Biochem. J. (1976) **155**, 255–263
Printed in Great Britain

Neutral Proteinases of Human Spleen

PURIFICATION AND CRITERIA FOR HOMOGENEITY OF ELASTASE AND CATHEPSIN G

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(*Received* 28 *November* 1975)

1. Human spleen was found to contain proteinases active against azo-casein at neutral and alkaline pH values. 2. The activity was stimulated by high ionic strength and some detergents. 3. Optimal extraction of the proteinases from the tissue was achieved with 1.0M-NaCl containing 0.1% Brij 35 and 0.1% trisodium EDTA. 4. The proteinases were efficiently adsorbed to insoluble material in the absence of salt in the initial stages of purification. 5. Two distinct proteinases were separated by chromatography on DEAE-cellulose, an elastase and a chymotrypsin-like enzyme designated cathepsin G. 6. Both enzymes were highly purified by further column chromatography. 7. The molecular weights of the enzymes were estimated by gel chromatography and sodium dodecyl sulphate-gel electrophoresis. 8. It was shown by isoelectric focusing and gel electrophoresis that both enzymes are cationic proteins that occur in multiple forms.

It has been known since the work of Hedin (1904) that bovine spleen contains proteolytic enzymes active at both acid and alkaline pH values. The major acid proteinase, now called cathepsin D, was studied by Anson (1939) and other acid peptidases of the bovine spleen were characterized by Fruton and co-workers (Tallan *et al.*, 1952). The interest of immunologists in the processing of protein immunogens in the spleen led to further work on cathepsin D (Lapresle & Webb, 1960; Press *et al.*, 1960), and also to the studies of Ziff and co-workers (Fehr *et al.*, 1970; LoSpalluto *et al.*, 1970) on the degradation of immunoglobulins by human spleen enzymes at both acid and neutral pH values. The purpose of the present study was to purify and characterize the enzymes responsible for the neutral proteinase activity of human spleen. Two endopeptidases were isolated, an elastase and a previously unnamed enzyme, now designated cathepsin G. These enzymes have been shown to be immunologically identical with the elastase and the chymotrypsin-like enzyme of human neutrophil leucocytes (Starkey & Barrett, 1976*a*,*b*).

Materials

Normal human spleen tissue was removed in the course of post-mortem examinations, freed from the capsule, minced and stored at −20°C until required.

Chemicals were obtained as follows: agarose, albumin (bovine serum, crystalline), albumin (egg white, grade V), Bz-DL-Phe-2-ONap*, carbonic anhydrase (bovine), ClAc-ONapAS-D (grade II), α-chymotrypsinogen A (bovine, 6 times crystallized, type II), cytochrome *c* (horse heart, type IIA), Fast Garnet (zinc chloride-stabilized diazonium salt of 4-amino-2′,3-dimethylazobenzene), lysozyme (egg white, 3 times crystallized, grade I) and soya-bean trypsin inhibitor (type II-S) from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; Brij 35 (polyoxyethylene dodecyl ether), cetyltrimethylammonium bromide, Hyamine 2389 [approx. 50% (w/v) aqueous solution of methyldodecylbenzyltrimethylammonium chloride and methyldodecylxylylenebistrimethylammonium chloride], Triton X-100 (iso-octylphenoxypolyethoxyethanol containing approx. 10 mol of ethylene oxide), myoglobin (horse heart) and hydroxyapatite from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.; DEAE-cellulose (DE-52) and CM-cellulose (CM-52) from Reeve Angel Scientific Ltd., London EC4V 6AY, U.K.; Sephadex G-75 and Blue Dextran 2000 from Pharmacia (G.B.) Ltd., London W5 5SS, U.K.; carrier ampholines from LKB Ltd., Bromma 1, Sweden; Coomassie Brilliant Blue R 250 from Serva, Heidelberg 1, W. Germany; ultrafiltration membranes from Sartorius Membranfilter G.m.b.H, 34 Gottingen, W. Germany.

Z-Ala-2-ONap was prepared by Dr. C. G. Knight, Strangeways Research Laboratory, Cambridge CB1 4RN, U.K.

Azo-casein was prepared by treating casein with diazotized sodium sulphanilate, the method being otherwise as described by Charney & Tomarelli (1947); the material had an $E^{1\%}_{366}$ (1 cm light-path) of 40.

* Abbreviations: Bz-DL-Phe-2-ONap, *N*-benzoyl-DL-phenylalanine 2-naphthyl ester; ClAc-ONapAS-D, 2-(chloroacetyl)-3-naphthoic acid *o*-toluidide; Z-Ala-2-ONap, *N*-benzyloxycarbonyl-L-alanine 2-naphthyl ester.

Methods

Enzyme assays

Activity against azo-casein. Proteolytic activity was measured with azo-casein as substrate, in incubation mixtures (1.0ml) containing 0.4ml of 1.25M-Tris/HCl buffer, pH7.5/2.5M-KCl, 0.35ml of enzyme diluted with 0.05% Brij 35 and 0.25ml of 6% (w/v) azo-casein. After 30min at 50°C the reaction was stopped by the addition of 3.0ml of 5% (w/v) trichloroacetic acid and the mixture filtered. The E_{366} of the trichloroacetic acid-soluble reaction products was determined, and ΔE_{366} values calculated by subtraction of blank values. Under these conditions ΔE_{366} was linear with time up to 45min of incubation and linear with enzyme concentration up to a value of 0.6. A unit of proteolytic activity was defined as that amount which would have given a ΔE_{366} of 1.0 in 30min at 50°C, if the response were linear to this value.

