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PARTIAL PURIFICATION AND PROPERTIES OF PORCINE PANCREATIC ELASTASE II

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SUMMARY

A highly active elastolytic enzyme was isolated in addition to the well-known elastase (pancreatopeptidase E, EC 3.4.21.11), from a crude elastase preparation. The enzyme was partially purified and some of its physical and kinetic properties were determined.

The approximate molecular weight was 21 900, the absorbance index ($A^{1\%}_{1cm}$) at 282 nm was 20.5, and the pH optimum was 8.7. The apparent K_m and k_{cat} for elastolysis were 8.9 mg/ml, and 11.4 min^{-1}, respectively.

In addition to the elastolytic activity, the enzyme showed a considerable general proteolytic activity and very high activity towards *N*-acetyl-L-tyrosine ethyl ester and benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester (NBA), which are all blocked by diisopropylfluorophosphate (DFP).

INTRODUCTION

In the previous paper [1] we described the purification of porcine pancreatic elastase (pancreatopeptidase E, EC 3.4.21.11) from the crude elastase preparation, by means of DEAE-Sephadex chromatography. By further modification of this method, we were able to separate a second highly active elastolytic enzyme that differs in many respects from pancreatopeptidase E and seems to be similar to "elastomucase" [2–4] or "elastomucoproteinase" [5], except that it has a very high activity against elastin. We called this enzyme "elastase II' using the name "elastase I" for pancreatopeptidase E.

This paper presents the partial purification of elastase II, and some properties of this enzyme in comparison with those of elastase I.

MATERIALS AND METHODS

The sources of materials and supplies were as follows: *N*-α-benzoyl-L-arginine

Abbreviations: BAEE, *N*-α'-benzoyl-L-arginine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; TPCK, tosyl-L-phenylalanyl chloromethyl ketone; NBA, benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester, DFP, diisopropylfluorophosphate.

ethyl ester (BAEE), diisopropylfluorophosphate (DFP), chymotrypsin, and trypsin from Koch–Light; *N*-acetyl-L-tyrosine ethyl ester (ATEE) from Sigma; tosyl-L-phenylalanyl chloromethyl ketone (TPCK) from Calbiochem; 3-phenylpropionic acid from T. Schuchardt, München; pancreatic trypsin inhibitor from Worthington. Benzyloxycarbonyl-L-alanine *p*-nitrophenyl ester (NBA) from Fluka, was kindly given by Dr. A. Koj, Jagiellonian University, Kraków, Poland.

Crude elastase was prepared from a commercial porcine pancreas powder (POLFA, Warsaw, Poland), according to the method of Loeven [4].

Elastin was obtained by the method of Partridge et al. [6] from ox nuchal ligament. The fraction of diameter below 0.1 mm was used throughout. In some experiments, elastin stained with resorcin fuchsin [7, 8] was used.

Disc polyacrylamide gel electrophoresis at pH 4.5 was carried out according to Reisfield et al. [9] using 15% gels.

Determination of molecular weight was performed by gel filtration on Sephadex G-100. Elastases, as well as trypsin were previously treated with diisopropylfluorophosphate [1] to avoid autolysis. Cytochrome *c*, diisopropoxy-trypsin, egg albumin, and ox serum albumin were used as standards assuming the molecular weights of 12 600, 24 000, 45 000 and 69 000, respectively.

Activity towards elastin was determined at 37 °C by the direct spectrophotometric method, described previously [10], with the following modification: 0.2 ml of 6.6 M formic acid, instead of 1 ml of 5 M NaCl, was added to the incubation mixture in order to stop the reaction. One elastolytic unit is defined as the amount of enzyme, that releases 1 mg of soluble elastolysis products during 30 min under the conditions described. Specific activity is expressed as units per mg of enzyme preparation or as units per one optical unit at 282 nm.

One optical unit is the amount of preparation which if dissolved in 1 ml would have an absorbance of unity at 282 nm. The elastolytic activity of chromatographic fractions was determined with resorcin fuchsin–elastin (40 mg in 5 ml) and expressed as absorbance at 560 nm. Activity towards casein was determined and expressed as described previously [1]. The method was based on the spectrophotometric determination (280 nm) of degradation products soluble in 9% trichloroacetic acid. NBA activity was determined at 30 °C, according to Janoff [11]. ATEE activity was measured at 30 °C, according to Schwert and Takenaka [12], except that borate buffer of pH 8.7, 0.015 M with respect to boric acid was used. One unit of the activity is defined as the amount of enzyme that hydrolyses 1 μmole of ATEE during 1 min under the conditions employed. BAEE activity was measured according to the method described by Rick [13], except that the substrate concentration of 0.83 mM, instead of 1.0 mM was used.

