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CHARACTERIZATION OF A CHEMOTACTIC AND CYTOTOXIC PROTEINASE FROM HUMAN SKIN

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Summary

A proteinase (EC 3.4.—.—) active at physiological pH has been isolated from human skin utilizing gel filtration and affinity chromatography techniques. The proteinase has a molecular weight of approx. 28 000 and it is inhibited by α_2-macroglobulin, α_1-antitrypsin, $C\bar{1}$ inactivator, soybean trypsin inhibitor and diisopropyl fluorophosphate. Injection of 1 ng of purified proteinase into rabbit skin induces polymorphonuclear leukocyte infiltration of the cutis. Inhibition of enzyme activity with diisopropyl fluorophosphate inhibits the chemotactic effect. Addition of 0.2 μg/ml of purified proteinase to fibroblast cultures kills the cells within minutes. This proteinase may play a significant role in modulating the inflammatory response after cellular injury.

Introduction

Proteolytic enzymes (EC 3.4.—.—) active at neutral pH have been extracted from the skin in a variety of species by high ionic strength buffers [1—9]. For a complete review see Lazarus and Hatcher [10]. Recently, Lazarus and Barrett [11] using a high ionic strength buffer extracted a proteinase from rabbit skin that was capable of inducing leukocyte infiltration of cutaneous tissue. The present study describes the isolation and characterization of a proteinase from human skin which causes polymorphonuclear leukocyte infiltration when injected into skin and it is extremely toxic to human fibroblasts. Such an enzyme could be a mechanism by which cellular injury induces a polymorphonuclear leukocyte response.

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Methods

Casein nach Hammarsten (E.M. Chemicals, Elmsford, N.Y.) (50 mg/ml) was suspended in distilled water, adjusted to pH 7.0 with 1 M NaOH and incubated at 65° C for 30 min. Hemoglobin (50 mg/ml) was prepared according to the method of Barrett [12]. 2 ml of each of these solutions was dialyzed against 0.1 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl, 0.02 M KCl and 0.05 M sodium acetate (4° C). 5 μCi of [^{3}H]acetic anhydride (New England Nuclear, Boston, Mass.) (400 Ci/M) was added to the casein and 10 mCi was added to the hemoglobin solution. The reaction mixture was vigorously agitated in a Vortex mixer, allowed to incubate for 1 h at 4° C and then exhaustively dialyzed against the starting buffer. The specific activities of the conjugated proteins were casein 3840 cpm/pmol assuming a molecular weight of 121 700 and the hemoglobin was 1450 cpm/pmol assuming a molecular weight of 64 500.

Proteolytic enzyme determinations. Neutral proteinase activity was determined by measuring the production of trichloroacetic acid-soluble radioactive peptides from [^{3}H]acetyl casein [13—16]. The radioactive proteinase assay was performed by taking 20 μl of the enzyme solution, 20 μl of 0.05 M sodium phosphate buffer, pH 7.8, and 20 μl of the casein solution (10 μg protein, 78 000 cpm) and incubating for 60 min at 40° C in a microfuge tube (Spinco Division, Beckmann Instruments Co., Palo Alto, Calif.). At the end of the incubation 50 μl of 3% (w/v) solution of unlabelled casein in 50 mM sodium phosphate buffer, pH 7.0, containing 1 M KCl was added to the tubes. The tubes were incubated for an additional 5 min at 40° C and then 100 μl of ice-cold trichloroacetic acid (6% w/v) was added. The contents of the tubes were vigorously agitated in a Vortex mixer and the mixture was then incubated for an additional 90 min in an ice slurry. The tubes were centrifuged at 10 000 rev./min in a Spinco microfuge (Spinco Division, Beckmann Instruments, Co., Palo Alto, Calif.), the top of each tube was sliced off and 100 μl of the supernatant was removed, added to 10 ml of Riafluor (New England Nuclear, Boston, Mass.) and the radioactivity determined in a Packard liquid scintillation spectrometer. Each value represents the mean of triplicate samples from which was subtracted the mean of three blanks containing heat-inactivated enzyme. Under our experimental conditions 20 μl of trypsin (twice crystallized, Worthington, Freehold, N.J.) solution (2.5 ng/ml) produced 750 cpm of trichloroacetic acid-soluble [^{3}H]acetyl peptides [14,15]. One unit of enzyme activity has been arbitrarily taken as the amount of enzyme which solubilizes 1000 cpm/h of [^{3}H]acetyl casein. Cathepsin D was measured with [^{3}H]acetyl hemoglobin at pH 3.5 [13, 16].

Protein determination. Protein determinations were performed according to the method described by Lowry et al. [17] utilizing bovine serum albumin (Sigma, St. Louis, Mo.) as a standard.

