Characterization of Two Acid Proteinases Found in Rabbit Skin Homografts

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Two types of acid proteinase activity found in rabbit skin homografts were characterized by studying the effect of temperature, pH and polyacrylamide-gel electrophoresis. Their chromatographic behaviour was characterized on DEAE-cellulose, Sephadex G-75, G-100 and G-200, and their molecular weights were estimated by gel filtration. One of the acid proteinases in the homograft resembled cathepsin D (EC 3.4.23.5) of normal skin. The other acid proteinase differed from cathepsin D with respect to heat inactivation, pH optimum and molecular weight; it was not inactivated on heating at 60°C for 60 min, its pH optimum was 2.5 and its molecular weight measured by Sephadex G-100 chromatography was 100000. In all these respects, the heat-stable proteinase resembles cathepsin E (EC 3.4.23.5) of rabbit polymorphonuclear leucocytes.

Proteinase activity with pH optima in the range pH2.0 to pH4.5 has been reported in several mammalian tissues, including the spleen (Press *et al.*, 1960; Lapresle & Webb, 1960), bone marrow (Lapresle & Webb, 1960) and the liver (Barrett, 1967).

Unlike the activities in the liver and spleen, the proteinase activity in the bone marrow was found to have two components with different pH optima (Lapresle & Webb, 1962). One of these, cathepsin D, has a pH optimum of 3.5; the other, cathepsin E, has a pH optimum of 2.5. Since the cellularity of the bone marrow differs from that of the spleen mainly with respect to the predominance of polymorphonuclear leucocytes, Lapresle & Webb (1962) concluded that cathepsin E was probably associated chiefly with this cell type, an inference that has since been corroborated by several workers (Stefanovic *et al.*, 1962; Cochrane & Aikin, 1966).

Preliminary observations reported previously from our laboratory revealed that as in the liver (Barrett, 1967) rabbit skin also possessed only one acid proteinase, data that are also consistent with those of Lazarus (1974). Its pH optimum was 3.0 and it was labile to heating at 60°C for 5 min (Jasani, 1973). After transplantation, however, rabbit skin was found to possess two acid proteinase activities, one that resembled the heat-labile enzyme found in non-grafted skin, and the other, with pH optimum of 2.5, that remained active despite heating at 60°C for up to 90 min. This latter activity will, therefore, be referred to as the heat-stable proteinase.

Since the pH optima of these heat-labile and heat-stable proteinases resemble those of cathepsin D

(pH3.5) and E (pH2.5) of Lapresle & Webb (1962), the present experiments were designed to establish the extent of the resemblance by means of chromatography and gel electrophoresis.

Materials and Methods

Samples of rabbit skin homografts were obtained by the method of Jasani & Lewis (1971) and immediately frozen. Bovine haemoglobin (twice crystallized) was purchased from Calbiochem, San Diego, CA, U.S.A. DEAE-cellulose (Whatman DE-52) was obtained from H. Reeve Angel and Co., London E.C.4, U.K. Sephadex G-75, G-100, G-200 and Blue Dextran 2000 were products of Pharmacia Fine Chemicals, Uppsala, Sweden. Egg albumin (grade VI), bovine γ -globulins (Cohn fraction II), bovine serum albumin (fraction V), Coomassie Brilliant Blue R, *NNN'*-tetramethylethylenediamine, acrylamide, *NN'*-methylenebisacrylamide and riboflavin were supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Tris/HCl buffer (0.05 M; pH8.0) was prepared according to the manufacturer's instructions from Trizma Base and Trizma HCl (Sigma). Stock acid mixture containing formic acid (23.1 ml; Analytical reagent, Fisons Scientific Apparatus, Loughborough, Leics., U.K.), orthophosphoric acid (33.6 ml; AnalaR grade, BDH Chemicals, Poole, Dorset, U.K.) and acetic acid (34.4 ml; Analytical reagent, Fisons) was diluted with water to 1 litre (Barrett, 1967).

