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NEUTRAL PROTEINASE OF RABBIT SKIN:

AN ENZYME CAPABLE OF DEGRADING SKIN PROTEIN AND INDUCING AN INFLAMMATORY RESPONSE

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SUMMARY

1. A neutral proteinase has been extracted from rabbit skin in the presence of 1 M KCl and purified 86-fold.

2. The enzyme was active at neutral pH; it was not inhibited by diisopropylphosphorofluoridate, thiol-blocking reagents or EDTA.

3. About 85% of the activity in skin was located in the dermis, the remainder in the epidermis.

4. Incubation of skin with enzyme resulted in the release of trichloroacetic acid-soluble peptides from the tissue.

5. Intradermal injection of the purified neutral proteinase into rabbits produced whealing within 15 min and acute leukocytic infiltration within 18 h. It is suggested that this enzyme may play a part in the initiation of an inflammatory reaction in skin.

INTRODUCTION

The role of individual tissue proteinases in the catabolism of connective tissue components is being studied by use of specific inhibitors [1]. By use of specific antisera against cathepsin D we have been able to define the role of this enzyme in the resorption of rabbit skin (refs 2 and 3, and Lazarus and Dingle, unpublished). During the course of these studies, a distinctive caseinolytic proteinase active at neutral pH was detected. This enzyme was maximally extracted in high concentrations of KCl, and was similar to the neutral proteinase described by Beloff and Peters [4] and further studied by Inderbitzin [5] and Fraki and Hopsu-Havu [6]. The present paper reports the extraction, partial purification, localization, and physiological activity of this neutral proteinase.

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MATERIALS

Male New Zealand White Rabbits 8–12 weeks of age were obtained from the Strangeways Research Laboratory breeding colony. The following materials were obtained from commercial sources as indicated: casein (Hammersten grade), cytochrome *c*, DFP (diisopropylphosphorofluoridate) and Folin-Ciocalteu reagent, BDH Chemicals Ltd. Poole, Dorset, BH12 4NN, U.K.; chymotrypsinogen A type II, crystalline bovine serum albumin and egg albumin, Sigma London Chemical Co. Ltd., Kingston upon Thames, Surrey, KT2 7BH, U.K.; Sephadex and Blue Dextran, Pharmacia (Great Britain) Ltd., London W5 5SS, and Arquad 2C-50, Armour Hess Chemicals Ltd., Leeds, LS1 4NR, U.K.

METHODS

Neutral proteinase assay

An 8% (w/v) solution of casein was made by suspending the protein in distilled water, adjusting the pH to 7.0 with 1 M NaOH and heating at 65 °C for 30 min. Each incubation mixture contained 50 μl of casein solution, 50 μl of 50 mM sodium phosphate buffer, pH 7.0, and 100 μl of the enzyme preparation in 50 mM sodium phosphate buffer containing 1 M KCl. The tubes were incubated for 1 h at 55 °C after which 4 ml of 2.75% (w/v) trichloroacetic acid was added to each. After 10 min the precipitated casein was removed by filtration through a Whatman No. 1 filter paper and the $A_{280\ nm}$ of each filtrate was read. One unit of activity was defined as that quantity which produced an increase in absorbance ($\Delta A_{280\ nm}$) of 1.00 in the assay during 1 h under the specified condition. In some experiments trichloroacetic acid-soluble peptide reaction products were determined by a modification of the Lowry et al. [7] procedure. To 1 ml of the filtrate 2.5 ml of modified alkaline copper reagent (10 mg of trisodium citrate dihydrate, 5 mg of $CuSO_4 \cdot 5H_2O$, 0.64 g of NaOH and 2.0 g of Na_2CO_3 per 100 ml) was added. When the mixtures had stood for 10 min at room temperature, 0.25 ml of Folin-Ciocalteu reagent (diluted 1:3 with distilled water) was added with immediate mixing, and $A_{700\ nm}$ was measured after 30 min. Tyrosine in 2.5% (w/v) trichloroacetic acid served as a standard. In other experiments 8% (w/v) bovine haemoglobin prepared according to the method of Barrett [8], or 8% (w/v) bovine serum albumin, replaced casein as substrates. All assays were made in duplicate and control values were obtained by adding the enzyme preparation to the incubation mixture after the introduction of trichloroacetic acid.