For the inhibition studies, the enzymes were preincubated with the inhibitors (20 min at room temperature) prior to the activity determinations. The inhibition by pancreatic trypsin inhibitor was studied at a weight ratio enzyme: inhibitor of 1:2. For the inhibition by DFP and 3-phenylpropionic acid, 200-μg samples of elastase were preincubated in 1 mM solutions of inhibitors which were also present in the incubation mixtures at the same concentrations. Treatment with TPCK was performed according to the method of Schoellmann and Shaw [14].

RESULTS

Partial purification of elastase II

The crude elastase preparation was subjected to column chromatography on DEAE-Sephadex A-50 medium. The elution profile is presented in Fig. 1. Activity towards elastin was found in the first and the second peak; the third very large peak and the material strongly adsorbed (not shown) were inactive. The material of the first peak was then dialysed against distilled water and lyophilized. The properties of this

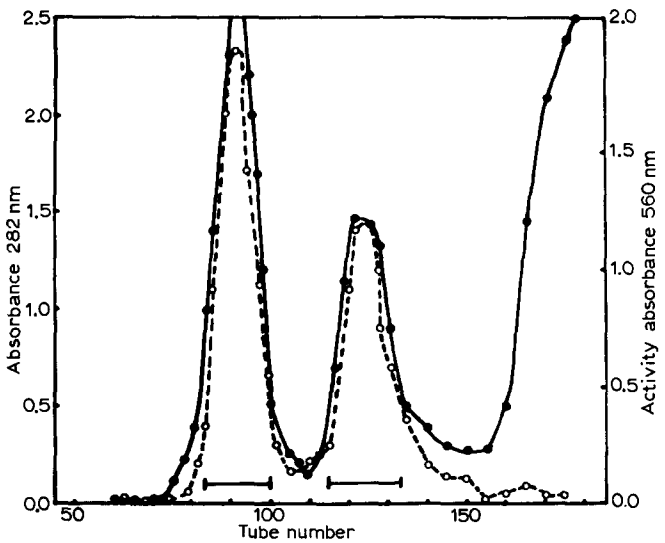

Fig. 1. Elution pattern of elastases from DEAE-Sephadex. The solution of crude elastase preparation (27 ml; $A_{282\ nm} = 7656$ (3 g); total activity 85 222 units) was applied to the column (4 cm × 45 cm) with DEAE-Sephadex A-50 medium, equilibrated and eluted (at 20 °C) with 0.05 M sodium carbonate buffer of pH 8.7, made in 3 M urea. Fractions of 5 ml were collected into tubes containing 0.35 ml of 1.2 M potassium formate buffer of pH 3.0. The downward flow rate was 60 ml/h. ●—●, $A_{282\ nm}$; ○—○, activity towards resorcin fuchsin–elastin. The solid bar indicates the fractions pooled.

enzyme corresponded to those of elastase I. The second peak (corresponding to elastase II) was concentrated, dialysed against 0.2 M ammonium acetate buffer at pH 4.4, and chromatographed on the Sephadex G-100 column (2.5 cm × 54 cm) in the same buffer. Small amount of an inactive material could be removed this way. Isolated enzyme was then dialysed against distilled water and freeze dried. A summary of the purification procedure is given in Table I.

Physical properties

The ultraviolet spectra of elastase II are presented in Fig. 2. Under mild alkaline conditions the main distinct peak at 282 nm, and two smaller ones at about 276 and 291 nm, and the minimum at 250 nm, are clearly visible. A considerable red shift of the minimum and a small increase of the absorbance at 291 nm were observed at high pH value.

TABLE I

PURIFICATION OF ELASTASE II

Steps	Total $A_{282\ nm}$	Total activity (units)	Specific activity (units/$A_{282\ nm}$)	Yield (%)
Crude elastase preparation	7656	85 222	11.1	100
DEAE-Sephadex A-50				
Elastase I	222	25 283	114	29.7
Elastase II	118	16 820	143	19.7
	340	42 103		49.4
Sephadex G-100				
Elastase II	94	14 288	152	16.8

The absorbance index ($A^{1\%}_{1cm}$ at 282 nm in 1 mM HCl) was also determined. The value of 20.5 was obtained, while the index for elastase I was 18.7.

