Substrate Specificity and Modification of the Active Centre of Elastase-Like Neutral Proteinases from Horse Blood Leucocytes

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Two proteinases (2A and 2B) purified from the granular fraction of horse blood leucocytes degrade casein (K_m values 12.8 and 6 mg/ml respectively) with the maximum activity at pH 7.4 and in the presence of 2M-urea. Urea-denatured haemoglobin, fibrinogen, albumin and resorcin/fuchsin-stained elastin are digested at a slower rate. The enzymes hydrolyse synthetic substrates of elastase, *N*-benzyloxycarbonyl-L-alanine 4-nitrophenyl ester (K_m 0.114 and 0.178 mM) and *N*-acetyl-tri-L-alanine methyl ester (K_m 5.55 and 0.98 mM), but they do not hydrolyse synthetic substrates of trypsin, chymotrypsin and thrombin. The examined proteinases are completely inhibited by 2 mM-di-isopropyl phosphor-fluoridate and show a sensitivity to butyl and octyl isocyanates similar to that of pancreatic elastase. The pH-dependence of their photoinactivation in the presence of Rose Bengal indicates the presence of histidine in the active centre. Proteinase 2A is rather insensitive to iodination by ICl as is pancreatic elastase, whereas proteinase 2B is totally inactivated after incorporation of five iodine atoms per enzyme molecule.

Substrate specificity of neutral proteinases from human polymorphonuclear leucocytes has been the subject of numerous studies and it appears at present that three groups of enzymes can be distinguished: collagenase (Lazarus *et al.*, 1968, 1972; Ohlsson & Olsson, 1973; Sopata & Dancewicz, 1974), elastase-like proteinase (Janoff & Scherer, 1968; Janoff, 1972, 1973; Ohlsson & Olsson, 1974; Schmidt & Havemann, 1974; Dewald *et al.*, 1975) and chymotrypsin-like proteinase (Mounter & Atiyeh, 1960; Schmidt & Havemann, 1974; Rindler-Ludwig & Braunsteiner, 1975; Gerber *et al.*, 1974). The situation is complicated by the fact that most of these enzymes occur in multiple molecular forms and show heterogeneity during polyacrylamide-gel electrophoresis or molecular sieving. However, the latter two groups share the property of being serine proteinases, since proteolytic activity of human leucocyte-granule extract at pH 7.4 is totally inhibited by di-isopropyl phosphorofluoridate (Mounter & Atiyeh, 1960; Janoff & Zeligs, 1968; Gerber *et al.*, 1974).

After isolating pure forms of two neutral proteinases from horse blood leucocytes we studied their catalytic properties and the structure of the active centre.

Materials and Methods

Reagents

Casein was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., haemoglobin for proteinase

determination (by the method of Anson) from Merck, Darmstadt, W. Germany; bovine fibrinogen (plasminogen-free) from Miles Laboratories Ltd., Stoke Poges, Slough, Bucks., U.K.; crystalline bovine serum albumin from Charles Druce Ltd., London, U.K.; *N*-benzyloxycarbonyl-L-alanine 4-nitrophenyl ester (Z-Ala-ONp) from Fluka, Lucerne, Switzerland; *N*-acetyl-tri-L-alanine methyl ester (Ac-Ala₃-OMe) from Cyclo Chemical Corp., Los Angeles, Calif., U.S.A.; trypsin (bovine, twice crystallized) from Schuchardt, München, W. Germany; α -chymotrypsin (thrice crystallized), *N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) and di-isopropyl phosphorofluoridate from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; pancreatic elastase (twice crystallized) from Sigma Chemical Co., St. Louis, Mo., U.S.A.; Rose Bengal, containing 84% of pure dye, from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; *N*-benzoyl-L-arginine methyl ester (Bz-Arg-OMe) from Reanal, Budapest, Hungary; *N*-benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt) from Calbiochem, Los Angeles, Calif., U.S.A.; *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine *p*-nitroanilide (Bz-Phe-Val-Arg-NHPhNO₂) from AB Bofors, Stockholm, Sweden. Na¹³¹I (carrier-free, 10 mCi/ml) was supplied by IBI, Swierk, Poland. Dr. W. Ardel of the Institute of Rheumatology, Warsaw, Poland kindly provided resorcin/fuchsin-stained elastin and pancreatopeptidase E (elastase I, EC 3.4.21.11). The isolation and properties of this enzyme were described by Ardel & Ksiezny (1970).