The following buffers were used to investigate the effect of pH: formic acid–NaOH, pH 3.0; acetic acid–NaOH, pH 4.0 and 5.0; sodium phosphate, pH 6.0 and 7.0; Tris–HCl, pH 8.0 and 9.0, and sodium carbonate–bicarbonate, pH 10.0. The final molarity of each buffer in the assay mixtures was 250 mM.

Enzyme assays

Collagenase was measured using the radioactive reconstituted collagen fibril assay [9]. Cathepsin B1 activity was assayed by hydrolysis of benzoyl-DL-arginine *p*-nitroanilide [10].

Protein determination

The method was essentially that of Lowry et al [7], crystalline bovine serum albumin being used as standard.

Distribution of neutral proteinase in skin

Clipped back skin from an 8-week-old rabbit was trimmed of fat, cut into 1–2-mm cubes and washed in three changes of cold phosphate-buffered saline (0.80 g NaCl, 0.02 g KCl, 0.02 g KH_2PO_4 and 0.12 g Na_2HPO_4 per 100 ml; pH 7.2) for 3 h. The tissue (4 g) was then incubated in 20 ml of a solution of 2.5 mg of crude trypsin (Flow laboratories, Irvine, Ayrshire, U.K., type 1:250) in 0.9% NaCl solution for 2 h at 25 °C. Dermis and epidermis were separated under a dissecting microscope, and the completeness of separation was monitored by histological examination. The separated epidermis, separated dermis, unseparated trypsin-treated skin (1 g) and a sample of skin from the same rabbit, which had been incubated in phosphate buffered saline alone (1 g) were washed five times in phosphate-buffered saline for 12 h and then homogenized in 10 vol. (w/v) of 50 mM sodium phosphate buffer, pH 7.5, 250 mM KCl. After 12 h of extraction the tissue was removed by centrifugation at 10 000 $\times$ *g* for 15 min and proteinase assays were performed as usual, and also in the presence 1 mM DFP.

Treatment of skin with neutral proteinase in vitro

The skin from the back of a freshly sacrificed 10-week-old male rabbit was trimmed of hair and subcutaneous fat. The tissue was cut into cubes (1–2 mm) and washed overnight in 3 changes of cold phosphate-buffered saline. The following morning the tissue was rapidly frozen and thawed 5 times in liquid nitrogen. Portions of tissue (1 g) were incubated in a total volume of 2 ml at a final concentration of 50 mM sodium phosphate buffer, pH 7.5, 250 mM KCl, with 20 mg of enzyme protein (15 units of activity). This amount of enzyme represented a 5-fold excess over the extractable endogenous activity of the tissue. Control tubes contained heat-inactivated enzyme and all experiments were performed in triplicate. The mixtures were incubated at 45 °C with gentle agitation for variable periods of time. Incubations were stopped by adding 4 ml of water at 0 °C, mixing vigorously and placing the tubes in ice. The tissue was removed by centrifugation at 10 000 $\times$ *g* for 15 min (4 °C) and 1.8 $\pm$ 0.1 ml was recovered from the incubations. To 1 ml of each of these solutions 4 ml of 3% trichloroacetic acid solution was added; after 15 min the larger molecular weight peptides which had precipitated were removed by filtration through a Whatman No. 1 filter paper, and the clear filtrate was assayed for protein as previously described. Similar experiments were performed with tissue which had been depleted of proteolytic enzymes; skin was frozen and thawed 5 times and extracted exhaustively with 50 mM sodium phosphate buffer, pH 7.5, 250 mM KCl [3], until no proteinase activity remained in the tissue.