Activity against elastin. Activity was measured using elastin–agarose plates at pH8.5 essentially as described by Schumacher & Schill (1972).

Activity against Z-Ala-2-ONap. Activity against Z-Ala-2-ONap was measured by a method based on that of Barrett (1972) for cathepsin B. The free naphthol liberated by hydrolysis of the substrate was coupled to the zinc-stabilized diazonium salt, Fast Garnet, to give a product absorbing at 520nm. This normally insoluble reaction product was kept in solution by the detergent Brij 35.

Activity was measured in incubation mixtures (2.0ml) containing 1.5ml of 0.1M-Tris/HCl buffer, pH8.5/2.5M-NaCl, 0.5ml of enzyme in 0.05% Brij 35 and 0.02ml of Z-Ala-2-ONap (5mg/ml of dimethyl sulphoxide). After incubation for 10min at 50°C the reaction was stopped by addition of 1.0ml of soya-bean trypsin inhibitor (1mg/ml of 1.0M-potassium phosphate buffer, pH7.8), and coupling of the free naphthol was carried out by addition of 1.0ml of Fast Garnet [0.6mg/ml of 4% (w/v) Brij 35]. The E_{520} of the coupled reaction products was determined. For some assays NaCl was omitted from the Tris buffer, as higher activities were obtained under these conditions.

E_{520} was found to be linearly related to naphthol concentration up to a value of 0.92 and to enzyme concentration up to a ΔE value of 0.65 (representing 0.13nkat, since the value of $\Delta\varepsilon_{520}$ for the colour reaction was found to be $3.3\times10^4\,M^{-1}\cdot cm^{-1}$). The nkat (nanokatal) is defined as that activity hydrolysing 1nmol of substrate per second (Florkin & Stotz, 1973). ΔE_{520} was linear with respect to time in assays with up to 15min incubation.

Activity against Bz-DL-Phe-2-ONap. Activity was measured by a method similar to that described for Z-Ala-2-ONap, the reaction product being the same.

Incubation mixtures (2.0ml) contained 1.5ml of 0.1M-Tris/HCl buffer, pH7.5, 0.5ml of enzyme in 0.05% Brij 35 and 0.02ml of Bz-DL-Phe-2-ONap (5mg/ml of dimethyl sulphoxide). After incubation for 10min at 50°C the reaction was stopped with soya-bean trypsin inhibitor and the products coupled with Fast Garnet as described above.

The assay was linear with respect to enzyme concentration up to ΔE_{520} of 0.35, and linear with respect to time up to 20min incubation. Activity was expressed in nkat.

Other methods

Protein determination. Protein concentrations were determined either by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard, or by measurement of E_{280}, proteins being assumed to have an $E^{1\%}_{280}$ (1cm light-path) of 10.0.

Column chromatography. All column chromatography was done at 4°C. All buffers contained 1% (v/v) preservative [10% (w/v) 1,1,1-trichloro-2-methylpropan-2-ol and 0.01% pentachlorophenol in butan-1-ol].

Sodium dodecyl sulphate-gel electrophoresis. A method based on that of Neville (1971) was used, the concentration of acrylamides in the separating gel being 15.1T×0.66C [terminology of Hjertén (1962)]. To avoid autolysis, samples of enzyme were precipitated with 10% (w/v) trichloroacetic acid at 4°C within 2h of elution from a column of Sephadex G-75. The precipitates were washed twice with 1% trichloroacetic acid and then redissolved at 100°C in upper reservoir buffer containing (50%, v/v) glycerol, 2% (w/v) sodium dodecyl sulphate and 150mM-2-mercaptoethanol or 1mM-iodoacetic acid.

To stain for protein, all electrophoresis gels were incubated at 55°C, for 1h in 0.1% Coomassie Brilliant Blue R 250 in methanol/acetic acid/water (5:2:3, by vol.). The gels were rinsed in the same solvent, destained at 55°C in 2–3 changes of methanol/acetic acid/water (1:2:7, by vol.), and stored in 5% (v/v) acetic acid.

Polyacrylamide-gel electrophoresis. The system was no. 729 of Jovin (1973), with minor modifications. The concentration of acrylamides in the separating gel was 15.1T×0.66C, and Triton X-100 (0.5%) was included in both separating and spacer gels.

When gels containing elastase were to be stained for enzymic activity, protein was first fixed with 4% (w/v) formaldehyde during 30min at 0°C. Each gel was then washed in 3×15ml of phosphate-buffered saline (0.80% NaCl, 0.02% KCl, 0.02% KH_2PO_4 and 0.12% Na_2HPO_4), pH7.2.

To stain for enzymic activity against Z-Ala-2-ONap or Bz-DL-Phe-2-ONap, gels were incubated at 40°C for 1h in reaction mixtures containing 6.0ml of 0.1M-potassium phosphate buffer, pH7.0, 2.0ml of

Fast Garnet (2mg/ml of water; freshly made up and filtered), and 0.1 ml of substrate (5mg/ml of dimethyl sulphoxide). The reaction mixture was replaced with a fresh solution after 30min. Gels were stained for enzymic activity against ClAc-ONapAS-D, by incubation at 40°C for 30min in reaction mixtures as above except that phosphate buffer, pH6.0, was used, and the complete reaction mixture was filtered before use to remove the heavy purple precipitate that formed. The reaction mixture was replaced with fresh solution after 15min. Gels were stored in 5% (v/v) acetic acid.