The skin homografts (100–250 mg wet wt.) removed between 3 and 8 days after transplantation, were chopped in the frozen state and homogenized with a Polytron mixer-homogenizer (Northern Media Supply, Brough, North Humberside, U.K.)

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in ice-cold 0.15M-sucrose medium (5.0ml) for 60s at maximum speed. This iso-osmotic medium contained the following electrolytes: MgCl_2 (9mM), KCl (75mM), Tris/HCl buffer (35mM) adjusted with Trizma HCl or Trizma Base, to pH 7.4 at 37°C. The initial homogenate was centrifuged at 4°C and 5000g for 15min (MSE Major centrifuge, eight-place rotor 6875) and the clear supernatant retained. The sediment from the first centrifugation was resuspended in 4.5ml of the sucrose medium and rehomogenized as above. Triton X-100 (0.5ml; 2%, v/v) was added to the homogenate, which was then agitated intermittently for 30min before centrifugation as above. The clear supernatant was combined with that obtained after the first homogenization. This procedure solubilized up to 90% of cytoplasmic as well as membrane-bound enzymes (Jasani, 1973). In the present study homogenates from several experiments were pooled to provide approx. 500ml of starting material.

Control skin was homogenized similarly though in larger amounts (0.75–1.5g in 9.0ml initially, and the sediment in 8.5ml of sucrose medium and 1.0ml of Triton X-100).

The pools were freeze-dried and the resultant powders suspended in 200ml of Tris/HCl buffer (0.05M; pH 8.0) and dialysed against this buffer (5litres) at 4°C for 16h. During dialysis a heavy precipitate appeared. On redissolving in Tris/HCl buffer, pH 8.0, it exhibited no proteinase activity.

Cathepsin assay

A modification of the procedure of Anson (1938) was used. Bovine haemoglobin (10mg/ml) was dissolved in 0.01M-HCl, filtered through Whatman no. 1 paper and adjusted to pH 2.5 with 1M-HCl before use.

FAP buffer (0.3M) was prepared by adjusting the pH of portions (50ml) of stock acid mixture (0.6M, pH 1.1) to the required value by adding 4M-NaOH and diluting to 100ml.

Incubation mixtures contained FAP buffer (0.3M, pH 2.5 or 3.5, 0.5ml), enzyme sample (0.2ml) and haemoglobin solution (0.4ml). For routine assay of fractions collected after column chromatography the samples were incubated at 37°C in a water bath, with continuous shaking for 16h. The reaction was terminated with ice-cold trichloroacetic acid (2.5ml; 7.5%, w/v). The tubes were kept at 4°C for 30min before centrifugation (1800g) at room temperature (20°C) for 10min in an MSE Multex centrifuge (rotor head 48 × 15ml; r_{av} , 17.5cm). The supernatants were decanted and A_{280} was recorded by using a Unicam SP.1800 spectrophotometer, with the reagent as blank.

Kinetic enzyme assays were used for the determination of pH optima within the range pH 1.5–4.5.

The incubation mixture consisted of buffer/substrate mixture (2.5ml) and tissue extract (0.5ml). Samples (0.5ml) were removed at 0, 20, 40 and 80min, added immediately to ice-cold trichloroacetic acid (3.0ml; 5%, w/v), centrifuged as above, and the A_{280} of the supernatants was recorded as before. The results were plotted to test linearity and for calculation of proteinase activity. At low enzyme concentrations, e.g. 200 units/ml of homogenate, the reaction was linear up to 120min, whereas at higher concentrations, e.g. 3000 units/ml, the reaction was linear only up to 10min.

To measure heat-stable proteinase activity, portions (0.2ml) of tissue extracts were heated at 60°C for 40min and then rapidly cooled. Buffer and substrate were then added and the assay was carried out as described above.

The results of both assays are expressed in arbitrary units of proteinase activity, one unit being equivalent to an increase in A_{280} of 0.001 unit/h.

