Human Lysosomal Elastase

CATALYTIC AND IMMUNOLOGICAL PROPERTIES

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1. The elastase of human spleen was shown to exhibit endopeptidase activity against azo-casein and elastin. 2. Activity against several synthetic substrates was detected, and benzyloxycarbonyl-L-alanine 2-naphthyl ester was found to be a good substrate for routine use. 3. The enzyme showed a broad pH optimum in the range of 8.2–9.2 against azo-casein and the synthetic substrate. 4. The effect of inhibitors on the spleen elastase showed it to be a serine proteinase with a specificity similar to that of porcine pancreatic elastase. 5. Specific antisera were raised against the enzyme, and it was shown to be immunologically identical with the lysosomal elastase of human neutrophil leucocytes.

In the previous paper (Starkey & Barrett, 1976a), the purification of two neutral proteinases from human spleen was described. One of these enzymes was active against elastin, and therefore was designated an elastase. Evidence described in the present paper shows that this is the elastase that is a component of the lysosomal (azurophil) granules of human neutrophil leucocytes (Dewald *et al.*, 1975). There is much interest in the possibility that this enzyme may play a part in such important physiological processes as the digestion of bacteria by phagocytes (Janoff & Blondin, 1973), the degradation of elastin in the arterial wall and emphysematous lung, the degradation of kidney basement membrane in glomerulonephritis, and the destruction of the articular cartilage in rheumatoid arthritis (Janoff, 1972a). In the present paper, we describe some properties of the lysosomal elastase, and compare it with the well-characterized elastase of porcine pancreas.

Materials

Lysosomal elastase was purified from human spleen as described by Starkey & Barrett (1976a); its specific activity was 1040 nkat/mg with Z-Ala-2-ONap* as substrate (measured in the absence of NaCl) and 105 unit/mg with azo-casein.

Chemicals in addition to those mentioned in the previous paper (Starkey & Barrett, 1976a) were obtained as follows: Ac-ONapAS-D, Bz-DL-Arg-Nan, elastase (hog pancreas, twice crystallized, type I; the results were not corrected for the presumed partial inactivity of this commercial enzyme), Glt-Phe-Nan, lima-bean trypsin inhibitor (type II-L), PhPr-ONapAS, soya-bean trypsin inhibitor (type II-S) and Tos-PheCH₂Cl from Sigma (London)

Chemical Co. Ltd., Kingston-upon-Thames, Surrey, KT2 7BH, U.K.; Ac-(Ala)₃-Nan, Boc-Ala-ONp, Bz-DL-Arg-2NNap, and Tos-LysCH₂Cl from Bachem, Liestal, Switzerland; Dip-F and Pronase (45000 P.U.K. units/g) from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.; Sephadex G-50 from Pharmacia (G.B.) Ltd., London W5 5SS, U.K.; Pms-F (B grade) from Calbiochem Ltd., London W1H 1AS, U.K.; bovine pancreatic trypsin inhibitor (Kunitz) (Trasylol) from Bayer AG, Wuppertal-Elbelfeld, Germany. Z-PheCH₂Br was a gift from Dr. Elliott Shaw, Brookhaven National Laboratory, New York, NY, U.S.A.; Ac-(Ala)₃-AlaCH₂Cl and