Isoelectric focusing. The method was essentially that of Barrett (1970), with staining for protein as described by Barrett (1973).

Results

Effect of detergents and salts on spleen neutral proteinase activity

The effect of detergents on the unfractionated proteolytic activity was tested by use of a 1.0M-KCl extract of a low-salt precipitate (see below). Activity (0.5 unit) was assayed in the presence of 0.4M-Tris buffer, pH7.5. At a concentration of 0.1%, two anionic detergents, sodium deoxycholate and octyl sulphate, decreased activity by 14–30%, whereas the non-ionic Brij 35 and Triton X-100 gave 14–20% stimulation, and the cationic Hyamine 2389 and cetyltrimethylammonium bromide caused 34–51% activation.

To investigate the effect of salt, incubations were made as above, but with 0.2M-Tris buffer, pH7.5, and 0.02–1.27M-KCl. A similar experiment was made in which the incubation mixture contained 0.1–0.5M-Tris/HCl buffer, pH7.5, and 0.02M-KCl. It was found that both KCl and Tris/HCl increased the proteolytic activity approx. linearly with concentration, so that molar salt gave 40% activation.

Purification of spleen elastase and cathepsin G

The results of the purification procedure are shown in Table 1. The complete purification scheme resulted in apparent purification factors of 2550 for elastase and 765 for cathepsin G. The stages of purification were as follows.

(*a*) *Solubilization of proteolytic activity.* As a result of preliminary experiments with a variety of salts and detergents, solubilization of activity was carried out by homogenization of minced human spleen (2kg) with 4 litres of 1.0M-NaCl containing 0.1% Brij 35, 0.1% trisodium EDTA and 2% (v/v) preservative (see the Methods section). The mixture was then centrifuged at 2075*g* for 1.5h, and the pellet discarded.

(*b*) *Autolysis.* The preparation was adjusted to pH4.5 with 1.0M-sodium formate buffer, pH3.0, and incubated overnight at 35°C. Autolysis improved the results of subsequent purification steps and would

Table 1. *Purification of elastase and cathepsin G from human spleen*

The values given are those of a single preparation from 2kg of tissue. For the first three stages protein was measured by the method of Lowry *et al.* (1951), and for the DEAE-cellulose and all subsequent stages, protein was measured by E_{280} determinations. Assays with Z-Ala-2-ONap were made without NaCl. The yield of activity against each substrate is expressed as a percentage of the activity solubilized from the tissue.

		Activity against Z-Ala-2-ONap			Activity against azo-casein			Activity against Bz-DL-Phe-2-ONap		
Stage	Protein (g)	Activity (μkat)	Specific activity (nkat/mg)	Yield (%)	Activity (kunit)	Specific activity (unit/mg)	Yield (%)	Activity (μkat)	Specific activity (nkat/mg)	Yield (%)
Homogenate	250	104.5	0.42	100	64.5	0.26	100	35.8	0.14	100
Autolysate	223	130.0	0.58	124	61.6	0.28	96	28.6	0.13	80
Salt extract of low-ionic-strength pellet	8.1	63.5	7.84	60.7	34.6	4.28	54	20.4	2.53	57
Elastase										
DEAE-cellulose	2.56	44.2	17.3	42.2	5.76	2.25	9	—	—	—
CM-cellulose	0.068	18.9	278	18.0	1.80	26.4	2.8	—	—	—
Sephadex G-75	0.007	7.3	1040	7.0	0.70	105.0	1.0	—	—	—
Cathepsin G										
DEAE-cellulose	37.5	—	—	—	8.53	0.23	13	8.5	0.23	24
Hydroxyapatite	0.068	—	—	—	2.0	29.2	3	1.03	15.1	3
Sephadex G-75	0.004	—	—	—	0.55	144.0	0.9	0.41	107.0	1.1

