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The Specificity of Proteinases from *Streptomyces griseus* (Pronase)

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Purification of pronase by ion-exchange chromatography gave four proteolytically active fractions. Fraction A_2 contained an endopeptidase that attacks poly L-valine. Fraction B contained an endopeptidase, an aminopeptidase and carboxypeptidases. The activities against hippuryl-L-arginine and hippuryl-L-phenylalanine could be inhibited to a considerable extent by di-isopropyl phosphorofluoridate and by EDTA. Fraction C contained an endopeptidase resembling bovine trypsin. The pure enzyme was completely inactivated by di-isopropyl phosphorofluoridate and pancreatic trypsin inhibitor and to about 90% by other naturally occurring trypsin inhibitors. Fraction D contained an apparently homogeneous endopeptidase, inhibited by di-isopropyl phosphorofluoridate, that adsorbed to and hydrolysed elastin. The activity of all these fractions was tested qualitatively against a wide range of small peptides and synthetic substrates.

Pronase, a commercial preparation from *Streptomyces griseus*, contains several proteinases and peptidases (Nomoto, Narashashi & Murakami, 1960*a*,*b*). Earlier reports described the isolation of a trypsin-like enzyme that hydrolyses N^{α}-benzoyl-L-arginine ethyl ester and is inhibited by di-isopropyl phosphorofluoridate (Hiramatsu & Ouchi, 1963; Wählby, Zetterqvist & Engström, 1965; Trop & Birk, 1968*a*) and naturally occurring trypsin inhibitors (Birk, 1968; Trop & Birk, 1968*a*). The isolation of other proteinases and peptidases has been reported as well (Wählby, 1968; Trop & Birk, 1968*a*,*b*; Narahashi, Shibuya & Yanagita, 1968). The present paper describes the separation, characterization and inhibition of the trypsin-like enzyme from pronase ('pronase trypsin'), of a new elastolytic enzyme ('pronase elastase') and of peptidases with activities similar to those of carboxypeptidase A, carboxypeptidase B and aminopeptidase.

MATERIALS AND METHODS

Chemicals. Pronase (B grade, lot no. 54909) was purchased from Calbiochem. Los Angeles, Calif., U.S.A. Trypsin, chymotrypsin, trypsinogen, chymotrypsinogen A and Kunitz's crystalline soyabean trypsin inhibitor were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Lima-bean trypsin inhibitor and egg-white trypsin inhibitor were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Pancreatic trypsin inhibitor was from Laboratoire Choay, Paris, France and soya-bean trypsin and α-chymotrypsin inhibitor AA was from Miles–Yeda, Rehovot, Israel. Elastase was prepared as described by Gertler & Hofmann (1967). Porcine pancreatic proelastase was kindly provided by Dr A. Gertler (see Gertler & Birk, 1969). N^{α}-Benzoyl-L-arginine amide, N^{α}-benzoyl-L-aginine ethyl ester, N^{α}-toluene-*p*-sulphonyl-L-arginine methyl ester, elastin and benzyldimethyl{2-[2-(*p*-1,1,3,3,tetramethylbutylphenoxy)ethoxy]ethyl}ammonium chloride (benzethonium chloride) were products of Mann Research Laboratories Inc., New York, N.Y., U.S.A. N^{α}-Benzoyl-DL-arginine *p*-nitroanilide hydrochloride was purchased from Serva Entwicklungslabor, Heidelberg, West Germany. The synthetic peptides and poly amino acids were kindly donated by Yeda Research and Development Co. Ltd., Rehovot, Israel.

Enzymic-activity assays. Proteolytic activity was determined at pH 7.6 by the casein-digestion method of Kunitz (1947) as described by Laskowski (1955). Elastolytic activity was assayed by the Congo Red–elastin method described by Gertler & Hofmann (1967) but at 37°C instead of 30°C. The precipitate was separated by centrifugation for 2 min at 3600*g*. The specific activity is defined as the reciprocal value of the time ($T_{0.5}$ min) required to obtain a solution giving $E_{495}^{1.2\,\text{cm}}$ 0.50 per mg of enzyme. The specific activity of pancreatic elastase assayed in parallel was 3.57.