Purification of proteinase

Extraction of tissue. Human skin was obtained from amputation limbs (approved by the human research sub-committee), cut into small pieces and placed into 15 volumes of ice-cold 0.05 M sodium phosphate buffer, pH 7.5, containing 1 M KCl and 0.1% Triton X-100. The suspension was homogenized on ice

for a total of 10 min with a Polytron SW 35 homogenizer (Brinkmann Instruments, Lucerne, Switzerland) being careful not to allow the temperature to rise above 17°C. Following incubation overnight at 4°C the suspension was centrifuged at 18 000 rev./min for 1 h and the supernatant was collected.

Ammonium sulfate fractionation. The enzyme-rich solution was brought to 30% saturation with saturated $(NH_4)_2SO_4$ at 4°C and the initial precipitate was removed by centrifugation at 30 000 × *g* for 30 min. The supernatant solution was brought to 85% saturation with saturated $(NH_4)_2SO_4$ (4°C), the precipitate was collected by centrifugation at 30 000 × *g* for 30 min and dissolved in a small volume of 0.05 M sodium phosphate buffer, pH 7.5, containing 1 M KCl and 0.1% Triton X-100.

Sephadex G-75 chromatography. An aliquot of the enzyme solution (4 ml) was applied to a column of Sephadex G-75 superfine gel (Pharmacia, Upsala, Sweden) (100 × 4.5 cm, 4 ml/h, 4°C), equilibrated and eluted with the above buffer. Fractions were collected and assayed for protein, cathepsin D and neutral proteinase as described above. In order to separate all the ammonium sulfate fractions, several runs on washed Sephadex G-75 were necessary.

Affinity chromatography. Soybean trypsin inhibitor (Worthington, Freehold, N.J.) was conjugated to Sepharose 4B (Pharmacia, Upsala, Sweden) according to the method of Cuatrecasas and Anfinsen [18]. Each ml of Sepharose 4B resin bound 3.7 mg of soybean trypsin inhibitor. This was determined by measuring the total unbound soybean trypsin inhibitor in solution after conjugation and subtracting it from that used in the starting solution. An aliquot (2 ml) of the smallest molecular weight proteinase (Fraction IV) from the Sephadex chromatography step were applied to the soybean trypsin inhibitor-Sepharose column (1 × 2.5 cm, 4°C, 21 ml/h) and eluted with 22.5 ml of 0.05 M sodium phosphate buffer, pH 7.5, containing 1 M KCl and 0.1% Triton X-100. The column was then eluted with the same buffer which had been adjusted to pH 2.0 with 6 M HCl and 1.4-ml aliquots were collected which were immediately adjusted to pH 7.5 with 6 M NaOH. The soybean trypsin inhibitor column was extensively washed with 0.05 M sodium phosphate, pH 7.5, containing 1 M KCl and 0.1% Triton X-100. The above procedure was repeated until all of Fraction IV was purified.

Characterization of proteinase by inhibition with [^{3}H]diisopropyl fluorophosphate followed by SDS-polyacrylamide gel electrophoresis. 1-ml fractions (5—50 μg protein) of the various Sephadex fractions or soybean trypsin inhibitor-Sepharose 4B affinity-purified enzyme were treated for 16 h at 4°C with 20 μl of [^{3}H]diisopropyl fluorophosphate (DFP) (Amersham, Chicago, Ill.) in propylene glycol (1 Ci/l, 3.9 Ci/mM). Unreacted DFP was removed by exhaustive dialysis against 0.05 M sodium phosphate buffer, pH 7.0, after which 1 ml of 0.02 M sodium phosphate buffer containing 2% SDS and 2% β-mercaptoethanol was added. The samples were heated at 100°C for 1 h, concentrated by dialysis (dialysis membrane exclusion limit 3500 molecular weight) against Aquacide II (Calbiochem, San Diego, Calif.) to 200 μl and then dialyzed against 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% β-mercaptoethanol and 0.1% SDS. The proteins were separated on SDS-polyacrylamide gel according to the method described by Weber and Osborn [19]. The gels were sliced at a uniform thickness of 1 mm and then each slice was incubated with 50 μl

of 3% H_2O_2 at 60° C for 16 h. 200 μl of Protosol (New England Nuclear, Boston, Mass.) was added and the radioactivity was counted in 10 ml of toluene scintillation fluid (4 g PPO, 0.2 g POPOP in 1000 ml toluene) in a liquid scintillation spectrometer.