Heat-treatment of crude and purified homogenate

To determine the heat-stability of the proteinase activity in crude homogenate the activities of samples of control (non-grafted) and homograft skin homogenates were assayed, at pH 2.9, without heating and also after heating for 5min at temperatures from 37°C to 90°C. The residual activity of each heat-treated sample was then calculated as a percentage of its activity without heat-treatment. To investigate the properties of the heat-stable component found in crude homogenates, samples were heated at 60°C for 40min and then subjected to further heating for 5min at temperatures from 37°C to 80°C before assay for proteinase activity.

The heat-stability of the purified enzyme found in peak II (Fig. 2) after DEAE-cellulose chromatography was determined by heating the extract at 60°C for 40min and then assaying samples after further heating at 60°C for periods of 10, 20, 30, 40, 50, 80, 110 and 165min. The activity remaining after total heating was expressed as a percentage of the activity of the unheated sample.

Protein determination

The protein concentrations of samples obtained at various stages of purification was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard. To obtain reproducible results, the samples were diluted to yield A_{750} values of between 0.1 and 0.2.

Ion-exchange chromatography

A column (3.0cm × 15.0cm) of DEAE-cellulose (Whatman DE-52) was prepared as recommended by the manufacturers and equilibrated with Tris/HCl buffer (0.05M; pH 8.0). The column was kept at 4°C,

the flow rate was controlled at 50 ml/h with a peristaltic pump (LKB Varioperpex 2120) and the eluate was collected in 10 ml fractions (LKB Ultrarac). The sample was pumped on to the top of the column and eluted with a linear NaCl gradient (0–0.5 M) in Tris/HCl buffer (total volume, 500 ml). The conductivity of the eluate fractions was measured with a conductivity meter (Radiometer, Copenhagen, Denmark) and the results were used to estimate the concentration of NaCl in the eluates. Eluates from chromatographic procedures were routinely monitored at 280 nm for protein concentration. Those fractions exhibiting significant absorbance at 280 nm were assayed for both heat-labile (pH 3.5) and heat-stable (pH 2.5) proteinase activities, as described above. All the fractions containing appreciable amounts of heat-labile or heat-stable activity were pooled separately, dialysed against Tris/HCl (0.05 M, pH 8.0) buffer and then freeze-dried and dissolved in water (10 ml).

Exclusion chromatography

Sephadex gel columns (3 cm × 92 cm) were prepared according to the manufacturer's instructions and equilibrated with Tris/HCl buffer (0.05 M; pH 8.0) containing NaCl (0.15 M). Sephadex G-75, G-100 and G-200 gels were used at 4 °C at flow rates of 20.0, 7.5 and 3.0 ml/h per cm² respectively. Heat-labile and heat-stable fractions from DEAE-cellulose chromatography were loaded by siphon on to the Sephadex column and eluted with Tris/HCl/NaCl buffer, by gravity. Fractions (4 ml) were collected and the protein concentration was monitored by A_{280} measurement. Those fractions containing protein were assayed for heat-labile and heat-stable proteinase activities. Fractions containing the two activities were again pooled separately, dialysed against Tris/HCl (0.05 M, pH 8.0) buffer and then freeze-dried and dissolved in water (10 ml).

The purified heat-labile and heat-stable proteinases were eluted in separate experiments on a previously calibrated Sephadex G-100 column (3.0 cm × 92 cm) by the method of Andrews (1965). The column was calibrated with a mixture containing Blue Dextran (2 mg, mol.wt. approx. 2000 000), bovine serum albumin (2 mg, mol.wt. 65 000–70 000), egg albumin (2 mg, mol.wt. 44 000–46 000) and bovine γ -globulin (2 mg, mol.wt. 150 000–170 000) in Tris/HCl (0.05 M, pH 8.0) containing NaCl (0.15 M). Fractions (4 ml) were collected, the A_{280} was measured, and a calibration curve of elution volume (V_e) against molecular weight plotted. The molecular weights of the two enzymes were then estimated on the basis of their elution volumes.