* Abbreviations: Ac-(Ala)₃-AlaCH₂Cl, *N*-acetyl-tri-L-alanyl-L-alanine chloromethyl ketone; Ac-(Ala)₃-Nan, *N*-acetyl-tri-L-alanine 4-nitroanilide; Ac-(Ala)₃-1-ONap, *N*-acetyl-tri-L-alanine 1-naphthyl ester; Ac-(Ala)₂-Pro-AlaCH₂Cl, *N*-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine chloromethyl ketone; Ac-(Ala)₃-OMe, *N*-acetyl-tri-L-alanine methyl ester; Ac-Ala-1-ONap, *N*-acetyl-L-alanine 1-naphthyl ester; Ac-ONapAS-D, 2-acetyl-3-naphthoic acid *o*-toluidide; Ac-Phe-2-ONap, *N*-acetyl-L-phenylalanine 2-naphthyl ester; Boc-Ala-ONp, *t*-butyloxycarbonyl-L-alanine 4-nitrophenyl ester; Boc-Ala-2-ONap, *t*-butyloxycarbonyl-L-alanine 2-naphthyl ester; Bz-DL-Arg-Nan, *N*-benzoyl-DL-arginine 4-nitroanilide; Bz-DL-Phe-2-ONap, *N*-benzoyl-DL-phenylalanine 2-naphthyl ester; ClAc-ONapAS-D, 2-(chloroacetyl)-3-naphthoic acid *o*-toluidide; Dip-F, di-isopropyl phosphorofluoridate; Glt-Phe-Nan, *N*-glutaryl-L-phenylalanine 4-nitroanilide; NapAS-D, 3-naphthoic acid *o*-toluidide; PhPr-ONapAS, 2-(phenylpropionyl)-3-naphthoic acid anilide; Pms-F, phenylmethanesulphonyl fluoride; Suc-(Ala)₃-Nan, *N*-succinyl-tri-L-alanine 4-nitroanilide; Tos-LysCH₂Cl, 7-amino-1-chloro-3-tosylamido-L-heptan-2-one; Tos-PheCH₂Cl, 1-chloro-4-phenyl-3-tosylamido-L-butan-2-one; Z-Ala-2-ONap, *N*-benzyloxycarbonyl-L-alanine 2-naphthyl ester; Z-PheCH₂Br, 1-bromo-4-phenyl-3-(*N*-benzyloxycarbonyl)amino-L-butan-2-one.

Ac-(Ala)₂-Pro-AlaCH₂Cl were gifts from Dr. J. C. Powers, Georgia Institute of Technology, Atlanta, GA, U.S.A. Pepstatin was a gift from Professor H. Umezawa, Institute of Microbial Chemistry, Tokyo, Japan. Boc-Ala-2-ONap and Ac-Phe-2-ONap were prepared by Dr. C. G. Knight in this laboratory. Turkey ovomucoid and chicken ovomucoid were prepared as described by Lineweaver & Murray (1947) and Barrett (1974) respectively. A preparation of human blood leucocytes, predominantly neutrophils, prepared by re-centrifugation of 'buffy coat' and osmotic erythrocytolysis, was a gift from Dr. J. Gordon, Department of Pathology, University of Cambridge, Cambridge, U.K. An antiserum against elastase of human neutrophil leucocytes was a gift from Dr. K. Ohlsson, Department of Clinical Chemistry and Surgery, Malmö General Hospital, Malmö, Sweden.

Methods

Methods not described by Starkey & Barrett (1976a) were as follows.

Enzyme assays

Activities against low-molecular-weight substrates are given in nkat (nanokatals), whereas those against azo-casein are in units.

Activity against ClAc-ONapAS-D. Incubation mixtures (2.0ml) contained 1.5ml of 0.1M-potassium phosphate buffer, pH6.0, 0.5ml of enzyme diluted with 0.05% Brij 35, and 0.02ml of ClAc-ONapAS-D (5mg/ml of dimethyl sulphoxide). After 10min at 30°C a 0.5ml sample was withdrawn from the incubation mixture and added to 2.0ml of 0.025M-potassium phosphate buffer, pH6.0, containing 1.5mg of soya-bean trypsin inhibitor, to stop further reaction. Coupling was then carried out by the addition of 1.0ml of Fast Garnet (0.6mg/ml of 4% Brij 35).

E_{520} values were measured 5min later. Standards containing 2 μ g and 5 μ g of chymotrypsin were run with each set of assays, and the activity of the unknown enzyme sample was related to that of chymotrypsin. Under the coupling conditions described above, NapAS-D gave a $\Delta\epsilon_{520}$ of $3.7 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The specific activity of chymotrypsin under these conditions was 1.45 nkat/mg.

Activity against other naphthol esters. Activity against Boc-Ala-2-ONap was measured as described for Z-Ala-2-ONap (Starkey & Barrett, 1976a) except that the pH of the buffer was 7.5. Activity against Ac-Phe-2-ONap was measured as described for Bz-DL-Phe-2-ONap (Starkey & Barrett, 1976a).