Esterolytic activity on benzoylarginine ethyl ester was determined as described by Schwert & Takenaka (1955), and on toluenesulphonylarginine methyl ester as described by Hümmel (1959). For determination of hydrolytic activity on benzoylarginine *p*-nitroanilide, the method of Erlanger, Kosowski & Cohen (1961) was modified. The reaction mixture was 0.48 mg of the nitroanilide and 10 μg of enzyme in 3 ml of 0.1 M-sodium phosphate buffer, pH 7.6 at 25°C, and activity was measured by following the E_{390}.

Amidolytic activity on benzoylarginine amide was determined by a 60 min incubation of 10 μg of enzyme with 3.6 μmol of the amide in 0.5 ml of 0.1 M-sodium phosphate buffer, pH 7.6 at 25°C; then a sample (100 μl) was taken out, diluted with 5 ml of water and 0.5 ml of Nessler's reagent (prepared as described by Hawk, Oser & Summerson, 1954) was added. The E_{400} was measured.

Aminopeptidase activity was assayed, with L-leucine amide as substrate, by a method based on that of Mitz & Schlueter (1958), with 0.5 M-tris buffer, pH 8.0, and 10 mM-L-leucine amide solution. After 60 min reaction a sample (100 μl) was removed and the NH_3 produced was measured as described above.

Carboxypeptidase activities were assayed on the following synthetic substrates: hippuryl-L-phenylalanine, hippuryl-L-arginine, benzyloxycarbonylglycyl-L-leucine, benzyloxycarbonylglycyl-L-serine, benzyloxycarbonylglycyl-L-tyrosine, benzyloxycarbonyl-L-alanyl-L-histidine, benzyloxycarbonyl-L-histidyl-L-phenylalanine and benzyloxycarbonyl-L-phenylalanyl-L-phenylalanine. Their specificity was determined chromatographically by t.l.c. on cellulose or Kieselgel G plates prepared as described by Wollenweber (1967) and Stahl (1967) and developed as described by Brenner, Niederwieser & Pataki (1967). The reaction with hippuryl-L-phenylalanine and hippuryl-L-arginine as well as with the various synthetic peptides was performed by adding 10 μg of enzyme to 1 ml of 10 mM substrate solution in 0.1 M-tris buffer, pH 8.0, containing 10 mM-$CaCl_2$ and 0.1 M-NaCl. After 15 h at 25°C, samples (10 μl) were withdrawn and spotted on the plates.

Extent of inhibition. The inhibition by different naturally occurring trypsin inhibitors was determined as described by Birk, Gertler & Khalef (1963), with 10 μl of inhibitor solutions in reaction mixtures with different inhibitor/enzyme ratios. Excess of inhibitor (30–50 μg/reaction mixture) was used to ascertain the lack of inhibition of certain enzymes. The extent of inhibition by di-isopropyl phosphorofluoridate was examined after preincubation of 1.5 mg of enzyme in 1 ml of 1 mM-di-isopropyl phosphorofluoridate–0.1 M-sodium phosphate buffer, pH 7.6, for 15 h at 4°C or for 1 h at 25°C. Inhibition by EDTA was tested by introducing EDTA to a concentration of 10 mM in the reaction mixture. The influence of benzethonium chloride was determined by adding this reagent (final concn. 0.16 mM) to the reaction mixture.