Inhibition studies. Inhibition studies on the purified enzyme were carried out with the following inhibitors at the final concentrations noted: α_2-macroglobulin (1 μM), α_1-antitrypsin (13 μM), $C\bar{1}$ inactivator (8 μM), Kunitz pancreatic trypsin inhibitor (30 μM), diisopropyl fluorophosphate (5 mM), tosyl-lysyl-chloromethylketone (Calbiochem, San Diego, Calif.) ($TosLysCH_2Cl$, 5 mM), tosyl-phenylalanine-chloromethylketone (Calbiochem, San Diego, Calif.) ($TosPheCH_2Cl$, 5 mM), ε-aminocaproic acid (1.5 mM), Trasyol (Calbiochem, San Diego, Calif.) (1000 kallikrein (KI) units/60 μl), Ac-$(Ala)_3$-Pro-CH_2Cl (0.3 mM), Ac-$(Ala)_4$-CH_2Cl (0.3 mM), *N*-ethylmaleimide (Aldrich Biochemicals, Cedar Knolls, N.J.) (MalNEt, 2 mM), disodium ethylene diamine tetraacetic acid (Fisher Scientific, Fairtown, N.Y.) (EDTA, 2 mM), pepstatin (2 mg/60 ml) and soybean trypsin inhibitor (9 μM). Enzyme fractions were preincubated with the specific inhibitor for 30 min after which proteolytic enzyme activity was assayed as described previously.

Polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed according to the method described by Davis [20]. The protein sample (50—200 μg of protein) was dissolved in 0.1 ml of 0.06 M Tris · HCl buffer, pH 8.9. A constant current of 3.0 mA per tube was applied until the Bromphenol Blue tracking dye reached the bottom of the tube. The gels were removed from the tubes, stained with Amido Black 10B (Sigma, St. Louis, Mo.) (1% (w/v) in 7% acetic acid) for 30 min and then destained in 7% (v/v) acetic acid. Polyacrylamide gel electrophoresis in SDS was performed according to the method of Weber and Osborn [19]. Bovine serum albumin, trypsin and ribonuclease B (Sigma, St. Louis, Mo.) were utilized as standards in the determination of the molecular weight.

Amino acid analysis. The amino acid analysis was performed on a Jelco model 6AH amino acid analyzer after enzyme samples had been hydrolyzed for 24 or 48 h with 6 M HCl.

Injection of human enzyme in rabbit skin and morphological evaluation. Quadruplicate aliquots (0.1 ml) of human proteinase purified by soybean trypsin inhibitor-Sepharose affinity chromatography and proteinase which had been inhibited with DFP were dialyzed against 0.05 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl. Triplicate samples of active and inactive enzymes were injected intradermally into separate sites on the backs of three different shaved albino rabbits.

Skin punch biopsies (4 mm) were taken from the test area at 6 and 20 h; the samples were fixed in formalin, embedded in paraffin and stained with hematoxylin and eosin (Fisher Scientific, Fairtown, N.Y.). Specimens were examined with a Zeiss photomicroscope.

Cytotoxicity studies. Human skin fibroblasts and fibroblasts derived from human umbilical veins in the 16th and 25th passage were grown to confluence in Eagle's modified media (Grand Island Biologicals, Grand Island, N.Y.) containing 10% fetal calf serum (Grand Island Biologicals, Grand Island, N.Y.) at 37° C in 7% CO_2 in air. The cells ($4 \cdot 10^4$ cells/cm^2 in 25 mm Falcon petri dish)

were washed three times with serum-free Eagle's modified media and then incubated in 1.5 ml of fresh Eagle's modified media without serum at 37° C in 7% CO_2 for 1.5 h. The media was then removed and triplicate plates of cells were treated with 0.3 ml fresh serum-free Eagle's media containing: (1) 0.1—1.0 μg/ml of skin proteinase purified by affinity chromatography and dialyzed against serum-free culture media, (2) skin proteinase (1 μg/ml) which had been inactivated with DFP (final concentration of DFP 5 mM, 1 h at 25° C followed by exhaustive dialysis against serum-free culture media, or (3) trypsin 0.1 μg to 10 mg/ml dissolved in serum-free culture media). Viability was measured after incubating the enzymes with cells for 15, 30 and 60 min by adding 0.1% eosin Y in phosphate-buffered saline and observing dye exclusion.