Injection of neutral proteinase

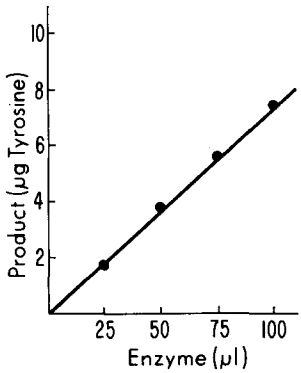
The back fur was removed from a 12-week-old rabbit with an automatic clipper and six injection sites were outlined with a gentian violet marking pen. 24 h later 0.1 ml of the most highly purified enzyme preparation (0.1 units per 0.1 mg) was injected intradermally into three areas and the same preparation which had been in-

activated by heating to 80 °C for 30 min was injected into the remaining three control sites. The reaction was closely monitored for 20 h.

RESULTS

Enzyme assay

The neutral proteinase assay was linear with respect to amount of enzyme used (Fig. 1) and duration of incubation (Fig. 2). The results shown were obtained using



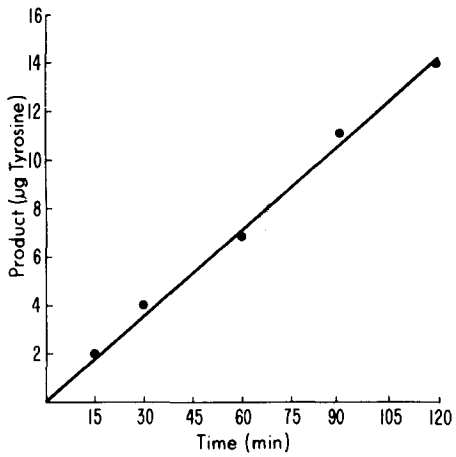


Fig. 1. Effect of enzyme concentration on the neutral proteinase assay. Increasing amounts of the enzyme preparation were included in the reaction volume and the effect on the release of trichloroacetic acid-soluble peptides was determined by the Lowry method.

Fig. 2. Effect of duration of incubation on the neutral proteinase assay. Assay mixtures containing a fixed amount of enzyme were incubated for varying periods of time. Trichloroacetic acid-soluble products were measured by the Lowry method.

the modified Lowry technique but similar results were obtained using direct measurement of $\Delta A_{280\ \mathrm{nm}}$. There was a slight loss of linearity above a $\Delta A_{280\ \mathrm{nm}}$ of 0.400 per h. Neutral proteinase activity, measured by both $\Delta A_{280\ \mathrm{nm}}$ and the Lowry technique, was dependent upon substrate concentration up to a final concentration of 0.4% casein (Fig. 3). A 5-fold excess of casein (2% final concentration) was routinely used in neutral proteinase determinations. To confirm the dependence of enzymic activity on exogenous substrate, autolytic experiments were performed. Acetone-fractionated neutral proteinase was incubated with buffer in the absence of substrate at 55 °C for 1 h; after the addition of trichloroacetic acid, the casein substrate was added. These autolytic samples liberated less than 10% of the total trichloroacetic acid-soluble material measured by the $\Delta A_{280\ \mathrm{nm}}$ or the Lowry method produced by the enzyme in the presence of substrate.

Purification of the enzyme

Extraction. Male New Zealand White Rabbits 8–12 weeks of age were clipped and then killed by blunt trauma to the neck. The dorsal trunk skin was immediately

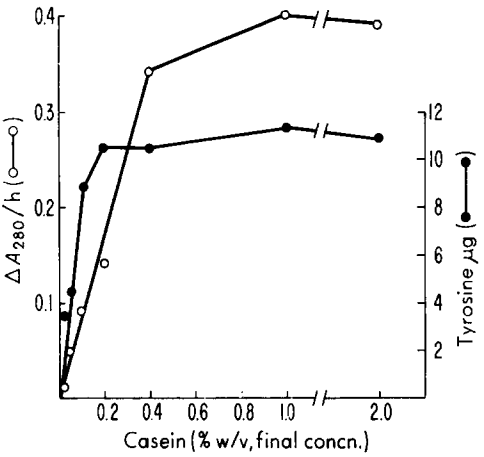


Fig. 3. Effect of casein concentration on neutral proteinase assay. Increasing concentrations of casein were included in the reaction mixture and the effect on the release of trichloroacetic acid-soluble peptides was determined by both the $A_{280\ nm}$ and Lowry methods.