Adsorption to elastin. The degree of adsorption of enzyme to elastin was examined by adding 50 μg of enzyme to 2 mg of Congo Red–elastin suspended in 1 ml of 50 mM-tris–HCl buffer at various pH values and shaking the suspension for 15 min at 25°C. Under these conditions the release of dye during the adsorption step is negligible. After centrifugation as described above the supernatant was removed and the precipitate was washed with 1 ml of the same buffer and centrifuged again. The supernatant and washings were combined with another 1 ml of non-reacted Congo Red–elastin suspension and made up to 3 ml with the same buffer. To the original precipitate another portion of buffer was added to make it up to 3 ml. Both samples were examined for elastolytic activity.

Ion-exchange chromatography. Enzyme purifications were carried out at 4°C on columns of CM-cellulose (Serva) or DEAE-cellulose (Serva) operated at flow rates of 60 ml/h and samples (3 ml) were collected. Freeze-dried preparations were stored at 4°C under desiccation.

Polyacrylamide-gel electrophoresis. This was performed at pH 4.5, as described by Reisfeld, Lewis & Williams (1962).

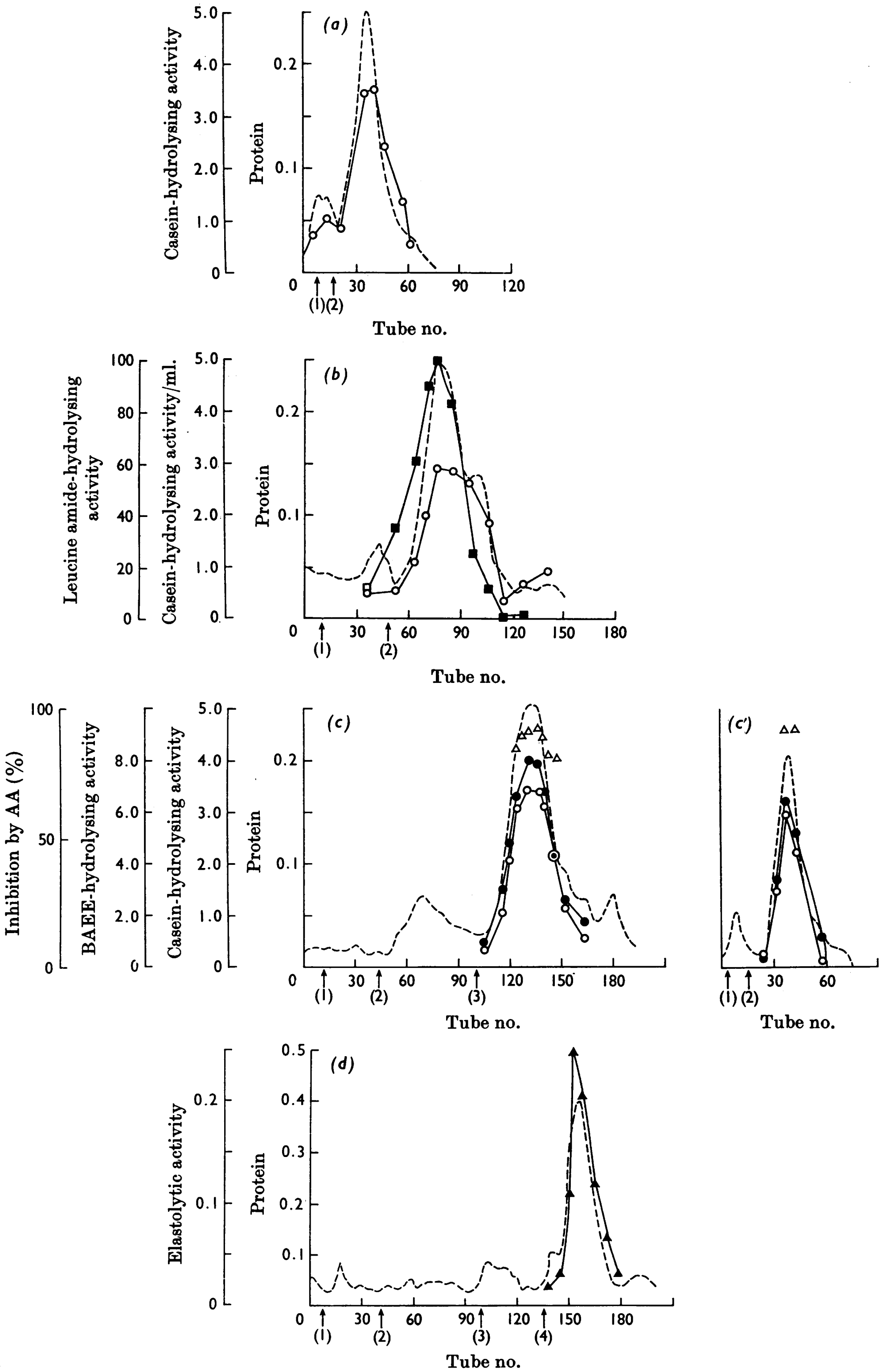
EXPERIMENTAL AND RESULTS

Enzyme separation and purification. A sample of 100 mg of pronase was separated on a CM-cellulose column with gradients from 10 mM-ammonium acetate, pH 4.6, to 0.2 M-ammonium acetate, pH 6.9, as described by Trop & Birk (1968*a*). Fractions A_1, A_2, B, C and D were dialysed against 10 mM-ammonium acetate buffer, pH 4.6 (containing 5 mM-calcium chloride in the case of fractions A_2 and B), and then freeze-dried.

Fraction A_1, which consisted of protein and pigment, was found to contain neither proteolytic nor esterolytic activities and has not been studied further.