Polyacrylamide-gel electrophoresis

Electrophoresis was performed in polyacrylamide gels (7.5%) prepared by the method of Davis (1964)

but without sample gels. Photopolymerization with riboflavin (0.0005%, final concentration) was used instead of initiation with ammonium persulphate (Barrett, 1970). A Tris/glycine discontinuous buffer system (see Davis, 1964) was used giving pH 8.5 and the current was 5 mA per tube. Samples (50 μ l) contained in sucrose solution (0.2 ml, 5% w/v) were applied directly on to the spacer gel, overlaid with buffer without mixing and a cotton pellet washed in buffer was then carefully inserted into the tube (Clarke, 1964). The electrophoresis was carried out in the apparatus described by Davis (1964). Bromophenol Blue (1 ml; 0.001%) was used as the tracer dye. After 1 h the gels were removed by rinsing the tubes under water and then fixed in trichloroacetic acid (20%, w/v) for 60 min with agitation. To identify protein, one of three gels run in parallel was stained with Coomassie Blue (Chrambach *et al.*, 1967). The other two gels were chopped into 2 mm segments. Each segment was suspended in 2 ml of Tris (0.04 M)/glycine (0.4 M) buffer, pH 8.5, and homogenized with a Polytron homogenizer for 30 s at maximum speed. After centrifugation (10 min at 1800 g; MSE Multex centrifuge), supernatant samples (200 μ l) were assayed for proteinase activity.

Results

Effect of temperature on rabbit skin proteinase activity

Results given in Fig. 1 show that the proteinase activity of rabbit control skin, although stable to heating at temperatures up to 50 °C for 5 min, underwent inactivation at higher temperatures. The critical temperature for inactivation, i.e. the temperature at which 50% of activity was lost in 5 min, was 55 °C, and virtually total inactivation had occurred at 60 °C.

Proteinase activity in the rabbit skin homograft homogenate, results for which are also summarized in Fig. 1, initially exhibited similar characteristics to that in the control skin. Heat inactivation resulted in up to 40% loss of activity at 60 °C. The remaining activity showed no further depletion, despite heating at temperatures between 60 °C and 70 °C, although decreasing rapidly at temperatures higher than 70 °C, negligible activity being detectable after heating at 80 °C for 5 min. The critical temperature of inactivation of this second component was approx. 75 °C.

When pretreatment of the homogenate, by heating at 60 °C for 40 min, was followed by further heating at different temperatures for 5 min, the heat-stable component of the homogenate remained unaffected up to 70 °C, but underwent rapid inactivation at higher temperatures, total loss of activity being evident at 80 °C.

Separation of the two proteinases by ion-exchange chromatography

Fig. 2 shows the elution pattern obtained when skin homogenate supernatant (approx. 120 ml) was applied to a column of DEAE-cellulose DE-52 and eluted with an NaCl (0–0.5M) gradient in Tris/HCl (0.05M, pH 8.0) buffer. Protein was eluted continuously from the column until about 300 ml of eluate had passed through with three major peaks being discernible. Assay of the column fractions for acid proteinase activity revealed the presence of two peaks of activity, proteinases I and II, eluted at 45 mm- and 165 mm-Cl⁻ respectively.

Heating the eluate fractions at 60°C for 40 min resulted in the loss of activity from peak I, but an increasing proportion of the activity of peak II remained unchanged.

Furthermore, assay revealed that proteinase I exhibited greatest activity at pH 3.5, whereas proteinase II was more active at pH 2.5.

Molecular-weight estimation

Fractions containing the two proteinases obtained by DEAE-cellulose chromatography were separately pooled. When applied to a calibrated column of Sephadex G-100 the peak-I proteinase was eluted as a single peak of activity with an elution volume (V_e)

Fig. 1. Effect of temperature on the acid proteinase activity of rabbit skin

Pooled control homogenate was concentrated 3-fold to adjust its proteinase activity to that of pooled homograft skin homogenate. Samples (0.2 ml) of non-grafted control skin (○) and homograft skin (Δ) were heated at various temperatures for 5 min and cooled rapidly. Homograft skin (▲), preheated at 60°C for 40 min, was further heated at various temperatures for 5 min and cooled rapidly. Proteinase activity was assayed at pH 2.9 and is expressed as the percentage of activity found in unheated homogenates, 100% corresponding to 3000 units/ml as reported previously (Jasani, 1973).