removed, cut into small pieces and ground in an ice cold tissue grinder. The average yield of skin from 2 rabbits was 125 g. The ground skin was homogenized in 7 vol. (w/v) of 50 mM sodium phosphate buffer, pH 7.5, containing 1 M KCl in a Polytron Homogenizer (type PT 20; Northern Media Supply, Ltd., 11 Blanket Row, Hull, England), at mark 10 for 1 min. During this process the temperature was kept below 10 °C by keeping the preparation in ice and by homogenizing in 10-s bursts followed by 30-s rests. The tissue was extracted for 12 h at 4 °C with slow stirring, after which the crude homogenate was centrifuged at 10 000 × *g* for 30 min and the tissue pellet discarded. All subsequent steps were carried out at 4 °C.

The concentration of KCl in the phosphate buffer was of critical importance in the extraction procedure (Fig. 4). Insignificant amounts of enzyme could be

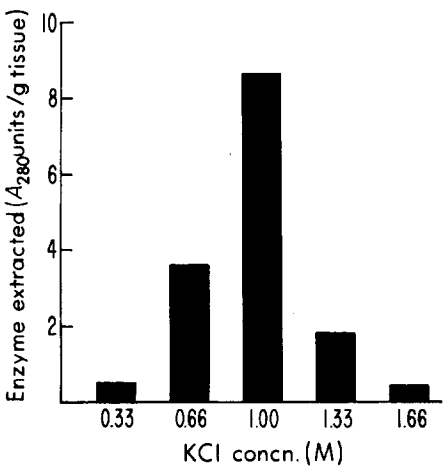


Fig. 4. Effect of the molarity of KCl on the extraction of the neutral proteinase. Portions of tissue (4 g) were extracted during 12 h at 4 °C with 30 ml of 50 mM sodium phosphate buffer, pH 7.5, containing varying amounts of KCl.

extracted with buffer alone whereas maximal extraction was found at a concentration of 1 M KCl. Above this concentration there was a significant increase in viscosity of the solution and a consequent decrease in the amount of recoverable supernatant after centrifugation. At 1.6 M KCl so much collagen and proteoglycan was extracted that essentially no supernatant was separated.

Arquad precipitation. To the supernatant, Arquad 2C-50 in the form of a 20% (v/v) emulsion [10] was added at the rate of 50 ml/l. After 30 min of gentle agitation, the precipitate was removed by centrifugation at 10 000 × *g* for 30 min at 15 °C. This step resulted in a modest but significant purification (Table I).

Acetone fractionation. To the cold Arquad supernatant solution, 0.4 vol. of anhydrous acetone at −10 °C was added during 10 min with constant stirring. The precipitate was collected at 10 000 × *g* for 15 min and discarded. A second portion of acetone equal to 0.7 vol. of the initial aqueous preparation was then added. The precipitate was collected and resuspended in 1/15 the volume of the original homogenate. The first acetone precipitate and the 1.1 vol. acetone supernatant had negligible enzymic activity and were discarded. The resuspended enzyme preparation was dialyzed exhaustively against three changes of 50 mM sodium phosphate buffer, pH 7.5, containing 1 M KCl. A precipitate which had negligible enzymic activity formed in the dialysis bag; it was removed by centrifuging and discarded. The enzyme preparation was concentrated in an Amicon Diaflo ultrafiltration cell with a UM-05 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.).

Sephadex chromatography. The concentrated enzyme preparation (5 ml) was applied to a bed (1.6 cm × 85 cm, 145 cm^3) of Sephadex G-100, equilibrated with 50 mM sodium phosphate buffer, pH 7.5, containing 1 M KCl. The column was eluted at 10 ml/h and 1.5-ml fractions were collected. Enzymically active fractions were pooled (Fig. 5), concentrated by ultrafiltration as above and reapplied to the column.