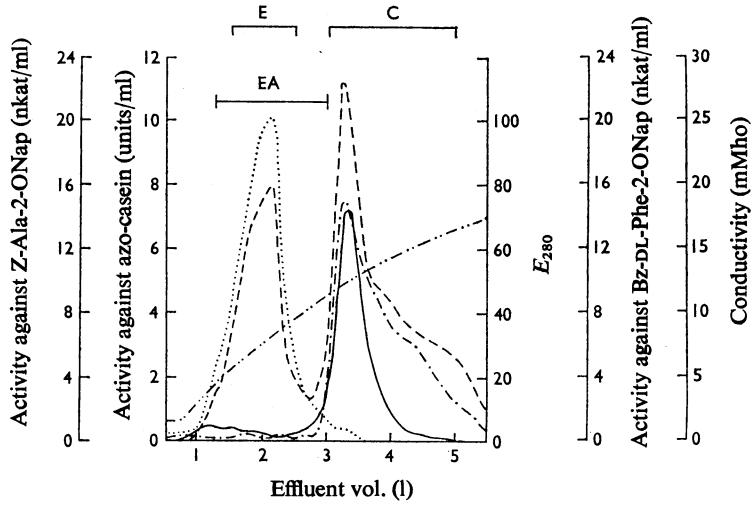


Fig. 1. *Chromatography of spleen extract on DEAE-cellulose*

The column (5cm×46cm; 903cm³) was eluted with a linear gradient (5 litres) of 0–0.6M-NaCl in 0.1% Brij 35/0.075M-Tris/HCl, pH7.8. The distribution of E_{280} (——), activity against azo-casein (– – – –), activity against Z-Ala-2-ONap (····), activity against Bz-DL-Phe-2-ONap (–·–·–·–) and conductivity (–··–··–··–) are shown. The distribution of activity against elastin is shown by the horizontal bar (EA). The fractions containing enzymic activity were combined to give elastase (E) and cathepsin G (C) pools for use in the further purification of each enzyme.

have inactivated any pathogenic viruses present in the human tissue.

(*c*) *Precipitation at low ionic strength and resolubilization.* The autolysate was centrifuged at 2075*g* for 1.5h and the pellet discarded. The supernatant, which retained some finely-divided insoluble material, was dialysed against 2×10vol. of 0.05M-sodium acetate buffer, pH5.1, for 48h. The mixture was again centrifuged at 2075*g* for 1.5h. The pellet contained 37% of the protein present before dialysis, and 99% of the proteolytic activity.

Extraction of enzymic activity from this pellet was attempted with various media; 2M-$MgCl_2$ was the only extractant found in which both enzymes were completely stable, and which gave good (62–77%) resolubilization of both. The pellet was homogenized in a minimum volume of 2M-$MgCl_2$ and adjusted to pH7.6 with Tris. The mixture was stirred at 4°C for 30min, and then centrifuged at 100000*g* for 30min. The procedure was repeated and the two supernatants were combined and filtered.

The step of dialysis to low ionic strength with subsequent salt extraction of the pellet achieved 15-fold purification (Table 1) with concentration of the enzymes.

(*d*) *DEAE-cellulose.* The $MgCl_2$ extract of the low-salt pellet was dialysed against 0.075M-Tris/HCl, pH7.8, containing 0.1% Brij 35. The detergent was necessary to prevent precipitation and inactivation of both enzymes. The dialysed extract was treated with 3×300g of DEAE-cellulose equilibrated with the buffer. The adsorbent was packed in a column (5cm×46cm), washed with buffer, and eluted with a gradient of increasing NaCl concentration. The results are shown in Fig. 1.

About 97% of the proteolytic activity of the sample, 80% of the activity against Z-Ala-2-ONap and all of the activity against Bz-DL-Phe-2-ONap were adsorbed on to the DEAE-cellulose. Two peaks of proteolytic activity were eluted from the column. The dip in azo-casein-degrading activity between the two peaks was shown by mixing experiments not to be due to the presence of an inhibitor.

The first peak of activity was clearly defined, and was eluted early in the gradient before, and well separated from, the major protein peak. These fractions were active against azo-casein, Z-Ala-2-ONap and elastin, but inactive against Bz-DL-Phe-2-ONap, and thus contained the spleen elastase activity.

The second peak of proteolytic activity was very broad, the exact distribution of activity varying between preparations. Sometimes the activity was resolved into two peaks, one eluting with the major protein peak and the other after. Activity against Bz-DL-Phe-2-ONap closely paralleled the azo-casein-degrading activity in these fractions; there was no activity against elastin or Z-Ala-2-ONap. This second proteinase peak was taken to represent the spleen

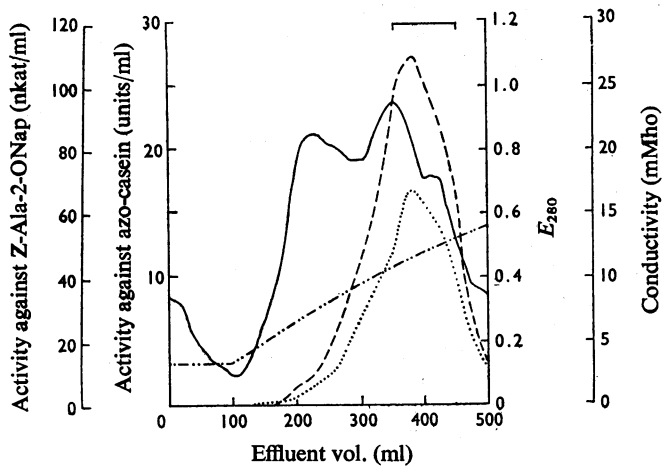


Fig. 2. *Chromatography of partially purified spleen elastase on CM-cellulose*

The column (2.5cm×22cm; 108cm³) was eluted with a linear gradient (500ml) of 0–0.4M-NaCl in 0.1% Brij/0.05M-potassium phosphate buffer. The distribution of E_{280} (——), activity against azo-casein (– – – –), activity against Z-Ala-2-ONap (· · · ·) and conductivity (–·–·–·–) are shown. The fractions indicated were combined to give the pool of elastase activity.

cathepsin G activity. An apparent decrease in specific activity at this stage (Table 1) was, in fact, attributable to the change from measurement of protein by the method of Lowry *et al.* (1951) to the use of E_{280}.

The material not adsorbed by the DEAE-cellulose contained a small part of the total elastase activity; the activity behaved exactly like the major component in further purification as described below, and was immunologically identical with it.