Fig. 3 shows the electrophoretic pattern of elastase II compared to those of elastase I and crystalline preparations of trypsin, α-, β- and γ-chymotrypsin. The electrophoretic mobility of elastase II was distinctly lower than that of elastase I, and both enzymes could be clearly separated from each other when placed in the mixture.

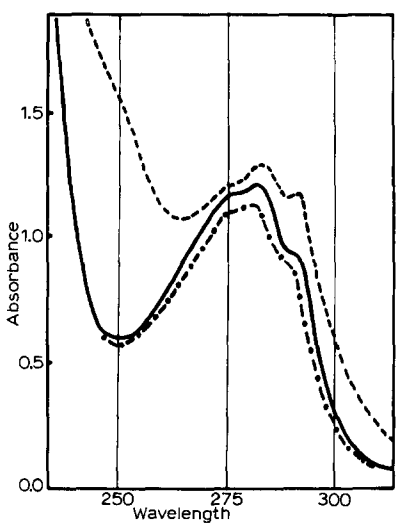

Fig. 2. Ultraviolet absorption spectra of elastase II. ———, 644 μg/ml, 0.015 M borate buffer with respect to boric acid, pH 8.7; — — —, 644 μg/ml, 0.2 M NaOH; -·-·-, 660 μg/ml, 0.05 M HCl.

Trypsin, as well as all chymotrypsin preparations migrated to the cathode faster than the elastases.

The approximate molecular weight of diisopropoxy-elastase II was estimated by the gel filtration method.

The plot of the relationship of elution volume void/volume, against the loga-

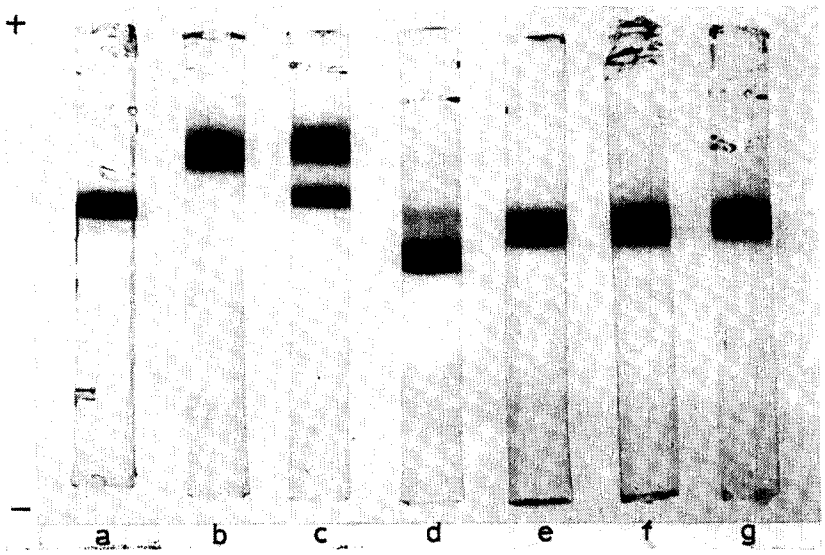

Fig. 3. Polyacrylamide gel electrophoresis of: a, elastase I; b, elastase II; c, elastase I + elastase II; d, trypsin; e, f and g, α-, β- and γ-chymotrypsin, respectively.

rithm of the molecular weight is presented in Fig. 4. From this plot, the value of 21 900 was calculated, while the corresponding value for diisopropoxy-elastase I was 21 100.