Fig. 2. Separation of the two proteinases by DEAE-cellulose chromatography

Elution of proteins (b, ■) and enzyme (a) from a column of DEAE-cellulose DE-52 by a gradient of NaCl (●) in Tris/HCl buffer (0.05M, pH 8.0, calculated from conductivity measurements). Eluate fractions (10 ml) were assayed for proteinase activity without heating at pH 3.5 (a, ----) and after heating at pH 2.5 (a, —). Fractions (9–15) comprising peak I and fractions (19–24) comprising peak II were pooled and retained for further purification.

Fig. 3. Purification of proteinase I by Sephadex G-75 chromatography

Gel filtration on Sephadex G-75 of the heat-labile acid proteinase activity of pooled homograft skin homogenate. Columns (3 cm × 92 cm) were equilibrated with 0.05 M-Tris/HCl buffer, pH 8.0, containing 0.15 M-NaCl. Proteinase activity was measured at pH 3.5 and is expressed as in Fig. 2. Fractions 17–22 were pooled and retained for further purification.

of 238 ml. This value was reproducible ± 2 ml over three separate column runs. This elution volume corresponded very closely to that of egg albumin ($V_e = 236.5 \pm 2.5$ ml). The peak-II proteinase on the same column was eluted as a single peak of activity with 185 ml. The value was reproducible ± 1 ml over three separate column runs and was between the elution volumes of bovine serum albumin ($V_e = 206 \pm 1$ ml) and of rabbit γ -globulins ($V_e = 161 \pm 1$ ml). The molecular weight of proteinase I was estimated to be 42000–43000 and that of proteinase II was estimated to be 100000.

Purification of proteinases I and II by exclusion chromatography

Active fractions containing peak-I proteinase from the Sephadex G-100 column described above were again pooled, freeze-dried, redissolved in water (15 ml) and reappplied to a column of Sephadex G-75. The elution pattern for this step (see Fig. 3) shows that the proteinase activity was eluted as a single fairly symmetrical peak (V_e 210 ml) corresponding to none of the three major protein peaks but eluted between the first two.

Proteinase II was purified by exclusion chromatography as described above for proteinase I but using Sephadex G-200. The elution pattern (see Fig. 4)

shows that the proteinase activity was eluted as a single symmetrical peak (V_e 287 ml) just before the last of the three major protein peaks.

Data summarized in Table 1 show the increase in specific activity of proteinases I and II after each chromatographic separation.

pH optima of proteinases I and II

After the three-step purification, the pH optimum (see Fig. 5) of proteinase I was 3.0 and that of proteinase II was 2.5.

Effect of temperature on purified peak-II proteinase from DEAE-cellulose

To establish whether the heat-stability of proteinase in peak II obtained from DEAE-cellulose chromatography was a property of the enzyme protein, the active fractions eluted from Sephadex G-200 were combined, freeze-dried and resuspended in 10 ml of distilled water, and heated at 60 °C up to 120 min. Apart from confirming the observations recorded previously with the crude homogenate (see Jasani, 1973), the results of this experiment showed that only a small decline in the activity (not more than 20%) occurs on heating the purified proteinase II up to 120 min.

Fig. 4. Purification of proteinase II by Sephadex G-200 chromatography

Gel filtration on Sephadex G-200 of the heat-stable acid proteinase activity of pooled homograft skin homogenate. A column (3cm×92cm) was equilibrated with 0.05M-Tris/HCl buffer, pH8.0, containing 0.15M-NaCl. Proteinase activity was measured at pH2.5 and is expressed as in Fig. 2. Fractions 31–37 were pooled and retained for further purification.