The elastase and cathepsin G were further purified separately.

Further purification of elastase

CM-cellulose. The pool of activity eluted from DEAE-cellulose was diluted with water (3vol.) and adjusted to pH7.0 with 1.0M-sodium formate buffer, pH3.0. The solution (4040ml) was treated with 2×50g of CM-cellulose, equilibrated with 0.05M-potassium phosphate buffer, pH7.4, containing 0.1% Brij 35. The adsorbent was packed as a column (2.5cm×22cm) and washed with buffer before elution with a gradient of increasing NaCl concentration. The results are shown in Fig. 2.

Azo-casein-degrading activity (85%) and 84% of the Z-Ala-2-ONap esterase activity of the original sample, together with 14% of the E_{280}-absorbing material, was adsorbed by the CM-cellulose. The NaCl gradient eluted a single peak of activity against azo-casein and Z-Ala-2-ONap. The active fractions were pooled, giving a 40% recovery of enzymic activity with a 14-fold purification over the sample adsorbed with CM-cellulose.

Gel chromatography on Sephadex G-75. The pool of active fractions eluted from CM-cellulose was concentrated by ultrafiltration to 17ml and run on a column (2.5cm×87cm) of Sephadex G-75 in 1.0M-NaCl/0.05M-potassium phosphate buffer, pH7.0, with recycling of the effluent. The results are shown in Fig. 3.

A single peak with activity against azo-casein and Z-Ala-2-ONap was eluted at 107% bed volume after recycling. The peak fractions were pooled, and the specific activity of the enzyme with each substrate was constant throughout the pooled fractions (85 units of azo-casein-degrading activity/mg and 280 nkat/mg activity against Z-Ala-2-ONap or 924nkat/mg assayed in the absence of NaCl). This active pool was concentrated by ultrafiltration and used as the source of pure spleen elastase in all subsequent experiments.

Further purification of cathepsin G

Chromatography on hydroxyapatite. The pool of cathepsin G activity eluted from the DEAE-cellulose (1560ml) was dialysed against 0.1M-potassium phosphate buffer, pH6.8, containing 0.1% Brij 35, and treated with 3×30g of hydroxyapatite equilibrated with the buffer. The hydroxyapatite was packed in a column (2.5cm×16cm) washed with buffer, and eluted with a linear gradient of increasing phosphate concentration. The results are shown in Fig. 4.

Only 12% of the E_{280}-absorbing material of the partially purified cathepsin G sample was adsorbed by the hydroxyapatite under these conditions,

whereas 75% of the enzymic activity was retained. The phosphate gradient eluted a single peak of activity against azo-casein and Bz-DL-Phe-2-ONap, well separated from the majority of protein eluted from the column.

Gel chromatography on Sephadex G-75. The pool of cathepsin G activity eluted from hydroxyapatite was concentrated by ultrafiltration to 17ml and run on a column (2.5cm×82cm) of Sephadex G-75 in 1.0M-NaCl/0.05M-potassium phosphate buffer, pH7.0. The results are shown in Fig. 5.

A single peak of activity against both azo-casein and Bz-DL-Phe-2-ONap was eluted at about 65% bed volume. The peak was well separated from the contaminating protein, and the specific activities against both substrates were essentially constant throughout the pooled fractions (150 units of azo-casein-degrading activity/mg and 120nkat of activity against Bz-DL-Phe-2-ONap/mg). The pool of active fractions was concentrated by ultrafiltration, and used as the source of pure cathepsin G in all subsequent experiments.

Electrophoretic properties and molecular weight of elastase and cathepsin G

Experiments were carried out to obtain evidence of the purity of the enzyme samples, and to determine some of their physical properties.

Polyacrylamide-gel electrophoresis. Elastase was run in gel electrophoresis at acid pH. The results are shown in Plate 1. Staining revealed four fast-moving protein bands each of which showed enzymic activity against Z-Ala-2-ONap and ClAc-ONapAS-D, the staining with the former being more diffuse, perhaps because of the greater solubility of 2-naphthol relative to naphthol AS-D.

Cathepsin G, run in the same way (Plate 1), gave four fast-moving protein bands of mobility comparable with that of lysozyme, and some slow-moving material. The fast-moving bands showed activity against ClAc-ONapAS-D and faint staining with Bz-DL-Phe-2-ONap. No enzymic activity was associated with the slow-moving protein. Control experiments with chymotrypsin also gave diffuse staining with Bz-DL-Phe-2-ONap under these conditions.

Isoelectric focusing. Samples of elastase and cathepsin G were subjected to isoelectric focusing in polyacrylamide gels containing ampholines pH3–10 and 7–9. The gels were cut into slices that were assayed for activity against azo-casein and naphthol

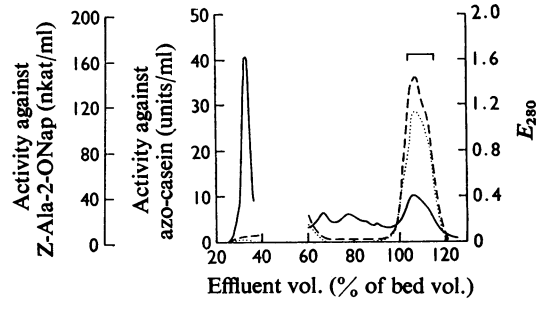


Fig. 3. *Chromatography of partially purified spleen elastase on Sephadex G-75*

The distribution of E_{280} (——), activity against azo-casein (----) and activity against Z-Ala-2-ONap (····) in the effluent from the column (2.5cm×87cm; 427cm³) are shown. The fractions indicated were pooled to give the pure elastase. The effluent between 37 and 60% bed volume was recycled through the column.