Activity against Boc-Ala-ONp. Incubation mixtures (3.0ml) contained 2.0ml of 0.01M-CaCl₂/0.15M-Tris/HCl, pH7.8, 1.0ml of enzyme diluted

with water and 0.05ml of Boc-Ala-ONp (10mg/ml of dimethyl sulphoxide). A reference mixture contained no enzyme. Both solutions were maintained at 37°C in a Unicam SP. 700 double-beam spectrophotometer. The release of 4-nitrophenol in the enzyme sample compared with the reference sample was measured by the increase in $E_{347.5}$, 4-nitrophenol having an extinction coefficient at this wavelength of $5.5 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Visser & Blout, 1972). Activity was expressed in nkat.

Activity against Bz-DL-Arg-2-NNap. Activity against this substrate was measured by the method described for trypsin by Barrett & Starkey (1973).

Activity against 4-nitroanilides. Incubation mixtures (2.0ml) contained 1.5ml of 0.2M-Tris buffer, pH7.5 or 8.5, 0.5ml of enzyme diluted with 0.05% Brij 35 and 0.05ml of substrate in dimethyl sulphoxide. After incubation at 50°C for 30min the reaction was stopped by addition of 5M-sodium formate buffer, pH3.0 (1.0ml), or 1mg of soya-bean trypsin inhibitor in water (1ml). Incubation mixtures for the assay of chymotrypsin contained 0.01M-CaCl₂. Substrate solutions used were Glu-Phe-Nan (40mg/ml), Bz-DL-Arg-Nan (40mg/ml), Bz-Tyr-Nan (5mg/ml) and Ac-(Ala)₃-Nan (1mg/ml). The release of 4-nitroaniline was followed as ΔE_{410} , and activity was expressed in nkat on the basis of a $\Delta\epsilon_{410}$ of $8.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Erlanger *et al.*, 1961). It was found that E_{410} was unaffected by pH within the range covered in these experiments.

Activity against ³H-labelled elastin. Elastin was labelled with ³H by the use of sodium boro[³H]-hydride and used in assays of elastinolytic activity, as described by Takahashi *et al.* (1973). The preparation of a uniform dispersion of the elastin was facilitated by the inclusion of 0.1% Triton X-100 in all reaction mixtures.

Inhibition studies

Assays of the proteolytic activity of lysosomal (0.3 unit) and pancreatic (3 μ g) elastases were made by the standard method. In general, the enzyme was preincubated with the inhibitor for 5min at room temperature before the addition of azo-casein substrate. The concentration of each inhibitor given in Table 3 is that in the final reaction. Inhibition by gold thiomalate and by mercaptosuccinic acid was measured in 0.1M-Tris/HCl buffer, pH7.5, without added KCl. For certain inhibitors [Dip-F, Tos-LysCH₂Cl, Tos-PheCH₂Cl, Z-PheCH₂Br, Pms-F, Ac-(Ala)₃-AlaCH₂Cl and Ac-(Ala)₂-Pro-AlaCH₂Cl] enzyme was preincubated with the inhibitor at 4°C for 18h, and the concentration of inhibitor stated is that present at the start of the preincubation. Incubation mixtures (0.75ml) contained 0.4ml of 2.5M-KCl/1.25M-Tris/HCl buffer, pH7.5, 0.25-ml of 0.1% bovine serum albumin in 0.02M-CaCl₂,

0.05 ml of enzyme diluted with 0.05% Brij 35 and 0.05 ml of inhibitor. Tos-LysCH₂Cl was freshly dissolved in water; Dip-F, Tos-PheCH₂Cl, Z-PheCH₂Br and Pms-F were dissolved in propan-2-ol, and Z-PheCH₂Br, Ac-(Ala)₃-AlaCH₂Cl and Ac-(Ala)₂-Pro-Ala-CH₂Cl were dissolved in dimethyl sulphoxide immediately before use.