Fraction A_2 (8 mg), in 10 ml of 1 mM-ammonium acetate buffer, pH 7.0, was applied to a column (1 cm × 10 cm) of DEAE-cellulose, previously equilibrated with the same buffer. Elution was first performed with the 1 mM-ammonium acetate buffer, pH 7.0, and continued with 10 mM-ammonium

Fig. 1. (*a*) Anion-exchange chromatography (DEAE-cellulose, pH 7.0) of fraction A_2; (1) elution with 1 mM-ammonium acetate buffer, pH 7.0; (2) elution with 10 mM-ammonium acetate buffer, pH 4.6. (*b*) Cation-exchange chromatography (CM-cellulose, pH 4.6) of fraction B; (1) elution with 50 mM-$CaCl_2$–10 mM-ammonium acetate buffer, pH 4.6; (2) elution with 5 mM-$CaCl_2$–28 mM-ammonium acetate buffer, pH 5.1. (*c*) Cation-exchange chromatography (CM-cellulose, pH 4.6) of fraction C; (1) elution with 10 mM-ammonium acetate buffer, pH 4.6; (2) elution with 28 mM-ammonium acetate buffer, pH 5.1; (3) gradient elution with respect to buffer concentration and pH by flowing in 80 mM-ammonium acetate buffer, pH 6.9, through a mixing chamber containing 80 ml of 28 mM-ammonium acetate, pH 5.1. (*c'*) Anion-exchange chromatography (DEAE-cellulose, pH 9.5) of chromatographed fraction C from Fig. 1(*c*); (1) elution with 5 mM-potassium carbonate buffer, pH 9.5; (2) gradient elution with respect to buffer concentrations and pH by flowing 10 mM-ammonium acetate buffer, pH 4.6, through a 80 ml mixing chamber containing the carbonate buffer. (*d*) Cation-exchange chromatography (CM-cellulose, pH 4.6) of fraction D; (1), (2) and (3) as in Fig. 1(*c*); (4) increasing gradient by flowing 0.2 M-ammonium acetate buffer, pH 6.9, into the mixing chamber. In all operations 3 ml fractions were collected. ----, protein concentration (E_{280}); ——, enzyme activities; ○, casein-hydrolysing activity/ml (E_{280}); ●, benzoylarginine ethyl ester-hydrolysing activity/ml (E_{253}); △, percentage inhibition by soya-bean trypsin and chymotrypsin inhibition AA; ▲, elastolytic activity (min^{-1}); ■, leucine amide-hydrolysing activity (μmol of NH_3 ml^{-1} h^{-1}).