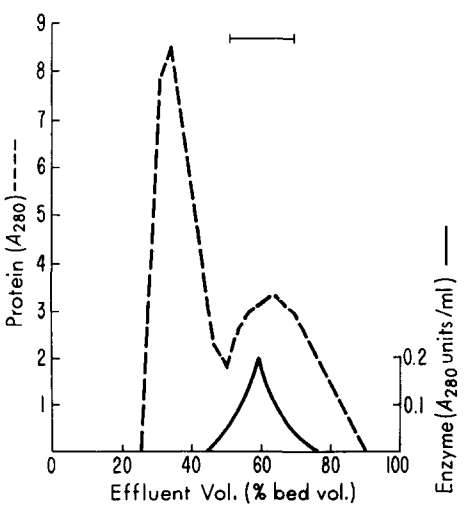


Fig. 5. Gel chromatography of the neutral proteinase. The enzyme sample was run on a column of Sephadex G-100 as described in the text, and the concentration of protein (– – –) and enzyme activity (———) in the effluent was determined. Active fractions which were combined and rechromatographed are marked by the horizontal bar.

TABLE I

PURIFICATION OF A RABBIT SKIN NEUTRAL PROTEINASE

	Protein (g)	Total activity (units)	Spec. act. (units/mg)	Purification	Yield (%)
Homogenate	28.8	1012	0.035	1	100
Arquad supernatant	18.83	921.4	0.048	1.3	91
Acetone (0.4–1.1 vol.) precipitate	7.76	1179	0.152	4.3	110
Sephadex G-100 (X2)	$6.8 \cdot 10^{-2}$	204	3.0	86	20

The active pool from the second chromatographic run showed an 86-fold purification from the homogenate and was used for the further experiments. The Sephadex column was calibrated for the measurement of molecular weight by running a sample (4 ml) containing Blue Dextran (4 mg), bovine serum albumin (15 mg), egg albumin (15 mg), chymotrypsinogen (5 mg), cytochrome *c* (6 mg) and $K_3Fe(CN)_6$ (4 mg). The elution position of the enzyme, immediately before cytochrome *c*, indicated a molecular weight of 15 000–20 000.

Properties of the partially purified neutral proteinase

The enzyme after rechromatography on Sephadex G-100 was 86-fold purified (Table I), but could be shown not to be homogenous since numerous bands were seen on analytical isoelectric focusing gels in the range pH 3–10 [8].

The partially purified enzyme showed a broad pH-dependence, with greatest activity at pH 7.5 (Fig. 6). Acidification to pH 4 resulted in precipitation of the enzyme

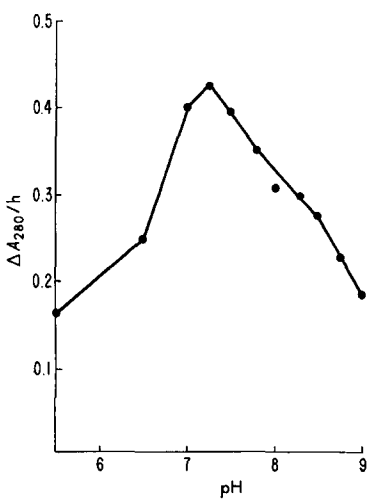


Fig. 6. The effect of pH on the activity of the neutral proteinase. Experimental details are given in the text.

TABLE II

ACTIVATION OF ACETONE-FRACTIONATED NEUTRAL PROTEINASE BY SALTS

The enzyme preparation, dialyzed against 50 mM sodium phosphate buffer, pH 7.5, showed no activity without added salt. The activity that reappeared after the addition of various salts (final concentration in assay 250 mM) is expressed as a percentage of that obtained with KCl.

Cation	Anion: Cl^-	I^-	Br^-
K^+	100	29	28
Na^+	49	0	0
Mg^{2+}	62	0	0

and only 60% of the activity could be redissolved from the precipitate. Incubation of the enzyme, in the absence of substrate, at 50 °C for 30 min resulted in a 50% fall in activity.

The $A_{280\ nm}$ values obtained in assays in which haemoglobin or albumin replaced casein were almost identical to those obtained with the normal substrate. The enzyme did not solubilize reconstituted collagen fibrils and did not hydrolyze benzoyl-DL-arginine *p*-nitroanilide in the presence or absence of 1 mM cysteine.