Raising of antisera

Serum 1. Purified human elastase (0.8 mg) dissolved in 1.0 M-NaCl/0.05 M-potassium phosphate buffer, pH 7.0, was adsorbed with 2 × 0.3 g (wet wt.) of ovininhibitor-Sepharose at 4°C for 1 h. The elastase-ovininhibitor-Sepharose complex was then washed with 50 ml of 1.0 M-NaCl in the phosphate buffer and 50 ml of 0.1% Brij 35 in the same buffer. The adsorbent was finally resuspended in phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.02% KH₂PO₄, 0.12% Na₂HPO₄) and stored at -20°C in three 1 ml samples each containing 0.25 mg of elastase bound to 0.2 g of ovininhibitor-Sepharose. One sample was mixed with an equal volume of Freund's Complete Adjuvant, divided into two portions and injected intramuscularly into the thighs of a rabbit. The procedure was repeated after 2 and 4 weeks. Blood was taken 2 weeks after the final injection.

Serum 2. A second antiserum against lysosomal elastase was raised by injection of precipitin lines formed between the pure enzyme (150 µg) and serum 1 (as described by Dingle *et al.*, 1971). The agarose containing immunoprecipitates was injected into a rabbit on three occasions exactly as described above.

Immunodiffusion

Plates were made and stained for protein as described previously (Barrett, 1974). For detection of enzymic activity, plates were incubated at 37°C in 0.075 M-potassium phosphate buffer, pH 6.0, containing 0.1 mM-Fast Garnet and 60 µg of Ac-ONapAS-D/ml. The Fast Garnet was prepared freshly by diazotization of 4-amino-2',3-methylazobenzene (Lillie & Pizzolato, 1969).

Results

Substrate specificity

Azo-casein. The reaction products of the digestion of azo-casein by purified elastase were analysed by gel chromatography on Sephadex G-50.

Azo-casein was digested with 1.3 units of elastase under the standard assay conditions, except in that the reaction was stopped by the addition of 0.25 ml of 12.5% (w/v) trichloroacetic acid. The mixture was filtered and a 0.5 ml sample of the filtrate run on a column (0.9 cm × 28 cm; 18 cm³) of Sephadex G-50 equilibrated with 0.2 M-NaCl/0.05 M-sodium acetate

buffer, pH 5.5. The volume and E_{366} of the fractions were measured. For comparison an incubation mixture (1.0 ml) containing 0.5 ml of Pronase (1 mg/ml), 0.25 ml of 0.04 M-CaCl₂/0.15 M-Tris/HCl buffer, pH 7.8, and 0.25 ml of 6% (w/v) azo-casein, was allowed to react at 37°C for 18 h before being stopped with trichloroacetic acid as above. No precipitate appeared, and the solution was applied to the column under the same conditions as those described for elastase. Undigested azo-casein was also chromatographed on Sephadex G-75.

It was found that the trichloroacetic acid-soluble products of elastase appeared as a complex peak at about 90% of the bed volume, whereas the products of action of Pronase (a mixture of endopeptidases and exopeptidases with broad specificity) were eluted later. Undegraded azo-casein was eluted much earlier. It was concluded that elastase had degraded azo-casein with an endopeptidase action, yielding products smaller than azo-casein itself, but larger than those produced by exopeptidases.

Elastin. Activity against elastin was measured in two ways. Elastin-agarose plates were used, and also the solubilization of ³H-labelled elastin was followed. Both methods were used in an examination of the effect of sodium dodecyl sulphate and 1.0 M-NaCl on the elastinolytic activity of the purified enzyme from spleen. Sodium dodecyl sulphate was found by Takahashi *et al.* (1973) greatly to enhance the elastinolytic activity of pancreatic elastase, whereas the activity is strongly inhibited by 1.0 M-NaCl (Gertler, 1971).

Sodium dodecyl sulphate had a complex effect on the size of the zones of clearing on the elastin agarose plates. The zones formed by small amounts of enzyme were larger in the presence of sodium dodecyl sulphate than in its absence, whereas larger amounts of enzyme produced smaller zones in the presence of sodium dodecyl sulphate than in its absence. NaCl (1.0 M) did not prevent digestion of the elastin, but caused the zones to become diffuse, so that precise measurement was impossible. Small amounts (0.05%) of neutral detergent, Brij 35, in enzyme samples placed in the wells, markedly enlarged the zones of lysis, even when sodium dodecyl sulphate was already present. The most satisfactory conditions found for routine assays of spleen elastase on elastin plates involved the use of 0.1 M-Tris/HCl buffer, pH 8.0, in the gel, with no sodium dodecyl sulphate or NaCl, but with at least 0.5% Brij 35 in the samples.