(a)
Casein-hydrolysing activity
Protein
Tube no.
(1)(2)
(b)
Leucine amide-hydrolysing activity
Casein-hydrolysing activity/ml.
Protein
Tube no.
(1)
(2)
(c)
(c′)
Inhibition by AA (%)
BAEE-hydrolysing activity
Casein-hydrolysing activity
Protein
Tube no.
(1)
(2)
(3)
(d)
Elastolytic activity
Protein
Tube no.
(4)

acetate buffer, pH 4.6, after emergence of the enzymically inactive first peak. A second fraction, possessing proteolytic activity, then emerged (Fig. 1*a*, tubes 29–46) which was dialysed against 5mM-acetic acid containing 5mM-calcium chloride for 18h and then freeze-dried.

Fraction B was found to contain carboxypeptidase A, carboxypeptidase B, aminopeptidase and proteinase activities, as assayed on hippuryl-L-phenylalanine, hippuryl-L-arginine, L-leucine amide and casein, respectively. For further purifications a sample (32mg) was dissolved in 35ml of 5mM-calcium chloride–10mM-ammonium acetate buffer, pH 4.6, and applied to a column of CM-cellulose (1cm × 10cm), previously equilibrated with the same buffer. Elution was first performed with 120ml of the same buffer and then with 5mM-calcium chloride–28mM-ammonium acetate, pH 5.1. The contents of tubes 60–92 (Fig. 1*b*) were combined, dialysed against 5mM-acetic acid–5mM-calcium chloride and freeze-dried. To separate the aminopeptidase activity from that of the carboxypeptidase and proteinase, 2mg of rechromatographed fraction B were dissolved in 20ml of 5mM-potassium carbonate buffer, pH 9.0, and shaken for 10min with a slurry of 20g of DEAE-cellulose in the same buffer. The DEAE-cellulose particles were then separated by centrifugation and washed twice with another portion (20ml) of the carbonate buffer. The combined supernatant and washings contained the bulk of the carboxypeptidase, the proteinase and part of the aminopeptidase activities. The remaining aminopeptidase (about 30%) was then eluted from the DEAE-cellulose with 20ml of 0.1M-acetic acid. The specific proteolytic activity on casein of this eluate decreased to about 10% and it was not inhibited by soya-bean inhibitor AA. It was highly active when assayed on leucine amide and fully inhibited by EDTA. Its activity on hippuryl-L-phenylalanine and hippuryl-L-arginine was negligible.

Fraction C, the trypsin-like enzyme, amounting to 18mg, still contained residual activities from fractions B and D. It was dissolved in 20ml of 10mM-ammonium acetate buffer, pH 4.6, and applied to a CM-cellulose column (1cm × 10cm) previously equilibrated with the same buffer. Elution was performed with 120ml of this buffer followed by 150ml of 28mM-ammonium acetate buffer, pH 5.1. A gradient of ammonium acetate concentration was then introduced by flowing in 80mM-ammonium acetate buffer, pH 6.9, through a mixing chamber containing 80ml of the above pH 5.1 buffer. Tubes 120–143 (Fig. 1*c*), which contained the bulk of trypsin-like activity, were combined, dialysed against 5mM-acetic acid and freeze-dried. A sample (3.5mg) of this preparation was dissolved in 10ml of 5mM-potassium carbonate buffer, adjusted to pH 9.5 and applied to a DEAE-cellulose column (0.75cm × 8.5cm) which had been equilibrated with freshly prepared pH 9.5 carbonate buffer. The column was washed with 70ml of the same buffer and a gradient was then introduced by flowing in a solution of 10mM-ammonium acetate, pH 4.6, through a mixing chamber containing 80ml of the above potassium carbonate buffer. Tubes 32–47 (Fig. 1*c'*) corresponding to the peak were combined and were found to contain the trypsin-like enzyme ('pronase trypsin') free of carboxypeptidase.