Horse leucocyte proteinases 2A and 2B were purified by subcellular fractionation, gel filtration and ion-exchange chromatography (Dubin *et al.*, 1976). Their specific activities ranged from 150 to 250 units/mg of protein for proteinase 2A and from 310 to 580 units/mg of protein for proteinase 2B (where 1 unit is defined as a ΔE_{280} of 0.1 unit in a trichloroacetic acid filtrate after incubation for 60 min with 5% casein and 2-M urea at pH 7.4). Octyl and butyl isocyanates were synthesized by Dr. Z. Moskal (Jagiellonian University, Kraków). All other compounds and reagents were high-purity preparations obtained from commercial sources.

Determination of enzymic activities

Caseinolytic activity of leucocyte proteinases and esterolytic activity against Z-Ala-ONp were determined as described in the preceding paper (Dubin *et al.*, 1976), but the final concentration of Z-Ala-ONp was 0.2 mM unless otherwise stated. Proteolytic activity of trypsin, chymotrypsin and elastase was measured in a similar way but in the absence of urea; each sample contained 0.4 ml of enzyme in 0.9% NaCl, 0.3 ml of 0.1 M-sodium phosphate buffer, pH 7.4, and 1.8 ml of 5% (w/v) solution of casein in this buffer. After 60 min incubation at 37°C, 4.5 ml of 10% (w/v) trichloroacetic acid was added and ΔE_{280} was determined in the filtrate.

The elastolytic activity was determined by incubating enzymes with 50 mg of resorcin/fuchsin-stained elastin in 5 ml of 0.05 M-borate buffer, pH 8.7, for 1 h at 37°C followed by filtration and measurement of E_{560} . Chymotrypsin and trypsin activities were determined with Ac-Tyr-OEt and Bz-Arg-OMe respectively as described by Schwert & Takenaka (1955) and chymotrypsin activity with Bz-Tyr-OEt as described by Zendzian & Barnard (1967). Hydrolysis of Ac-Ala₃-OMe was measured at pH 7.4 by the colorimetric method described by Bieth & Meyer (1973) with final substrate concentration of 2 mM, and hydrolysis of Bz-Phe-Val-Arg-NHPhNO₂ was determined at pH 7.5 as ΔE_{405} (Z. Latallo, personal communication).

Inhibition of enzymes by active site-specific reagents

Inhibition of proteinases by di-isopropyl phosphorofluoridate was carried out as described by Zendzian & Barnard (1967) by incubating 100–300 μ g of enzymes in 0.1 M-sodium phosphate buffer, pH 7.4, for 5 min with 2 mM-di-isopropyl phosphorofluoridate; the residual caseinolytic activities were then determined.

For studying inhibition of proteinases by alkyl isocyanates, all stock solutions and dilutions of isocyanates were made in anhydrous acetone immediately before use. The actual concentration of isocyanates was determined with benzylamine as

described by Brown & Wold (1973). Proteinases were diluted with 0.1 M-Tris/HCl buffer, pH 7.5, to the concentration of 2–6 μ M and incubated for 10 min with butyl and octyl isocyanate at the reagent/enzyme molar ratio of approx. 50:1. The final concentration of acetone in the reaction mixture never exceeded 5%. Appropriate amounts of acetone without isocyanate were added to control samples. After incubation, samples (0.1–0.3 ml) were taken from the reaction mixture and the caseinolytic activity was determined.

Photochemical inactivation of proteinases was carried out as described by Westhead (1965), Fishman *et al.* (1973) and Rybarska & Ostrowski (1974) in the following manner: samples (3 ml) containing 100–200 μ g of enzyme and 30 μ g of Rose Bengal in 0.1 M-Tris/acetate buffer (pH 5–9) were placed in a water-jacketed glass vessel cooled with running tap water (approx. 15°C) and magnetically stirred. The vessel was illuminated by using a 300 W slide projector bulb as light source at the distance of 9 cm. At different time-intervals (5–30 min) portions (0.2 ml) of the reaction mixture were withdrawn and caseinolytic activity was determined. The values obtained were expressed as a percentage of initial enzymic activity and plotted on a semi-logarithmic scale. The slopes of the curves were used for calculation of the inactivation rate constants for particular pH values to obtain the final graph showing pH-dependence of photoinactivation.