The radiochemical assay of Takahashi *et al.* (1973) proved extremely sensitive and allowed better quantitation than did the plate assay; 1 mg of spleen elastase showed activity equivalent to approx. 300 µg of porcine pancreatic elastase. This value may be compared with the finding of Janoff *et al.* (1971) that 1 mg of partially purified neutrophil elastase was equivalent to 25 µg of the pancreatic enzyme, in an

Table 1. Activity of lysosomal and pancreatic elastases against low-molecular-weight substrates

Assay conditions were as described in the Methods section. The activity against Z-Ala-2-ONap was measured in the absence of salt. n.d., Not determined.

Substrate	Concn. (mM)	pH	Enzymic activity (nkat/mg of enzyme)	
			Lysosomal elastase	Pancreatic elastase
Boc-Ala-ONp	0.54	7.8	627	327
Boc-Ala-2-ONap	0.80	7.5	39.8	12.1
Z-Ala-2-ONap	0.14	8.5	1014	90
ClAc-ONapAS-D	0.14	6.0	65.2	n.d.
Ac-(Ala) ₃ -Nan	0.06	8.5	5.9	n.d.

Table 2. Effect of salts on the activity against azo-casein of purified elastase

Enzymic activity is expressed as a percentage of that in the absence of added salt.

Addition	Ionic strength (mol/l)	Proteolytic activity (%)
None	0.09	100
1.0M-NaCl	1.09	119
1.0M-KCl	1.09	113
0.5M-CaCl ₂	1.59	133
0.5M-MgCl ₂	1.59	191
1.0M-NaSCN	1.09	169
0.5M-Na ₂ SO ₄	1.59	86

Fig. 1. pH-dependence of the enzymic activity of elastase

The activity against azo-casein (●) (0.43 unit of enzyme) and against Z-Ala-2-ONap (○) (0.1 nkat of enzyme) is expressed as a percentage of the maximal activity with that substrate. All buffers [sodium acetate (a); KH₂PO₄/K₂HPO₄ (b); Tris/HCl (c); glycine/NaOH (d)] (0.1 M) contained 1.0M-NaCl for azo-casein or 2.0M-NaCl for Z-Ala-2-ONap experiments.

assay with elastin-orcin. The accuracy of both comparisons clearly depends upon the purity of the commercial pancreatic enzyme used, however. In our work the inclusion of sodium dodecyl sulphate in the incubation mixtures (0.25 mg/ml of elastin) had no marked effect on the solubilization of radioactivity, either for the spleen enzyme or pancreatic elastase. This difference from the results of Takahashi *et al.* (1973) may possibly be due to the presence of a neutral detergent, Triton X-100, in all of our assays,

although the work of Jordan *et al.* (1974) suggests that this would not substitute for the anionic detergent.

There was a clear difference between the pancreatic and spleen elastases in that the former were 90% inhibited by 1.0M-NaCl, whereas the latter was only 50% inhibited.

Low-molecular-weight substrates. Lysosomal elastase was inactive against Bz-DL-Arg-Nan, a substrate of trypsin, and several substrates of chymotrypsin (Bz-DL-Phe-2-ONap, Glt-Phe-Nan, Ac-Phe-2-ONap). The enzyme showed high activity against the alanine esters tested, being two- to ten-fold more active than commercial pancreatic elastase on a protein basis (Table 1). The lysosomal enzyme differed from the pancreatic in being particularly active against Z-Ala-2-ONap, and this factor clearly contributed to our successful use of this substrate in routine assays. A histochemical ester substrate, ClAc-ONapAS-D, was also hydrolysed.

pH-dependence of activity

The pH-dependence of the proteolytic and esterase activities of purified lysosomal elastase was measured (Fig. 1). Elastase has a broad pH optimum in the range 8.2–9.2, with both azo-casein and Z-Ala-2-ONap, the activity at pH 7.0 being about 80% of the activity at the optimal pH. This agrees well with the published results for the elastase of human neutrophil leucocytes, which has been found to have a pH optimum of 8.5 with fibrinogen and elastin (Ohlsson & Olsson, 1974), 8.0–11.0 with haemoglobin, elastin, casein and histone (Pryce-Jones *et al.*, 1974) and 8.0 with haemoglobin (Schmidt & Havemann, 1974).

