of porcine pancreatic chymotrypsin A (Folk & Cole, 1965) and ox chymotrypsin C (Sanger & Tuppy, 1951), are also given. Large arrows, bonds split rapidly; small arrows, bonds split slowly; broken arrows, bonds split very slowly.

Examination of the remaining peptides of the exhaustive digest allowed us to identify further sites of cleavage by elastase II (Fig. 2). Tyrosine, phenylalanine and leucine were found to contribute carboxyl group of the cleaved bond. Histidine and serine were also identified on prolonged digestion. From amino side of the scissile bond, various residues were identified.
Specifcity of dog leucocyte elastase

Five major peptides of the 60-min insulin digest were isolated and found to contain the following residues: 19 - 30, 1 - 12, 13 - 18, 1 - 14 and 1 - 9 (Fig. 3). Two latter peptides were recovered in smaller amounts. This suggested two major and two minor cleavage sites: Val₁₂-Glu₁₃, Val₁₈-Cys(O₂H)₁₉, Ala₁₄-Leu₁₅ and Ser₉-His₁₀, respectively. Examination of the 24-h digest confirmed these findings. Peptides

![Diagram of peptide cleavage sites](image)

Fig. 3. Specificity of action of dog leucocyte elastase. For comparison, specificities of pancreatic elastase I (Sampath Narayanan & Anwar, 1969) and human granulocyte elastase (Blow, 1977) are given. Designations as in Fig. 2.

containing residues 1 - 14 and 1 - 9 were found in higher amounts than those recovered from the 60-min digest, whereas the contents of peptides with residues 1 - 12 as well as those composed of residues 19 - 30 were independent from the digestion time. Traces of peptides composed of residues 1 - 8, 13 - 15 and 17 - 20 were also detected suggesting very slow and rather unspecific cleavages: Gly₉-Ser₁₀, Leu₁₅-Tyr₁₆, Tyr₁₆-Leu₁₇ and Gly₂₀-Glu₂₁.

DISCUSSION

It has been established that specificity of elastase II differs distinctly from that of elastase I (Sampath Narayanan & Anwar, 1969) as well as of human (Blow, 1977) and dog (Fig. 3) granulocyte elastases.

The results presented confirm our earlier suggestions on similarity of elastase II to chymotrypsins (Ardelt, 1974, 1975) and are in accordance with the results of Gertler et al. (1977) obtained with elastin as a substrate.
Therefore, it seems reasonable to classify porcine elastase II as a new pancreatic chymotrypsin that acquired an ability to degrade elastin, rather than as "elastase". It is worth of notice that recently Lamy et al. (1977) described a new chymotrypsinogen-like proenzyme with proelastolytic activity, which they called "chymotrypsinogen D". Its activated form appeared to be similar to elastase II. However, the two enzymes do not seem identical because of the differences in amino acid composition, especially in the content of leucine, methionine and half-cystine.

Specificity of dog leucocyte proteinase appeared very similar to those of human granulocyte elastase (Blow, 1977) and porcine pancreatic elastase I (Sampath Narayanan & Anwar, 1969). Thus, the enzyme may be regarded as a "true" elastase, but some minor differences are also noticeable. Like human granulocyte elastase, the canine enzyme does not hydrolyse the bond between Gly\textsubscript{22} and Phe\textsubscript{24}, which is readily cleaved by pancreatic elastase I. Moreover, the bond formed by Ala\textsubscript{14} and Leu\textsubscript{15}, i.e. one of the three major sites of cleavage by pancreatic elastase I, was found to be relatively resistant to dog leucocyte enzyme. We were unable to detect cleavage at Cys(O\textsubscript{2}H)\textsubscript{7}-7 residue found with human granulocyte elastase (Blow, 1977), but we obtained evidence for negligible hydrolysis of the neighbouring bond Gly\textsubscript{9}-Ser\textsubscript{9} and of three other bonds.

In spite of the different specificities, porcine pancreatic elastase II and dog leucocyte elastase are capable of degrading elastin, a very inert structural protein stabilized by strong covalent cross-links.

It became clear that dog (and human) leucocyte elastase as well as porcine pancreatic elastase might destabilize elastin structure by degrading the alanine sequences, but porcine pancreatic elastase II might exert the same effect by hydrolysis of Tyr-X and Phe-X bonds adjacent to desmosine or isodesmosine. Polypeptide fragments between cross-links contain large proportion of valine and leucine, and, therefore, can also be hydrolysed by dog leucocyte elastase as well as by porcine pancreatic elastase II.

The question why chymotrypsin C, which exhibits practically the same specificity as elastase II, is inactive towards elastin, remains unanswered. Possibly, the extremely cationic character of elastase (Ardelt, 1975) facilitates binding of the acidic substrate or, which is even more probable, the enzymes differ subtly in the structure of the binding sites.

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REFERENCES


SPECYFICZNOSC ELASTAZ: DEGRADACJA UTLENIONEGO LANCUCHA β INSULINY PRZEZ ELASTAZĘ II TRZUSTKI WIEPRZOWEJ I ELASTAZĘ LEUKOCYTÓW PSA

Streszczenie

Trzustkowa elastaza II (EC 3.4.21.1) - trzustkowa proteaza z aktywnością elastolityczną — hydrolizuje utleniony lančuch βinsuliny, rozszczepiając głównie wiązania Leu₁₁ - Val₁₈, Phe₁₉-Phe₂₄, Phe₂₅-Tyr₂₆ i Tyr₂₇-Thr₂₈. Elastaza leukocytów psa, działając na ten sam substrat, rozszczepia Q₄₄ przed wszystkim wiązania Val₁₃-Glu₄₁ i Val₁₇-Cys₃₄. Przedstawione wyniki dostarczają dowodów na pokrewieństwo elastazy II i chymotryptyn (EC 3.4.21.1 lub 3.4.21.2) oraz pokrewieństwo enzymu leukocytów psa z elastazą granulocytów człowieka i elastazą trzustki wieprzowej (EC 3.4.21.11).

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