Modification of proteinases by iodination was carried out in the following way: ICl stock solution was prepared as described by McFarlane (1964) and its molarity accurately determined by titration with sodium thiosulphate. Before protein iodination ICl was diluted with 2 M-NaCl to 0.002–0.004 M and carrier-free Na¹³¹I was added (final radioactivity approx. 5 μ Ci/ml). Calculated amounts (usually 3 nmol) of proteolytic enzyme in 0.7 ml of 0.1 M-Tris/HCl buffer, pH 8.5, were mixed with 5–100 μ l of this ICl solution. Control samples contained enzyme and 5–100 μ l of 2 M-NaCl (without ICl). After 5 min incubation at room temperature (20°C), 0.2 ml of 0.2 M-KH₂PO₄ was added to each sample and then portions (0.2 ml) were withdrawn (in duplicate) for determination of caseinolytic activity as well as total and protein-bound radioactivity in a well-type USB2 scintillation counter. The molar ratio ICl/enzyme usually ranged from 5 to 100 with the observed efficiency of iodination between 50 and 10%. The highest efficiency of iodination was found within the molar ratio 5–20, but above this value it decreased, indicating that iodine-binding sites of the enzyme are saturated with excess of the reagent. From the efficiency of iodination and molar ratio the number of iodine atoms incorporated per enzyme molecule was calculated. The obtained values were used to plot the percentage of remaining caseinolytic activity against the number of iodine atoms in the enzyme molecule.

Results

Preliminary studies (Chudzik, 1972; Dubin *et al.*, 1974) indicated that degradation rate of casein or haemoglobin by horse leucocyte proteinases was almost doubled in the presence of 2–3M-urea. In the present experiments protein substrates were dissolved in 5M-urea in a suitable buffer and then diluted with enzyme at the start of incubation to a final urea concentration of 2–3M.

Fig. 1 shows the pH optimum of casein hydrolysis by leucocyte proteinases. The highest degradation rates were observed in the pH range 7.0–7.7 for both enzymes. It is noteworthy that at pH 5.5 or 9.0 casein is still degraded at approximately 25% of the maximum rate, so it is clear that leucocyte proteinases 2A and 2B show a rather broad pH optimum.

Fig. 1. pH optimum of proteinase 2A (Δ) and 2B ($\blacktriangle$)

Hydrolysis of 5% casein solution in the universal buffer of Britton & Robinson (see Dawson *et al.*, 1969) containing 5M-urea was measured and maximum activity was assumed to be 100%. The final urea concentration in incubated samples was 2.08M.

When comparing hydrolysis of various proteins at pH 7.4, casein was found to be the best substrate for the two proteinases (Table 1). However, some differences in enzymic activity are evident since proteinase 2A preferentially digests casein and haemoglobin, whereas proteinase 2B digests casein and fibrinogen.

Hydrolysis of casein was investigated in detail and it was found that proteinase 2A shows a lower affinity toward this substrate than proteinase 2B, as indicated by their K_m values (12.8 and 6.0mg/ml respectively).

Both proteinases are able to digest fuchsin-stained elastin although at a much slower rate than pancreatopeptidase E. When the ratios of elastolytic/caseinolytic activities were calculated after dividing $100 \times \Delta E_{560}$ by ΔE_{280} for a given enzyme solution it was found that pancreatopeptidase E shows the value of 13.45, leucocyte proteinase 2A, 3.64; 2B, 2.27; leucocyte proteinase 1 (crude preparation), 2.31; and commercial trypsin, 0.35. Further studies are required to determine the kinetics of elastin degradation by leucocyte proteinases but it appears that these enzymes are much less active against this protein in comparison with pancreatic elastase.

Further data on substrate specificity of leucocyte proteinases were obtained after analysing the rate of hydrolysis of two synthetic substrates of elastase, Z-Ala-ONp and Ac-Ala₃-OMe (Table 2). It is evident that the former compound is readily decomposed by all leucocyte proteinases and elastase I, but it is also hydrolysed by chymotrypsin and trypsin. On the other hand, Ac-Ala₃-OMe is hydrolysed highly specifically, in agreement with other reports (Gertler & Hofmann, 1970; Feinstein *et al.*, 1973). Detailed kinetic parameters of Ac-Ala₃-OMe hydrolysis by horse leucocyte proteinases and pancreatic elastase I are presented in Table 3. Assuming that k_{cat}/K_m ratio is an indicator of the biological efficiency of enzyme, it can be concluded that Ac-Ala₃-OMe is a rather poor substrate for proteinase 2A and better for proteinase 2B, but both leucocyte enzymes are less active than pancreatic elastase. On the other hand, purified proteinases 2A and 2B, as well as the full extract of horse leucocyte granules, did not hydrolyse synthetic

Table 1. Degradation rates of various proteins by purified horse leucocyte proteinases 2A and 2B

5% solutions of proteins were digested at pH 7.4 in the presence of 2M-urea (see the Materials and Methods section). Specific activities are expressed in proteolytic units/mg of enzyme. Relative activities are expressed with casein as a reference substrate.