Effect of inorganic ions on activity

The purified elastase (0.4 unit) was assayed against azo-casein in incubation mixtures containing 0.1M-Tris/HCl buffer, pH 7.5, together with the various

Table 3. *Effect of potential inhibitors on human lysosomal elastase and porcine pancreatic elastase*

Enzymic activity is expressed as a percentage of activity in the absence of inhibitor. Inhibitors marked (a) were made up as stock solutions in dimethyl sulphoxide and those marked (b) were dissolved in propan-2-ol, before being diluted into the incubation mixture. The final concentration of solvent was 4% (v/v). All other inhibitors were in aqueous solution. Assays were of proteolytic activity, except for those marked (c), which were with the ester substrate. n.d., Not determined.

Compound	Final concn. (mM)	Activity (%)	
		Lysosomal elastase	Pig pancreatic elastase
Dip-F (b)	1.0	2	4
Tos-LysCH ₂ Cl	1.0	86	101
Tos-PheCH ₂ Cl (b)	1.0	109	88
Z-PheCH ₂ Br (a)	1.0	90	79
(b)	0.5	91	83
Pms-F (b)	1.0	0	0
Ac-(Ala) ₃ -AlaCH ₂ Cl (a)	0.33	22	6
	0.10	47	4
	0.01	91	7
	0.001	95	31
Ac(Ala) ₂ -Pro-AlaCH ₂ Cl (a)	1.0	4	4
	0.1	12	2
	0.01	70	3
	0.001	89	9
CaCl ₂	5.0	102	104
ZnSO ₄	1.0	68	63
EDTA	5.0	100	63
Cysteine	5.0	62	57
4-Chloromercuribenzoate	0.1	100	101
4-Nitrophenyl-4'-guanidinobenzoate	0.1	98	n.d.
Heparin	5 mg/ml	100	106
Gold thiomalate	1.0	n.d.	108
	0.5	19(c)	n.d.
	0.05	40(c)	n.d.
	0.005	79(c)	n.d.
Mercaptosuccinate	1.0	104	n.d.
Pepstatin	0.001 mg/ml	100	n.d.
Soya-bean trypsin inhibitor (Kunitz)	1 mg/ml	3	37
	0.1 mg/ml	27(1c)	67(86c)
Lima-bean trypsin inhibitor	0.1 mg/ml	63	78
Bovine pancreatic trypsin inhibitor (Kunitz)	0.5 mg/ml	35	92
Turkey ovomucoid	0.1 mg/ml	8	4
Chicken ovomucoid	0.1 mg/ml	14(c)	0

salts at 0.5–1 M concentration. The results are given in Table 2. Activity was strongly stimulated by 1.0M-NaSCN and 0.5M-MgCl₂; 0.5M-Na₂SO₄ caused slight inhibition.

Inhibitors

The results of inhibition studies are presented in Table 3. Both elastases were completely inhibited by Dip-F and Pms-F. These inhibitors had previously been shown to be effective against the pancreatic enzyme (Hartley & Shotton, 1971) and Pms-F had been reported to inhibit the neutrophil elastase (Schmidt & Havemann, 1974). The halomethyl ketones of lysine and phenylalanine, designed for trypsin and chymotrypsin (Shaw, 1970), were ineffective against the elastases. The effectiveness of the alanine chloromethyl ketones designed by Powers & Tuhy (1973) against the pancreatic enzyme was confirmed, but the lysosomal enzyme proved to be less susceptible, in agreement with the results of Tuhy & Powers (1975). The finding of Janoff (1972b) that the lysosomal elastase is less affected by EDTA than that from pancreas was confirmed. In our hands, the lysosomal enzyme proved even more susceptible to inhibition by gold thiomalate than was found by Janoff (1970), who reported that 2.6mM-gold thiomalate inhibited the activity of the neutrophil enzyme against elastin and Boc-Ala-ONp 63 and 40% respectively. The inactivity of mercaptosuccinate (thiomalate) in our work shows that inhibition was due to the gold itself. The inhibitory activity of gold thiomalate was abolished by 1.0M-KCl.