Table 1. Effect of DEAE-cellulose and exclusion chromatography on the specific activity (units/mg of protein) of proteinases I and II of rabbit skin homografts

One unit of activity is equivalent to an increase in A_{280} of 0.001/h.

	Proteinase I (heat-labile)	Proteinase II (heat-stable)
Initial activity	235	130
Activity after:		
DEAE-cellulose	1033	1043
Sephadex G-100	3636	1550
Sephadex G-75	4284	—
Sephadex G-200	—	1882
Total yield (% of initial)	14.5	11

Polyacrylamide-gel electrophoresis

In the final product obtained after the three-step purification, the major peak of heat-labile enzyme activity was found in the protein band nearest the origin (see Fig. 6a). The minor peak of activity in the second band was too low to conclude the presence of an isoenzyme without further data. The third protein band was devoid of enzyme activity.

The major peak of heat-stable enzyme activity was found in the protein band with intermediate mobility (Fig. 6b). The two minor peaks of activity were again too low to conclude the presence of isoenzymes without further data, and of them only one corresponded to a protein band.

Discussion

Heat-labile proteinase

The acid proteinase activity present in rabbit control skin was labile to heating at 50°C and was totally inactivated by heating at pH6.8 to 60°C for 5 min (Fig. 1). A similar pattern of heat-lability was found for the purified acid proteinase activity associated with peak I obtained after DEAE-cellulose chromatography of the rabbit homograft skin homogenate (Fig. 2). The enzyme closely resembles cathepsin D found in (a) bovine spleen (Press *et al.*, 1960), (b) bovine uterus (Woessner & Shamberger, 1971), (c) rabbit polymorphonuclear leucocytes (Cochrane & Aikin, 1966), (d) pig spleen (Cunningham & Tang, 1976) and (e) human, rabbit and chicken liver (Barrett, 1971).

The peak-I proteinase was eluted from the DEAE-cellulose column at 45mM-Cl⁻, and this indicates that the enzyme is isoelectric at near-neutral pH. In this respect the enzyme resembles cathepsin D of rabbit polymorphonuclear leucocytes (Cochrane & Aikin, 1966) and human and chicken liver (Barrett, 1970).

The molecular weight of the heat-labile proteinase was estimated to be 42000–43000 by the method of Andrews (1965). This value exactly corresponds to that obtained for cathepsin D (see Barrett, 1971). It differs, however, from the values found for the enzymes, by the method of Andrews (1965), by Cunningham & Tang (1976) in pig spleen (35000), by Turk *et al.* (1966) in bovine liver and spleen (37000), by Lapresle (1971) in rabbit spleen (52000)

Fig. 5. Effect of pH on the activity of proteinase I and proteinase II

pH-optimum curves of the purified heat-labile (●) and heat-stable (■) acid proteinases from rabbit skin homografts with haemoglobin as substrate.

and in bone marrow (58000), and by Keilova (1971) in bovine spleen (55000–58000).

Since for enzymes from the same tissue and species different values have been reported by different investigators using the gel-filtration method, the question arises of whether the reported differences reflect errors of determination. An important variable has been identified by Woessner (1971), who found that the reference protein ovalbumin was eluted in different places under different conditions, even though bovine uterus cathepsin D was eluted reproducibly.

The pH-optimum curve of the heat-labile enzyme (Fig. 5) resembles that of cathepsin D of rabbit and chicken liver (Barrett, 1967, 1970), of bovine spleen (Press *et al.*, 1960; Turk *et al.*, 1968; Keilova, 1971) and of bovine uterus (Woessner & Shamberger, 1971) with the same substrate.

The heat-labile enzyme has a relatively low electrophoretic mobility at pH 8.5, thus supporting the suggestion that the enzyme has, like cathepsin D, a low negative charge at near-neutral pH.

Heat-stable proteinases

Up to 60% of the acid proteinase activity found in rabbit homograft skin was stable to heating at 60°C for 45 min, but was totally inactivated by heating at 80°C for 5 min. A similar pattern of heat-stability was also found for the purified acid proteinase activity associated with peak II after DEAE-cellulose chromatography (Fig. 2). As explained above, the effect of temperature on the enzyme activity in peak II of Fig. 2 varied, depending on the extent to which the fractions were contaminated with the heat-labile proteinase.