Substrate	Proteinase 2A		Proteinase 2B	
	Specific activity	Relative activity	Specific activity	Relative activity
Casein	170	100	580	100
Haemoglobin	156	92	183	32
Fibrinogen	82	48	398	69
Serum albumin	62	36	108	19

Table 2. Comparison of esterolytic and caseinolytic activities of horse leucocyte proteinases and some pancreatic proteinases

Rates of hydrolysis of 0.2–0.025 mM solutions of Z-Ala-ONp were determined at pH 6.5 and that of 2.0–0.2 mM solutions of Ac-Ala₃-OMe at pH 7.4. The initial velocities were used for calculating K_m by the Lineweaver–Burk plot. Caseinolytic activity was determined at pH 7.4, with urea for leucocyte proteinases and without urea for pancreatic enzymes (see the Materials and Methods section). The ratios of appropriate esterolytic/proteolytic activities (E/P) were calculated by dividing the rates of Z-Ala-ONp hydrolysis (at 0.2 mM) or Ac-Ala₃-OMe hydrolysis (at 2 mM) by caseinolytic activity of the enzyme solution. NMA, No measurable activity.

Proteinase	Z-Ala-ONp		Ac-Ala ₃ -OMe	
	K_m (mM)	100 × (E/P)	K_m (mM)	100 × (E/P)
Leucocyte proteinase 1	0.714	1.95	1.60	7.52
Leucocyte proteinase 2A	0.114	0.94	5.55	5.19
Leucocyte proteinase 2B	0.178	1.76	0.98	5.84
Pancreatic elastase I	0.133	0.83	1.02	25.00
α -Chymotrypsin	0.143	0.26	66.70	0.37
Trypsin	0.083	0.19	NMA	—

Table 3. Kinetic parameters of Ac-Ala₃-OMe hydrolysis by horse leucocyte proteinases and pancreatic elastase I

The hydrolysis rate of Ac-Ala₃-OMe (2.0–0.2 mM) was determined at pH 7.4. The Michaelis constant was calculated from the Lineweaver–Burk plot, and k_{cat} from the maximum velocity and concentration of the enzyme.

Enzyme	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)
Leucocyte proteinase 2A	5.55	9.6	1730
Leucocyte proteinase 2B	0.98	13.4	13700
Pancreatic elastase I	1.02	50.6	49200

substrates of trypsin (Bz-Arg-OMe), chymotrypsin (Bz-Tyr-OEt, Ac-Tyr-OEt) and thrombin (Bz-Phe-Val-Arg-NHPhNO₂).

Some information on the structure of the active centre of leucocyte proteinases was obtained by using specific inhibitors and reagents that modify definite amino acid residues. It was found that 2 mM-diisopropyl phosphorofluoridate completely blocked both caseinolytic and esterolytic (Z-Ala-ONp) activity of purified proteinases 2A and 2B, and of the whole-granule extract. Hence it may be concluded that these enzymes belong to the serine-proteinase group.

Brown & Wold (1971, 1973) introduced alkyl isocyanates as active site-specific reagents for studying the structure of the substrate-binding pocket of serine proteases. They observed that octyl isocyanate preferentially inactivated chymotrypsin, and butyl isocyanate showed greater efficiency toward elastase, whereas trypsin was little affected at the concentrations of inhibitors used. We fully confirmed these observations (Table 4) although commercial elastase slightly deviated from the predicted pattern. Leucocyte proteinases 2A and 2B show a great similarity to elastase I. This provides a further proof that neutral

Table 4. Differential inhibition of some proteinases by butyl isocyanate and octyl isocyanate

The enzymes were diluted with 0.1 M-Tris/HCl buffer, pH 7.5, to a concentration of 2–6 μ M and incubated with butyl isocyanate (BuNC) or octyl isocyanate (OcNC) at a molar ratio reagent/enzyme of 50:1. The residual proteolytic activity then was determined and BuNC/OcNC ratio was calculated.