Kinetic properties

The pH dependence of elastase II activity is presented in Fig. 5. A definite maximum at pH 8.7 was observed for the elastolytic, as well as for the ATEE activity

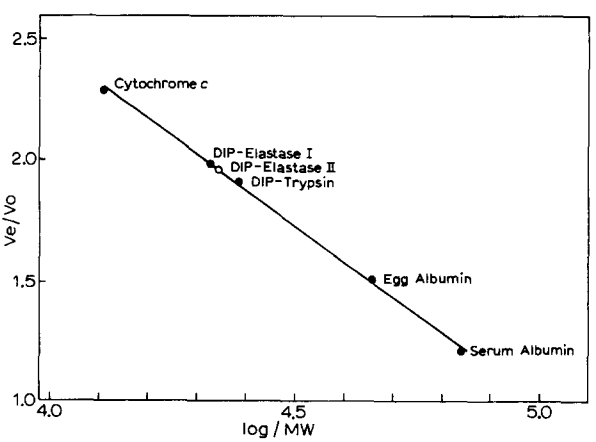

Fig. 4. Determination of molecular weight by gel filtration on Sephadex G-100. Diisopropoxy derivatives of elastase II, elastase I and trypsin as well as other standard proteins (1 mg in 1 ml) were applied to the Sephadex G-100 column (1.7 cm × 29 cm), equilibrated and eluted at room temperature with 0.05 M sodium carbonate buffer, pH 8.7. Fractions of 2 ml were collected at a downward flow rate of 5 ml/h.

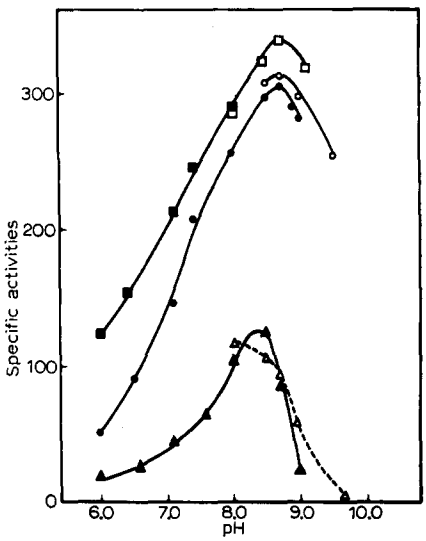

Fig. 5. Effect of pH on enzyme activity. The buffers used were: 0.03 M Tris–Acetate, (solid figures) and 0.015 M borate with respect to boric acid (open figures). (●, ○), elastolytic activity of elastase II; (■, □), ATEE activity of elastase II; (▲, △), ATEE activity of α-chymotrypsin.

of the enzyme. The same optimum has been previously determined for elastase I [1]. The activity of chymotrypsin was distinctly lower.

The apparent K_m and k_{cat} values for elastolysis by elastase II were then determined and compared to those for elastase I (Table II). The K_m value was several times higher than that of elastase I, a higher substrate concentration was thus necessary in the assay system (30 mg/ml) to provide 81–83% of the maximal velocity.

TABLE II

KINETIC CONSTANTS FOR ELASTOLYSIS

The Michaelis constant was calculated from the Lineweaver–Burk plot. It is expressed in mg/ml because of insolubility of the substrate, and, therefore, its unknown molecular weight. K_{cat} was calculated from the maximal velocity and concentration of the enzyme.

Enzyme	K_m (mg/ml)	k_{cat} (min^{-1})
Elastase II	8.9	11.4
Elastase I	1.3*	5.9
	0.95**	

* The result from this work.
** According to the previous paper [1].

Elastase II was also compared to elastase I with respect to the activity towards elastin and casein (Table III).

A distinctly higher activity of elastase II towards both substrates was noted. The difference was particularly well pronounced in the case of caseinolytic activity. Elastase II also appeared to be active towards NBA, a synthetic substrate for elastase [1,

TABLE III

ACTIVITY AGAINST ELASTIN AND CASEIN

The reaction mixture contained 150 mg of elastin and 17 μg of elastase II or 30 μg of elastase I in 5 ml of borate buffer, pH 8.7, 0.015 M with respect to boric acid. For the caseinolytic activity, 30 mg of casein and 2 μg of elastase II or 7 μg of elastase I in 2 ml of the same buffer were used.

Enzyme	Specific activity	
	Elastolytic units/mg	Caseinolytic units/mg
Elastase II	312	4.3
Elastase I	171	1.1

The specific activity (expressed as the change of absorbance at 347.5 nm per min per mg of the enzyme) amounting to 3.6 was determined. The corresponding value for elastase I was 8.1.