The proteinase activity associated with peak II was adsorbed strongly on DEAE-cellulose column and it was eluted at 165 mM-Cl⁻. This indicates that the enzyme has a high negative charge at pH 8.0. In this respect, it resembles cathepsin E. For example, Lapresle & Webb (1962) found that cathepsin E of rabbit bone marrow was eluted from a DEAE-cellulose column at 150–200 mM-Cl⁻, and Cochrane & Aikin (1966) found that cathepsin E of rabbit polymorphonuclear leucocytes was eluted similarly.

The molecular weight of the heat-stable proteinase was estimated to be 100 000 by the method of Andrews (1965). This value corresponds exactly to that found for rabbit bone-marrow cathepsin E by Lapresle (1971) using the same method. The estimate, however, does not agree with that for cathepsin E of bovine spleen reported by Turk *et al.* (1968). They estimated the molecular weight of bovine spleen proteinase, by ultracentrifugation instead of gel chromatography, to be 305 000. They elected to use ultracentrifugation because, in their experience, cathepsin E was excluded from Sephadex G-200.

The elution behaviour of the enzyme of rabbit skin homograft differs from that of bovine spleen reported by Turk *et al.* (1968), but resembles that of cathepsin E from rabbit bone marrow (Lapresle & Webb, 1962) and rabbit polymorphonuclear leucocytes (Cochrane & Aikin, 1966) in that it is not even excluded from Sephadex G-100 and this strongly indicates a molecular weight of less than 150 000.

Polyacrylamide-gel electrophoresis revealed the heat-stable proteinase to possess a high electrophoretic mobility at pH 8.5, which confirms the finding that like cathepsin E the enzyme has a high negative charge at near-neutral pH.

The pH-optimum curve of the heat-stable proteinase resembles that of cathepsin E of rabbit polymorphonuclear leucocytes (Cochrane & Aikin, 1966) and bone marrow (Lapresle & Webb, 1962).

However, the heat-stability of the cathepsin E-like proteinase of rabbit skin homografts appears to be greater than that reported for cathepsin E of rabbit bone marrow (Lapresle & Webb, 1962) or rabbit polymorphonuclear leucocytes (Cochrane & Aikin, 1966).

Fig. 6. Distribution of (a) heat-labile and (b) heat-stable proteinase in a polyacrylamide electrophoresis gel. The samples were the purified heat-labile and heat-stable enzyme preparations. In each case, after electrophoresis of three identical samples, one gel was stained for protein and the others chopped into 2mm segments for proteinase assay at (a) pH3.5 and (b) 2.5. Proteinase activity is expressed as in Fig. 2.

Light- and electron-microscopic examination of rabbit skin homografts reveals that beside a 5–6-fold increase in the number of epidermal cells there is a pronounced increase in the number of a variety of mononuclear cells, including immune lymphocytes, monocytes, macrophages, blast cells and plasma cells (Wiener *et al.*, 1964; Bullock *et al.*, 1977). Significantly, the polymorphonuclear leucocytes also are present from the outset and become more numerous with the onset of destructive changes in the graft (Wiener *et al.*, 1964; Rothwell & Papadimitriou, 1972). On the other hand, few if any polymorphonuclear leucocytes are present in the normal skin. This, and the fact that the cathepsin E-like activity is not present in lymphocytes, lymph nodes or macrophages (Lapresle, 1971), suggests that the heat-stable proteinase detectable in homografts may originate in the polymorphonuclear leucocytes.

From the practical viewpoint, results shown in Fig. 1 imply that heating at 60°C for 40 min does not significantly decrease the enzyme activity with optimum pH2.5 (peak II). For routine application, therefore, the heat-stable enzyme activity can conveniently be measured by heating crude homogenate at 60°C for 40 min.

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