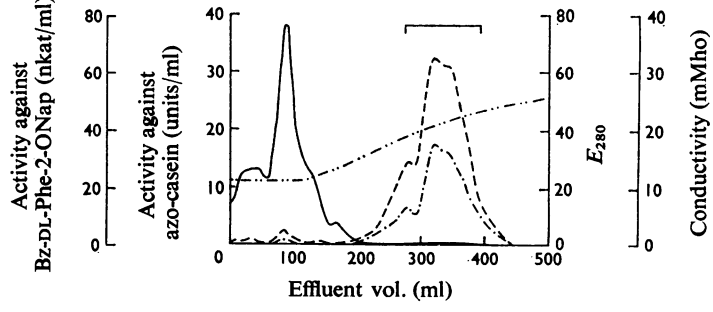
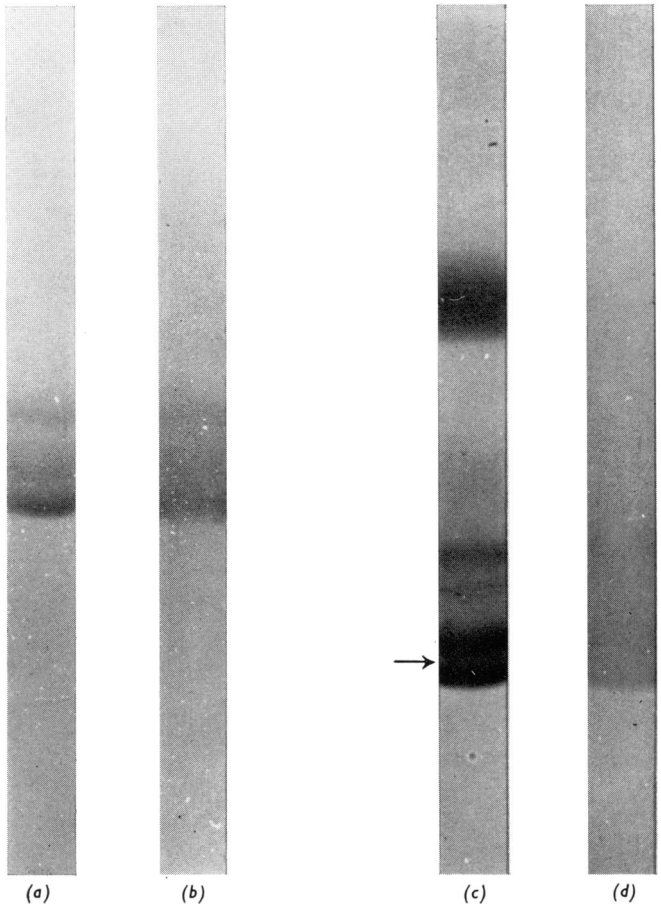


Fig. 4. *Chromatography of partially purified cathepsin G on hydroxyapatite*

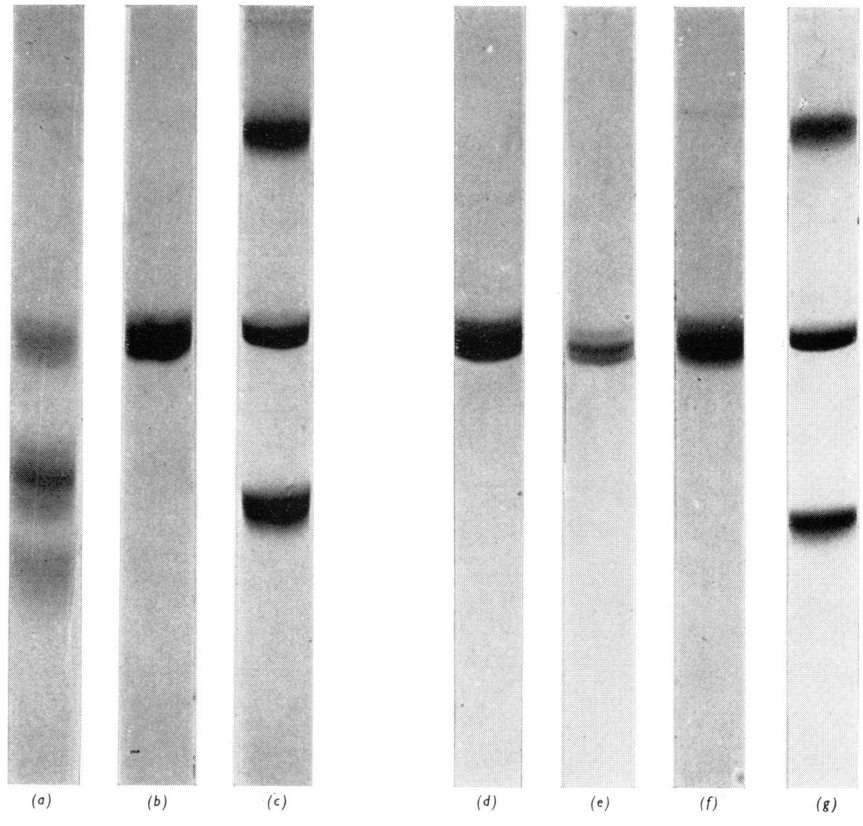
The column (2.5cm×16cm; 79cm³) was eluted with a linear gradient (500ml) of 0.2–0.7M-potassium phosphate buffer, pH6.8, containing 0.1% Brij 35. The distribution of E_{280} (——), activity against azo-casein (----), activity against Bz-DL-Phe-2-ONap (-·-·-·-) and conductivity (-··-··-··-) are shown. The fractions indicated were combined to give the pool of cathepsin G activity.