The effect of KCl on activity

Enzyme samples at various stages of purification were dialyzed against 50 mM sodium phosphate buffer; various salts were then added to the solution to give a final concentration of 250 mM and activity was measured 30 min later (Table II). KCl was the salt found most effective in the activation of the acetone-fractionated neutral proteinase. Only preparations purified by gel chromatography showed significant activity in phosphate buffer alone and even with these the activity was doubled at 250 mM KCl (Fig. 7). These data, like previous reports [6, 11–15] could suggest that the KCl acts by dissociating the enzyme from a complex with an inhibitor; attempts to detect such an inhibitory component in fractions from the Sephadex column by measuring their effect on the activity of the enzyme were unsuccessful.

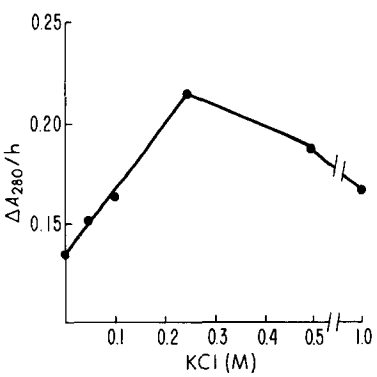


Fig. 7. The effect of KCl concentration on the activity of the purified neutral proteinase. The release of trichloroacetic acid-soluble peptides from casein was measured in assays made at various concentrations of KCl, as described in the text.

The effect of various potential inhibitors

No appreciable inhibition or activation (i.e. greater than 10%) was detected when the partially purified enzyme was assayed in the presence of Tos-LysCH_2Cl (1 mM), Tos-PheCH_2Cl (1 mM), *p*-chloromercuribenzoate (1 mM), trisodium EDTA (1 mM), pepstatin (200 μg/ml), 6-aminohexanoic acid (10 mM), soyabean trypsin inhibitor (1 mg/ml), or NaCN (10 mM). DFP (1 mM), which inhibited the caseinolytic activity of trypsin (50 μg/ml), did not inhibit neutral proteinase after 1 h incubation.

Distribution of neutral proteinase in skin

Skin incubated with trypsin (see Methods) gave 50% more soluble proteinase activity than skin incubated with buffer alone, when homogenized. All of the neutral proteinase activity in the unseparated tissue could be accounted for in the pellets of separated dermal and epidermal tissue. Separation of 2.1 g of skin yielded 1.80 g of dermal tissue, containing 85% of the total proteinase, and 0.30 g of epidermis containing the remaining 15% of activity.

The possibility that trypsin was responsible for some of the proteolytic activity of the separated skin components was excluded by the finding that DFP completely inhibited the trypsin preparation but not the skin extract and the skin extracts did not hydrolyze the trypsin substrate benzoyl-DL-arginine *p*-nitroanilide.

Effect of neutral proteinase on skin in vitro

Repeated freezing and thawing of tissue causes release of lysosomal enzymes which can cause autolytic degradation of protein. The addition of a 5-fold excess of acetone-precipitated, partially purified neutral proteinase to autolyzing tissue resulted in a 12-fold increase in the production of trichloroacetic acid-soluble peptides (Fig. 8);

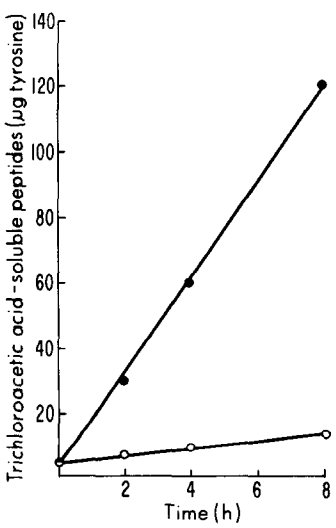


Fig. 8. The effect of neutral proteinase on the release of trichloroacetic acid-soluble peptides from skin. Samples of skin were incubated as described in the text with partially purified enzyme (●—●) or with the heat-inactivated enzyme (○—○).