Fraction D, the elastolytic enzyme, which was still accompanied by contaminants from fraction C, was further purified by submitting a solution of 19mg of the fraction in 12ml of 10mM-ammonium acetate buffer, pH 4.6, to rechromatography on CM-cellulose under the same conditions as described for fraction C (Fig. 1*c*). After elution with 130ml of the above gradient, the concentration of the ammonium acetate was increased by flowing 0.2M-ammonium acetate buffer, pH 6.9, into the mixing chamber. Tubes 149–160 (Fig. 1*d*), which contained high elastolytic activity, were combined, dialysed against 5mM-acetic acid and freeze-dried. This reaction is designated 'pronase elastase'.

The purity of the fractions, as checked by immunological tests (R. R. Avtalion & M. Trop, unpublished work) and by electrophoresis in polyacrylamide gel was as follows: 'pronase trypsin' and the aminopeptidase appeared as homogeneous single bands, fraction A_2 and 'pronase elastase' were accompanied by slight impurities; and fraction B was heterogeneous.

Enzyme specificities and characteristics. Fraction A_2 hydrolyses proteins and synthetic substrates (Table 1) and deserves further study. The ability to hydrolyse polymers of hydrophobic residues is particularly noteworthy.

Since fraction B as a whole includes carboxypeptidase A, carboxypeptidase B, aminopeptidase and proteinase activities, which as yet are not fully separable, its activities on the specific substrates have been tested with whole fraction B as obtained by additional chromatography on CM-cellulose (tubes 60–92, Fig. 1*b*).

The action of fraction B on synthetic substrates is given in Table 1. The so-called activities of carboxypeptidase A and carboxypeptidase B on hippuryl-L-phenylalanine and hippuryl-L-arginine respectively are partly inhibited by 1mM-di-isopropyl phosphorofluoridate, by 10mM-EDTA, or by dialysis against distilled water. The hydrolysis of L-leucylglycine and of L-leucine amide is not affected by di-isopropyl phosphorofluoridate at all but is completely inhibited by dialysis against distilled water and EDTA. Carboxypeptidase A activity as well as aminopeptidase activity are enhanced by addition of benzethonium chloride to the reaction mixture, as could be seen from the t.l.c. analyses. The proteinase activity of

Table 1. *Specificity of pronase and of its fractions as shown by t.l.c.*

The rate of hydrolysis is indicated semi-quantitatively by the number of plus signs. Arrows indicate the bonds hydrolysed in the substrates shown. Abbreviations: Z-, benzyloxycarbonyl; Hip-, hippuryl.

	Extent of hydrolysis by						
Substrate*	Pronase	Fraction A_2	Fraction B	'Pronase trypsin'	'Pronase elastase'	Trypsin (bovine)	Elastase (porcine)
Gly-↑Val	±	−	+	−	±	−	−
Gly-↑Leu	+	+	++	−	±	−	−
Ala-↑Gly-Gly	+	++	++++	−	+	−	±
Gly-↑Gly-Gly	±	−	+	−	±	−	−
Leu-↑Gly	+	+	++	−	±	−	−
Leu-↑Trp	++	±	++++	±	+++	−	−
Leu-NH_2	±	+	+++	−	−	−	−
Gly-Phe-↑Ala	±	+	+++	±	+	−	−
Gly-↑Phe	−	±	+	−	−	−	−
Gly-Phe-↑Phe	++	±	++++	±	+	−	−
Hip-↑Phe	+	+	++++	±	±	−	−
Z-Gly-↑Leu	+	+	++	−	+	−	−
Z-Gly-↑Ser	±	±	++	−	+	−	−
Z-Gly-↑Tyr	+	+	++	−	±	−	−
Hip-↑Arg	±	±	++	−	−	−	−
Z-Gly-↑Phe-NH_2	++	++	+++	−	+	−	−
Z-Ala-↑His	±	+	+	−	++	−	−
Z-His-↑Phe	+	+	+++	−	−	−	−
Z_2-Lys-↑Val	+	−	++	−	+	−	+
Z_2-Lys-↑Gly	±	−	±	−	±	−	±
Z_2-Lys-↑Phe	+	−	+++	−	+	−	±
Z_2-Lys-↑Ser	−	−	−	−	−	−	−
Z-Met-↑Gly	±	−	+	−	±	−	±
Z-Phe-↑Phe	+	+	+++	±	±	−	−
Poly Val	+	++	+++	−	+	−	+
Poly (Ala,Leu)	++	++	++++	−	+	−	+
Poly Lys	++	±	±	++++	+	++++	−
Poly Tyr	++	−	++	±	+	−	±