Soya-bean trypsin inhibitor gave moderate inhibition of pancreatic elastase, and powerful inhibition of the lysosomal enzyme. Bovine pancreatic trypsin inhibitor also inhibited the lysosomal enzyme more strongly than the pancreatic, whereas both enzymes were powerfully inhibited by turkey ovomucoid and chicken ovomucoid, previously shown to inhibit the pancreatic enzyme by Gertler & Feinstein (1971).

Ovoinhibitor was covalently linked to CNBr-activated Sepharose 4B and shown to be suitable for affinity chromatography of spleen elastase. The enzyme was tightly bound at pH 7.5, but eluted at pH 3.

Antisera to lysosomal elastase

Initial attempts to raise an antiserum to elastase by injection of rabbits with highly purified human elastase were unsuccessful, in that a polyvalent antiserum resulted. It has been shown that by binding an antigen to an immunoadsorbent and injecting the antigen-immunoadsorbent complex, antisera can be raised in response to the injection of very small amounts of antigen (Stevenson, 1974). This technique was adapted for use with elastase by adsorbing the enzyme to ovomucoid linked to Sepharose, in the hope that this would further purify the enzyme and allow a specific antiserum to be raised with a small amount of antigen (see the Methods section.)

Serum 1 gave a single precipitin line against pure lysosomal elastase and crude spleen extract. The

antiserum also contained antibodies to chicken ovinhibitor, but human serum contains no cross-reacting protein (Barrett, 1974). Serum 2 also was a monovalent antiserum.

The reactivity of the sera raised against the elastase with the enzyme was demonstrated by the inhibition of lysis of elastin in agarose gel (Plate 1). Immunoglobulin G was partially purified from the two antisera and three control sera by two precipitations from $2.0\text{M}-(\text{NH}_4)_2\text{SO}_4$. Both control sera and antisera showed inhibition of elastinolysis attributable to the proteinase inhibitors normally present in serum. With the immunoglobulin preparations, inhibition was obtained only for the antisera, however.

The antisera were used to establish the relationship of the spleen elastase to the neutrophil enzyme. The antisera against elastase of human neutrophil granulocytes (Ohlsson & Olsson, 1974) and spleen elastase gave an immunological reaction of complete identity when spleen elastase served as antigen in double diffusion. Moreover, the spleen enzyme showed complete identity with the antigen in an extract of human blood leucocytes (Plate 2). The precipitin lines were stained for activity against Ac-ONapAS-D. These results leave no doubt of the identity of the spleen enzyme with that of leucocytes.

Discussion

The porcine pancreatic elastase is an endopeptidase of particularly broad substrate specificity, and the indications are that the human spleen enzyme also is active against a wide range of proteins, including cartilage proteoglycan and collagen (M. C. Burleigh, P. M. Starkey & A. J. Barrett, unpublished work). The specific activity of the spleen elastase against elastin is low compared with that of the porcine pancreatic enzyme, and the results of Janoff *et al.* (1971) suggest that the neutrophil and macrophage elastases are also considerably less active against elastin than the pancreatic enzyme. It should be noted that human pancreas contains an elastase-like enzyme with little or no elastinolytic activity (Mallory & Travis, 1975).

It was found that endopeptidase activity against azo-casein was slightly stimulated by $1.0\text{M}-\text{NaCl}$, whereas this somewhat inhibited activity against elastin. The elastases from pig pancreas, *Streptomyces griseus* and *Flavobacteria* sp. were prevented from digesting elastin by high salt concentrations, whereas digestion of casein was unaffected (Gertler, 1971; Shio *et al.* 1974). All of the elastases, including that of human spleen, are basic proteins, and electrostatic binding to the negatively charged elastin seems essential for activity (Jordan *et al.*, 1974); this interaction is prevented by salt. It has been suggested that the elastase-like human protease E is not elastinolytic, because it is anionic (Mallory & Travis, 1975).