Enzyme	Remaining activity (%)		BuNC/OcNC ratio
	Butyl isocyanate	Octyl isocyanate	
Trypsin	86	87	0.99
Chymotrypsin	30	5	6.00
Elastase (Sigma)	80	97	0.83
Elastase I (Ardelt)	14	86	0.16
Leucocyte proteinase 2A	24	82	0.29
Leucocyte proteinase 2B	11	72	0.15

proteinases from horse polymorphonuclear leucocytes are elastase-like enzymes.

It is known that pancreatic serine proteinases also contain histidine in the active centre. A rather specific modification of the histidine residue depends on its photo-oxidation at an appropriate pH in the presence of sensitizing dyes such as Methylene Blue or Rose Bengal (Westhead, 1965; Fishman *et al.*, 1973). Initial experiments were carried out with trypsin and elastase I in the pH range 5–9, and then identical determinations were completed with partly purified proteinase 2 [a mixture of proteinases 2A and 2B after Sephadex G-75 column chromatography; cf. Dubin *et al.* (1976)]. The results are shown in Fig. 2. It is clear that at pH 5–6 the photoinactivation rate of all examined enzymes is low but then increases rapidly up to pH 8. The experimental curves resemble the theoretical ionization curve of imidazole (Westhead, 1965;

Fig. 2. Influence of pH on photo-oxidation of trypsin (Δ), leucocyte proteinase 2 ($\bullet$) and pancreatic elastase I ($\circ$) in the presence of Rose Bengal

Fig. 3. Inactivation of trypsin (—), pancreatic elastase I (---), leucocyte proteinase 2A (Δ) and 2B ($\circ$) during iodination

The curves for trypsin and pancreatic elastase are based on a large number of measurements, but the experimental points are omitted for the sake of clarity.

Rybarska & Ostrowski, 1974; Murachi & Okimura, 1974). This fact strongly suggests the involvement of histidine in the catalytic centre of leucocytic proteinases. It is noteworthy that the curve obtained for leucocyte proteinases is almost identical with that of trypsin, but slightly differs from that of elastase I.

Exposure of enzyme molecule to ICl leads to modification of tyrosine residues, although cysteine, histidine, and to a smaller extent methionine and trypto-

phan, may also be affected (Filmer & Koshland, 1964). Fig. 3 shows the relationship between the number of iodine atoms incorporated per enzyme molecule and the progressive inactivation of proteinases. It is evident that 50% inactivation of trypsin is achieved after incorporation of approximately five iodine atoms, whereas elastase I requires ten iodine atoms for such inactivation. With two iodine atoms bound/enzyme molecule a slight activation of elastase was observed. The number of experimental points obtained for leucocyte proteinases is too small to permit the drawing of inactivation curves, but it is clear that proteinase 2A resembles elastase I whereas proteinase 2B is even more sensitive to iodination than trypsin. The experiments do not prove that tyrosine is involved in the active centre of the examined proteinases, but the observed differences in sensitivity to iodination bear direct implications to enzyme structure and catalytic mechanism. Moreover, it is evident that when radioiodination of these proteinases is attempted for metabolic studies, the number of incorporated iodine atoms should not exceed two.

Discussion

Neutral proteinases purified from horse blood leucocytes show a broad specificity and broad pH optimum and thus may be involved in the degradation of various tissue proteins in both physiological and pathological conditions. Since they also attack elastin and some synthetic substrates of pancreatic elastase they should be classified as elastase-like cellular proteinases. The observed sensitivity to active-site-specific inhibitors indicates that their active centre includes serine and histidine residues.

In the preceding paper (Dubin *et al.*, 1976) we reported some differences in molecular parameters of proteinases 2A and 2B. It appears that these structural alterations are responsible for the observed differences in catalytic properties (Tables 1–3) and in sensitivity to iodination of both enzymes (Fig. 3). All these facts suggest that proteinase 2A and 2B represent distinct proteins rather than multiple forms of the same enzyme.

Our experiments indicate that horse blood polymorphonuclear leucocytes contain only one class of leucocyte neutral proteinases, i.e. elastase-like enzymes, in distinction to human leucocytes (Ohlsson & Olsson, 1973, 1974; Schmidt & Havemann, 1974). Hence postulated species-dependent differences in the enzymic pattern of leucocyte neutral proteinases (Davies *et al.*, 1971; Dewald *et al.*, 1975) seem to be confirmed.

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