* The following substrates were not hydrolysed at all by the above enzymes: Gly-Gly, Ala-Asn, Z-Pro-Pro, Z-Pro-Trp, GSH, Z-Phe-Gly, Z-Gly-NH_2, poly Gly, poly Ala, poly His, poly Glu, poly Asp, poly Pro, poly Trp.

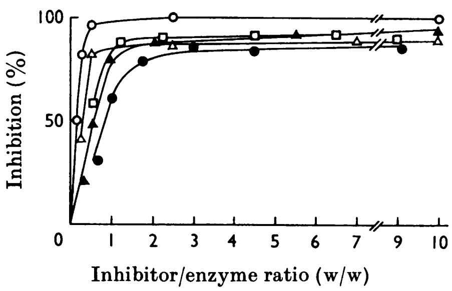


Fig. 2. Inhibition by naturally-occurring trypsin inhibitors of the hydrolysis of casein by 'Pronase trypsin'. The reaction mixtures contained 15 μg of enzyme and the indicated amounts of the following inhibitors: ○, pancreatic trypsin inhibitor; △, lima-bean trypsin inhibitor; □, Kunitz's crystalline soya-bean trypsin inhibitor; ●, egg-white trypsin inhibitor; ▲, soya-bean trypsin and α-chymotrypsin inhibitor AA.

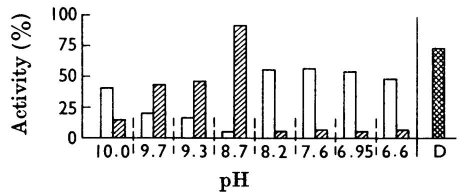


Fig. 3. pH-dependence of adsorption of fraction D (elastolytic enzyme) to Congo Red–elastin examined by shaking, for 15 min at 25°C, 50 μg of enzyme with 2 mg of Congo Red–elastin suspended in 1 ml of 50 mM-tris–HCl buffer of various pH values, centrifugation and washing of the separated precipitate with 1 ml of the same buffer. The supernatant and washings were combined with another 1 ml of non-reacted Congo Red–elastin suspension and made up to 3 ml with the same buffer. To the original precipitate another portion of buffer was added to make up to 3 ml. Both samples were assayed at pH 9.7 for their elastolytic activity: adsorbed elastolytic activity (■), compared with unadsorbed fraction D (□), or with fraction D incubated at pH 9.7 under the described experimental conditions (■). Activity is expressed as a percentage of the activity of untreated 50 μg of fraction D.

fraction B on casein is inhibited by 10 mM-EDTA to the extent of 78%.