Table IV summarizes the results of the ATEE activity determinations and the inhibition of this activity by pancreatic trypsin inhibitor. Elastase II was found to be more active than α-chymotrypsin, while elastase I showed only a negligible activity towards this substrate. The latter is in accordance with the previously obtained result [1]. Moreover, elastase II was not at all inhibited by pancreatic trypsin inhibitor, while a considerable inhibition of elastase I as well as of α-chymotrypsin was observed. Elastase II appeared to be almost inactive against BAEE (0.1% trypsin equivalence); a slightly higher result was previously recorded [1] for elastase I.

TABLE IV

ACTIVITY TOWARDS *N*-ACETYL-L-TYROSINE ETHYL ESTER AND ITS INHIBITION BY PANCREATIC TRYPSIN INHIBITOR

The incubation mixture contained 4.5 μmoles of the substrate and 2 μg of elastase II (or 50 μg of elastase I, or 7.0 μg of α-chymotrypsin) in 3.2 ml of borate buffer pH 8.7, 0.015 M with respect to boric acid.

Enzyme	Specific activity (ATEE units/mg)	Inhibition by pancreatic trypsin inhibitor (%)
Elastase II	338	0
Elastase I	3.6	51
α-Chymotrypsin	91	75

To further characterise elastase II and to differentiate between this enzyme and elastase I as well as chymotrypsin, the inhibition of all enzymes by DFP, 3-phenylpropionic acid and TPCK was studied. 1 mM DFP blocked the elastolytic, caseinolytic and NBA activity of both elastases, as well as the ATEE activity of elastase II. 3-phenylpropionic acid, a well-known reversible inhibitor of chymotrypsin had no effect on elastolysis with both elastases when used at a concentration of 1 mM. TPCK, a potent inactivator of chymotrypsin (over 95% inhibition) was without effect on the elastolytic activity of elastase I (even if used in a greater than 30-fold molar excess), but caused a slight inhibition (9%) of elastolytic, as well as ATEE activity of elastase II.

DISCUSSION

Introduction of 3 M urea into the equilibrating buffer for DEAE-Sephadex chromatography, enabled the separation of another elastolytic enzyme in addition to the known elastase I (pancreatopeptidase E). The question arose, whether this elastase originates from the peak of elastase I, or from the material retarded on the column when the chromatography without urea is performed [1]. A sample of elastase I, prepared according to the previous method [1] was, therefore, rechromatographed on DEAE-Sephadex under the new conditions. The elution profile showed only one almost symmetrical peak. This result strongly suggests, that elastase II is recovered from the material retarded on the column.

Disc polyacrylamide gel electrophoresis revealed a high degree of purity of the elastase II preparation. Electrophoretic mobility of elastase II is distinctly lower than that of elastase I, but unexpectedly both enzymes have the same molecular weight. Thus, the difference in electrophoretic mobility is perhaps a consequence of a difference in a charge and/or shape of the molecules.

The ultraviolet spectrum of elastase II exhibits the typical features of tryptophan; there is no evidence of a considerable contribution of tyrosine. On the other hand, the spectrum of elastase I [1] resembles that of tyrosine. A considerable similarity of the spectra of elastase II and α-chymotrypsin, as well as of those of elastase I and trypsin, should also be noted.

Kinetic experiments revealed further differences between the two elastases. A considerably higher substrate concentration is needed for the assay of elastase II, thus the determination of V should be preferably recommended for more exact experiments. The latter value was found to be considerably higher in comparison to that of elastase I.

Inspection of the pH profiles showed, that in spite of the same optimal value, the pH dependence for elastase II is not so sharp as it was found for elastase I [1]. 82% of the maximal activity is retained at pH 8.0 and over 90% at pH 9.0, while the activity of elastase I falls at these pH values to 48 and 58%, respectively.

The activity towards ATEE is the next property of elastase II that differentiates this enzyme from elastase I which is almost inactive towards this substrate. This activity could not be separated from elastolytic activity by any chromatographic technique tested, including cationic and anionic exchangers as well as hydroxyapatite. Moreover, both activities behaved in a parallel manner during the inhibition studies. It seems thus probable, that both activities are catalysed by the same molecule.

The specific ATEE activity of elastase II is several times higher than that of α-chymotrypsin, and therefore, cannot be due to a contamination with the latter enzyme. Moreover, the inhibition studies showed a distinctly different character of the ATEE activity of both enzymes.

The results obtained, seem to characterise elastase II as the potent serine protease with a high activity against elastin. The enzyme differs in many respects (specificity, physical and kinetic properties) from elastase I, as well as from other pancreatic proteases.

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