The regions in elastin susceptible to proteolytic attack are rich in alanine residues, and a variety of alanine derivatives have been used as low-molecular-weight substrates for the elastases. Ac-(Ala)₃-OMe has been used for both pancreatic and leucocyte enzymes (Gertler & Hofman, 1970; Janoff & Basch, 1971), as has Boc-Ala-ONp (Janoff, 1969; Visser & Blout, 1972). Ac-Ala-1-ONap and Ac-(Ala)₃-1-ONap were used by Sweetman & Ornstein (1974) to stain electrophoresis gels for the activity of elastase-like esterases of human neutrophil leucocytes, but apart from our work, alanine naphthol esters have not been used in test-tube assays. Ac-(Ala)₃-Nan and other nitroanilides have been used for the pancreatic elastase (Bieth & Wermuth, 1973; Feinstein *et al.*, 1973), whereas Suc-(Ala)₃-Nan has been employed by Bieth *et al.* (1974). The substrate used in our work, Z-Ala-2-ONap, combines stability greater than that of the nitrophenol ester with sensitivity greater than that of the nitroanilides or the nitrophenol ester (A. J. Barrett, P. M. Starkey & C. G. Knight, unpublished work). It is also suitable for staining electrophoresis gels.

In the present work, the purified lysosomal elastase was found to be active against ClAc-O-NapAS-D, a non-specific substrate also hydrolysed by trypsin, chymotrypsin and the chymotrypsin-like enzymes of neutrophil leucocytes and mast cells (Rindler *et al.*, 1973; Starkey & Barrett, 1976b; Gomori, 1953). The effects of potential inhibitors on the elastase showed it to be a serine proteinase more like pancreatic elastase than trypsin or chymotrypsin.

Gold thiomalate has been used clinically to treat rheumatoid arthritis. It is concentrated in the lysosomes of cells in an inflamed joint (Persellin & Ziff, 1966). Oronsky *et al.* (1973) have shown that the degradation of cartilage proteoglycan by an uncharacterized neutral proteinase of human leucocytes is 85% inhibited by 0.1mM -gold thiomalate. We have shown that both the lysosomal elastase and cathepsin G, also present in neutrophils, are inhibited by gold thiomalate (Starkey & Barrett, 1976b).

It seems likely that all of the spleen elastase described here is derived from neutrophil leucocytes, in which cells it is present in the azurophilic granules (Dewald *et al.*, 1975). Platelets are abundant in the spleen and have been reported to contain an elastase (Legrand *et al.*, 1973), but a recent study (J. Gordon & A. J. Barrett, unpublished work) has failed to show any reaction of the anti-(spleen elastase) serum with components of highly purified human platelets. Other cell types in the spleen do not show histochemical reactivity with ClAc-ONapAS-D, a substrate of spleen elastase (Li *et al.*, 1972; A. Kennedy & A. J. Barrett, unpublished work). Under certain conditions, macrophages contain an elastase, but this has different properties from the neutrophil enzyme (Janoff *et al.*,

EXPLANATION OF PLATE I

Effect of anti-(spleen elastase) serum on lysis of elastin in agarose gel

The elastin-agarose plate was prepared as described by Starkey & Barrett (1976a). The vertical troughs contained spleen elastase, and the central, vertical row of wells contained normal rabbit serum 1(a), antiserum 1(b), normal serum 2(c), antiserum 2(d) and normal serum 3(e). The wells on either side contained immunoglobulin G isolated from these sera, in amounts similar to that in the whole sera.

EXPLANATION OF PLATE 2

Identity of spleen elastase with that of human blood leucocytes

A double immunodiffusion plate on which the samples were (a) purified spleen elastase (0.34 μg), (b) an extract of human blood leucocytes in 1.0M-NaCl containing 0.1% Brij 35 (340 μg of protein) and (c) antiserum to spleen elastase. The plate was stained for protein.

1971; Werb & Gordon, 1975), and it was not detected in spleen.

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