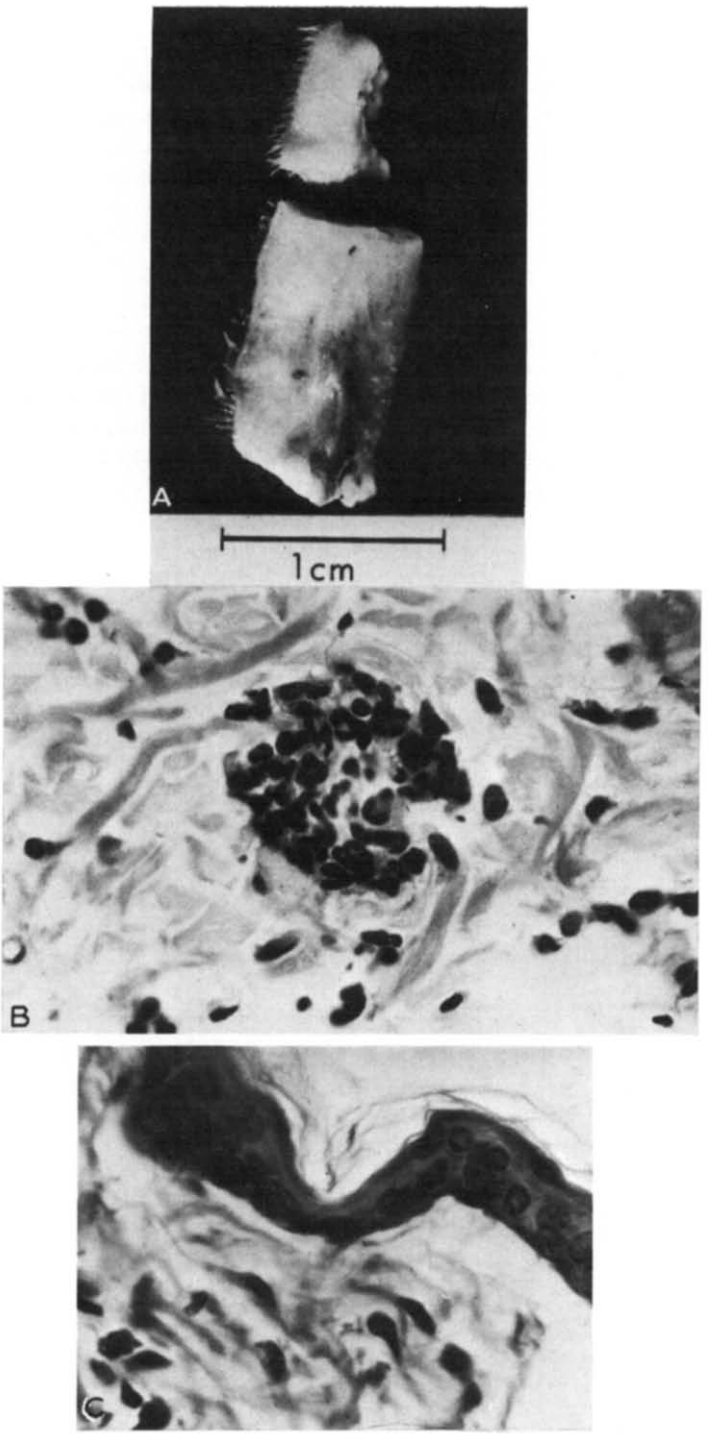


Plate 1. Effect of injection of purified neutral proteinase into rabbit skin. (A) Macroscopic appearance of rabbit skin removed 18 h after injection of the enzyme (below) or the heat-inactivated enzyme (above). (B) Micrograph ($\times 480$) of a wax-embedded section of skin after the intradermal injection of neutral proteinase, stained with haematoxylin and eosin. An acute polymorphonuclear leucocytic infiltration with necrosis is seen. (C) Micrograph ($\times 480$) of a skin section from the periphery of the lesion that followed injection of skin proteinase. There is dermal-epidermal separation that appears independent of leukocytic infiltration.

presumably, the neutral proteinase acted synergistically with other endogenous enzymes in the degradation of protein macromolecules in vitro. Neutral proteinase by itself is capable of degradation of skin protein. Incubation of neutral proteinase with enzyme-depleted skin resulted in the release of 20 μg of trichloroacetic acid-soluble tyrosine in 2 h.