The similarity in activities of bovine trypsin and 'pronase trypsin' is further substantiated by their action on various synthetic substrates (Table 1), from which it can be seen that blocking of the ε-amino group of the lysine residue in a peptide renders the substrate immune to hydrolysis by both enzymes. The activity of 'pronase trypsin' on all the specific synthetic substrates examined (e.g. benzoylarginine amide, benzoylarginine ethyl ester, toluene-*p*-sulphonyl methyl ester, benzoylarginine *p*-nitroanilide and poly L-lysine) and on peptides is inhibited by 1 mM-di-isopropyl phosphorofluoridate and by naturally occurring trypsin inhibitors. The degree of inhibition of the enzyme, assayed against casein, at different inhibitor/enzyme ratios is given in Fig. 2. Only pancreatic trypsin inhibitor causes complete inhibition at relatively low inhibitor/enzyme ratios, whereas the other inhibitors reach a maximal inhibition of 80–93%, which cannot be exceeded by higher inhibitor concentrations. The activity of 'pronase trypsin' on benzoyl-DL-arginine *p*-nitroanilide is increased by benzethonium chloride to about 140%.

'Pronase elastase' hydrolyses elastin to the extent of $T_{0.5}$/mg 1.61 min at the optimum pH, 9.6. The action of fraction D upon various synthetic substrates is given in Table 1. This enzyme, which is almost entirely unaffected by natural trypsin inhibitors, is completely inhibited by di-isopropyl phosphorofluoridate, as are pancreatic elastase and other 'serine' enzymes. Attention should be drawn to the strong inhibition (about 85%) of the elastolytic activity of 'pronase elastase' by benzethonium chloride, compared with 75% inhibition of pancreatic elastase. The elastolytic enzyme is adsorbed on elastin. Fig. 3 shows the pH-dependence of this adsorption.

DISCUSSION

The separation and isolation from pronase of proteinases and peptidases with different specificities may provide additional tools for determination of protein primary structure. The common specificities of some of the pronase enzymes and pancreatic proteases and peptidases give rise to the questions: Do they also originate from zymogens? Do they possess a similar structure in general and around the active site in particular?

Since the activities studied could be demonstrated in pronase immediately without any activation and since no increase in activity was noted during the purification procedure, it may be concluded that they are not present there as proenzymes. This does not rule out the possibility of their synthesis in *Streptomyces griseus* as zymogens that are activated immediately upon secretion or during the preparation procedure of the commercial pronase. Their presence in pronase in the active form does not facilitate their separation because of autolysis. The difficulty has presumably been minimized by employing acidic separation conditions that are not favourable for enzyme action, by operating in the cold and by decreasing separation time to a minimum. Repeated fractionations from different batches of pronase gave fractions of similar specific activities and specificities. Hence the active sites, if not the whole enzyme molecules, have not been damaged.

'Pronase trypsin' activates proelastase (A. Gertler, M. Trop & Y. Birk, unpublished work), trypsinogen and chymotrypsinogen A and hydrolyses poly L-lysine in a similar manner as does trypsin; both yield lysine, dilysine, trilysine and tetralysine as main products of hydrolysis of poly L-lysine.

The similarity in size (Wählby, 1968), in activities and in inhibition by naturally occurring trypsin inhibitor, of the trypsin-like enzyme from pronase to that of pancreatic trypsin suggests a possible similarity in composition and structure of the active site (Wählby & Engström, 1968).

When comparing reports on the specificities of pronase enzymes the variability of different pronase preparations should be pointed out. Thus pronase (B grade) showed only very little chymotrypsin-like activity when assayed against *N*-acetyltyrosine ethyl ester and, this activity could not be detected in any of the separated fractions (Trop & Brik, 1968*a*,*b*) whereas Ryan (1966) clearly demonstrated the presence, in another pronase preparation, of α-chymotrypsin-like enzyme. Different immunodiffusion patterns were also noted for different pronase preparations (R. R. Avtalion & M. Trop, unpublished work). These differences may perhaps be attributed to the frequent mutations so common to Actinomycetes (Waksman, 1967), or to variations in conditions of growth.

Attention is drawn to benzethonium chloride as an activator for the activity of the 'pronase trypsin' and as an inhibitor for the elastolytic activity of the 'pronase elastase'.

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