EXPLANATION OF PLATE 1

Gel electrophoresis of the spleen proteinases

Samples were (*a* and *b*) elastase (10 μg) and (*c* and *d*) cathepsin G (15 μg). Gels (*a*) and (*c*) were stained for protein, whereas gels (*b*) and (*d*) were stained with ClAc-ONapAS-D and Fast Garnet to show enzymic activity. The arrow indicates the mobility of chicken lysozyme run simultaneously. The direction of migration was downwards towards the cathode.



EXPLANATION OF PLATE 2

Sodium dodecyl sulphate-gel electrophoresis of spleen proteinases

Samples (*a–c*) were elastase (9 μg) in mercaptoethanol, elastase (8 μg) in iodoacetate and standards (bovine serum albumin, carbonic anhydrase and myoglobin, 2.5 μg of each) respectively. Samples (*d–g*) were cathepsin G (13 μg and 3 μg) in mercaptoethanol, cathepsin G (13 μg) in iodoacetate and standards as above respectively. The direction of migration was downwards towards the cathode.

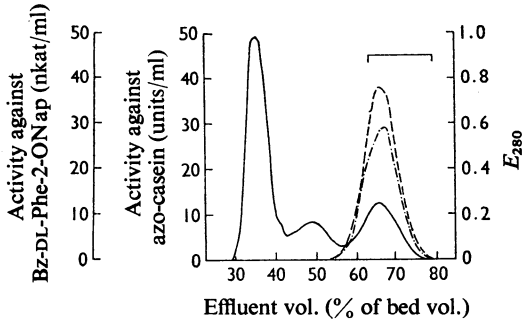


Fig. 5. *Chromatography of partially purified cathepsin G on Sephadex G-75*

The distribution of E_{280} (——), activity against azo-casein (– – – –) and activity against Bz-DL-Phe-2-ONap (–·–·–·–) in the effluent from the column (2.5cm×82cm; 402cm³) are shown. The fractions indicated were pooled to give the pure cathepsin G.

esters and reactivity with antisera to the purified enzymes. Uncut gels were stained for protein. The recovery of enzymic activity after focusing was low (10–25%), but all protein, enzymic activity and immunologically reactive material detected were at the alkaline ends of the gels.

Determination of molecular weights by gel chromatography. A column (1.5cm×88cm) of Sephadex G-75, equilibrated with 1.0M-NaCl/0.05M potassium phosphate buffer, pH7.0, was calibrated with Blue Dextran (mol.wt. 2000000), ovalbumin (mol.wt. 43000), α-chymotrypsinogen A (mol.wt. 27500), cytochrome *c* (mol.wt. 11700) and potassium ferricyanide and used to estimate the molecular weights of the spleen proteinases (Andrews, 1964). The value obtained for elastase was 20000 and that for cathepsin G 17000.

Electrophoresis in the presence of sodium dodecyl sulphate

Elastase run under reducing conditions gave multiple protein bands arranged in three groups (Plate 2). The slowest-moving band corresponded to a mol.wt. of approx. 27000, the middle group corresponded to a mol.wt. of 20000–15000 and the fastest-moving group corresponded to a mol.wt. of 13000–10000. Under non-reducing conditions elastase gave only two bands (scarcely resolved in Plate 2) corresponding to mol.wt. of 30000 and 27500. We conclude that many molecules of the enzyme had undergone autolytic cleavage of the polypeptide chain, but that the resulting fragments were linked by disulphide bonds.

Cathepsin G (see Plate 2) under reducing conditions gave one major band of mol.wt. 28000 with a minor band of slightly lower mobility. No low-molecular-weight material was present. Under non-reducing conditions cathepsin G gave three bands corresponding to mol.wt. 30000, 28500 and 27000, the two faster-moving bands being the more intense.

Discussion

Azo-casein proved to be an extremely useful substrate for the assay of neutral proteinase activity: not only has the substrate a high extinction coefficient, but the yellow colour of the products minimizes interference from other u.v. absorbing material. Assays were made at pH7.5 rather than 7.0 in order to eliminate the activity of cathepsin B. The azo-casein-degrading activity was enhanced by neutral and cationic detergents, and also by salts, whereas anionic detergents were inhibitory.