Injection of neutral proteinase into rabbit skin

The injection of 0.1 ml of 86-fold purified neutral proteinase (0.1 unit per 0.1 mg) into rabbit skin resulted in the production of a 3-cm wheal within 15 min. At 18 h the skin which had been injected with active enzyme was twice as thick as that at control sites which had been injected with heat-inactivated enzyme (Plate 1a). Histological examination of the tissue revealed an acute polymorphonuclear leukocytic infiltration (Plate 1b) and dermal-epidermal separation below the basement membrane; this separation was quite marked even in areas where there was no infiltration by inflammatory cells (Plate 1c). Sites injected with inactivated enzyme showed rare polymorphonuclear leukocytes, but no dermal-epidermal separation.

DISCUSSION

Caseinolytic activity in skin was first shown by Sexmith and Petersen in 1918 [16]. Beloff and Peters [4] demonstrated that a casein-hydrolyzing activity could be effectively extracted from skin in the presence of 5% (w/v) KCl; they postulated that salt dissociated the enzyme from a plasma inhibitor. Subsequently, several investigators have used salts in the extraction of neutral proteinases (for review see ref. 13).

Cathepsin D has been found in rabbit skin and is responsible for proteolytic digestion of hemoglobin below pH 5 and of casein at pH 5.5 [2, 3]. This enzyme is found primarily in epidermis as demonstrated by immunocytochemical techniques (Lazarus, G.S. and Poole, A.R., unpublished). Quantitative removal of cathepsin D by immunoprecipitation from skin extracts revealed a small but reproducible independent peak of caseinolytic activity at neutral pH [3].

Neutral proteinase was maximally extracted from skin by 1 M KCl in 50 mM sodium phosphate buffer, pH 7.5. Enzymic activity was dependent on casein concentration, so that the activity could not be attributed to contamination by ribonuclease [17] or xanthine oxidase activity [18]. The neutral proteinase was capable of degrading albumin and haemoglobin, but not collagen or benzoyl-DL-arginine *p*-nitroanilide. The enzyme is of low molecular weight and unusually stable to heat in the presence of substrate. The properties of the neutral proteinase as we know them at present do not allow it to be placed in one of the four classes of proteolytic enzymes recognized by Hartley [19], since it was not inhibited by DFP, EDTA, thiol-blocking reagents or pepstatin.

Our most highly purified enzyme preparation, which was still inhomogeneous, was activated by KCl, and may have retained an ionically complexed inhibitor, in spite of its low apparent molecular weight in gel chromatography.

The majority of the enzyme extracted from skin comes from the dermal connective tissue but the exact cellular source is unknown. It is possible that the enzyme is derived from polymorphonuclear leukocytes. A KCl-activatable neutral proteinase has been found in the granule fraction of rabbit polymorphonuclear leukocytes [20],

but it differed from the enzyme we have studied in being inactive against hemoglobin and being inhibited by soyabean trypsin inhibitor. Hayashi et al. [21] isolated from Arthus reaction sites of rabbit skin a proteinase that produced inflammatory changes when reinjected. It now seems that this enzyme, too, was derived from polymorphonuclear leukocytes, but it is distinguished by being activated by cysteine and inhibited by *p*-chloromercuribenzoate [22]. It also seems unlikely that our enzyme is the chymase from mast cells since that enzyme is inhibited by DFP [23].

Neutral proteinase is capable of degrading skin protein in vitro as shown by our autolysis experiments. In vivo, intradermal injection of the enzyme results in edema formation within 15 min and leucotaxis and dermal epidermal separation within 18 h. Such a reaction might be brought about by activating the complement or kinin systems. The elucidation of the mechanism of action of this enzyme may be of importance in understanding the processes of protein catabolism and inflammation in skin.

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