Purification

The extraction of neutral proteinase activity from the spleen was most efficient when a high ionic strength was combined with the presence of detergent. The low solubility of the enzymes in the absence of salt was exploited during the purification by dialysis to low ionic strength, and subsequent extraction of the pellet with 2M-$MgCl_2$. This procedure resulted in a 15-fold purification, with concentration of the activity. In the work of others, the requirement of salt for solubility of several proteolytic enzymes has been used in their purification. Thus, in the purification of the chymase of rat mast cells, Kawiak *et al.* (1971) allowed the enzyme to become adsorbed to Sephadex G-25 at low ionic strength and subsequently eluted the proteinase with NaCl. Rindler-Ludwig & Braunsteiner (1975) extracted elastase from human leukaemic granulocytes with 0.15M-NaCl before solubilizing the chymotrypsin-like enzyme in 1.0M-NaCl.

Anion-exchange chromatography on DEAE-cellulose resolved the proteolytic activity into two well-separated components, which could be distinguished by the use of the low-molecular-weight substrates Z-Ala-2-ONap and Bz-DL-Phe-2-ONap. Subsequent work with the purified enzymes showed that both are strongly cationic, and their adsorption to DEAE-cellulose at pH7.8 must therefore be regarded as anomalous. This was emphasized by the fact that part of the activity against Z-Ala-2-ONap was unadsorbed by the ion-exchanger, although the protein responsible was immunologically identical with the adsorbed enzyme. Janoff (1970) found the leucocyte elastase to pass unretarded through DEAE-cellulose at pH8.6, whereas the collagenase was retarded. The adsorption of part of the elastase activity, and all of the cathepsin G, in our work, was probably due to their association with soluble polyanionic

material, possibly nucleic acid, in the crude preparation, with the reversible formation of complexes of net negative charge. Since the enzymes behaved as expected for basic proteins during subsequent chromatography, it seems likely that they were eluted from the DEAE-cellulose column by displacement from the putative polyanions. The very high degree of purification achieved on CM-cellulose and hydroxyapatite was presumably due to the fact that the anomalous adsorption of the enzymes on DEAE-cellulose had resulted in their separation from other proteins of similar charge.

The proteinase active against Z-Ala-2-ONap was shown to be active also against elastin, and in later work (Starkey & Barrett, 1976*a*) to be immunologically identical with the elastase of normal and leukaemic human granulocytes. The second proteinase, active against Bz-DL-Phe-2-ONap, is immunologically identical with the chymotrypsin-like enzyme of human neutrophils, which has not been named previously, but is designated 'cathepsin G' by Starkey & Barrett (1976*b*). These enzymes have not previously been purified from a solid tissue.

Electrophoretic and chromatographic properties

It was found possible to stain polyacrylamide electrophoresis gels for activity of elastase and cathepsin G by incubation with the appropriate ester substrate and Fast Garnet. The lysosomal elastase appeared as four bands of activity and protein after electrophoresis at pH 3.5. A similar number of bands of activity was obtained by Janoff (1973), Ohlsson & Olsson (1974), Schmidt & Havemann (1974) and Sweetman & Ornstein (1974) with elastase from human neutrophil leucocytes.

The pattern of enzymic activity given by cathepsin G in disc-gel electrophoresis is almost identical with that of the chymotrypsin-like proteinase of human neutrophil leucocytes described by Rindler-Ludwig & Braunsteiner (1975), Li *et al.* (1973) and Schmidt & Havemann (1974). The high cathodal mobility of cathepsin G, comparable with that of lysozyme, showed it to be an extremely basic protein.

In addition to four cationic protein bands demonstrating activity against ClAc-ONapAS-D, the cathepsin G showed a diffuse, slow-moving protein band devoid of enzymic activity. Similar material present in a preparation of the neutrophil chymotrypsin-like enzyme was shown by Rindler-Ludwig & Braunsteiner (1975) to be a product of autolysis.

The results obtained in isoelectric focusing confirm that both elastase and cathepsin G are basic proteins with isoelectric points of 9 or above. This agrees with the isoelectric point of approx. 10 reported for human neutrophil leucocyte elastase by Janoff & Basch (1971), who also reported very low recoveries of activity after isoelectric focusing.

From their elution volumes on Sephadex G-75, the mol.wts. of elastase and cathepsin G appeared to be 20000 and 17000 respectively, values similar to those obtained by Schmidt & Havemann (1974) for the enzymes of human neutrophils. It seems likely that despite the presence of 1.0M-NaCl, both enzymes were adsorbed by the Sephadex, so that the molecular weights obtained by this method were low. The values of 30000 and 27500 that we obtained for elastase by sodium dodecyl sulphate-gel electrophoresis may be compared with those of 36500, 34000 and 33000 obtained by Ohlsson & Olsson (1974) for forms of the elastase of neutrophils. Our estimates of mol.wt. for the forms of cathepsin G by sodium dodecyl sulphate-gel electrophoresis were 30000, 28500 and 27000. The chymotrypsin-like enzyme from human neutrophils was found by Gerber *et al.* (1974) to give two bands of apparent mol.wt. 30000 and 26000.

Evidence presented in the following papers (Starkey & Barrett, 1976*a*,*b*) shows that both elastase and cathepsin G are neutral proteinases that occur in human neutrophil leucocytes. Their cellular origin in the spleen has yet to be established. Together, the enzymes are responsible for all of the neutral proteinase activity of human spleen detectable under our conditions of assay.

We thank Dr. P. Stovin, Department of Pathology, Papworth Hospital, Cambridge, U.K., for the supply of tissue. Financial support was received from the Arthritis and Rheumatism Council. We thank our colleagues for much helpful discussion.

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