Leucine incorporation into trichloroacetic acid-precipitable protein [21] was also used to determine protein synthetic ability of enzyme-treated cells. The cell cultures were incubated with skin enzyme or trypsin in various concentrations in serum-free media for 1 h at 37° C in 7% CO_2. 2 ml of Eagle's modified media containing 10% calf serum was then added. 10 μCi [^{3}H]leucine (New England Nuclear, Boston, Mass.) (specific activity 50 Ci/mM) was added to each culture and the cultures were incubated at 37° C in 7% CO_2 for 16 h. The media was removed from the culture dishes, the plates were washed with Eagle's modified media and then the cells were removed from the culture dish by brief incubation with 0.25% trypsin/0.04 M EDTA in phosphate-buffered saline. The cells were washed onto a 0.45 μm Millipore filter which was then rinsed with 0.85 M NaCl (twice), 5% trichloroacetic acid and methanol (twice). The filters were solubilized in scintillation vials by incubation in Hyamine at 60° C for 2 h. Toluene scintillation fluid was added and the samples were counted in a Packard liquid scintillation spectrometer.

Results

Purification of the skin proteinase. The purification scheme is shown in Table I. Ammonium sulfate precipitation was required for concentration of the starting material but this step resulted in a large decrease in enzymatic activity. Attempts at concentration by ultracentrifugation were unsuccessful because of the viscosity of the solution. Lyophilization of the crude homogenate resulted in complete loss of activity. Sephadex G-75 chromatography produces four peaks (Fractions I—IV) of neutral proteolytic activity (Fig. 1). The lysosomal proteinase cathepsin D which was totally inhibited by pepstatin [22] eluted just before Fraction IV. The proteolytic activity in Fraction I was partially inhibited by DFP whereas Fractions II, III, and IV were completely inhibited by DFP. In order to determine how many components which react with DFP were present in Fractions I, II, III and IV the peaks were reacted with [^{3}H]-DFP and separated by SDS-polyacrylamide gels (Fig. 2). The results indicated that Fraction I contained at least three components which reacted with DFP. Fractions II and III also contained three components. By contrast, Fraction IV contained a single component which reacted with DFP. The DFP reacting component in Fraction IV possessed the same electrophoretic mobility as one of the components in Fractions I, II and III. The proteolytic activity of Frac-

TABLE I

THE PURIFICATION OF HUMAN SKIN NEUTRAL PROTEINASE

	Total units	Total protein (mg)	Specific activity (units/mg protein)	Purification
Human skin extract	327 000	683	480	—
Ammonium sulfate precipitation (30—85% saturation)	83 000	203	410	—
Sephadex G-75	55 000	47	1 170	2.4
Soybean trypsin inhibitor-Sepharose	38 000	0.3	126 000	254

tions I, II and III are being purified further and will be reported in a separate communication.

Fraction IV was further purified by soybean trypsin inhibitor-Sepharose 4B affinity chromatography (Fig. 3). Most of the protein applied to the soybean trypsin inhibitor-Sepharose 4B column was eluted in the void volume of the column. Some proteolytic activity did not bind to the column; rechromatography of this material on fresh affinity columns did not result in binding of the proteolytic enzyme to the soybean trypsin inhibitor affinity column. Bound proteinase was eluted from the column by adjusting the pH to 2 with HCl. The initial peak of proteolytic activity eluted by lowering the pH was utilized for further studies.

Electrophoretic studies. SDS-polyacrylamide gel electrophoresis of the first peak of affinity-purified enzyme demonstrated a single component with an apparent molecular weight of approx. 28 000. Electrophoresis in SDS of the [^{3}H]DFP-labelled protein also resulted in a single ^{3}H-labelled component with the same electrophoretic mobility as the unlabelled affinity pure enzyme. Electrophoretic analysis of the purified enzymes in the polyacrylamide without

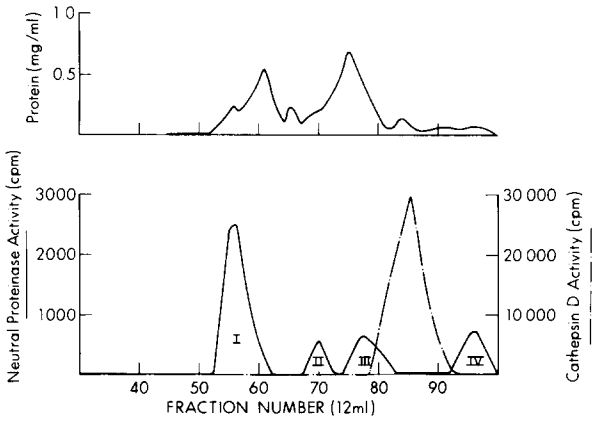

Fig. 1. Chromatography of an aliquot of human skin extract on Sephadex G-75 (100 × 4.5 cm, 4°C, 4 ml/h). Elution was performed with 0.05 M phosphate buffer, pH 7.5, containing 1 M KCl and 0.1% Triton X-100. Neutral proteinase activity using [^{3}H]acetyl casein (———), cathepsin D activity utilizing [^{3}H]-acetyl hemoglobin (— · — ·—) per 10 μl sample are presented.

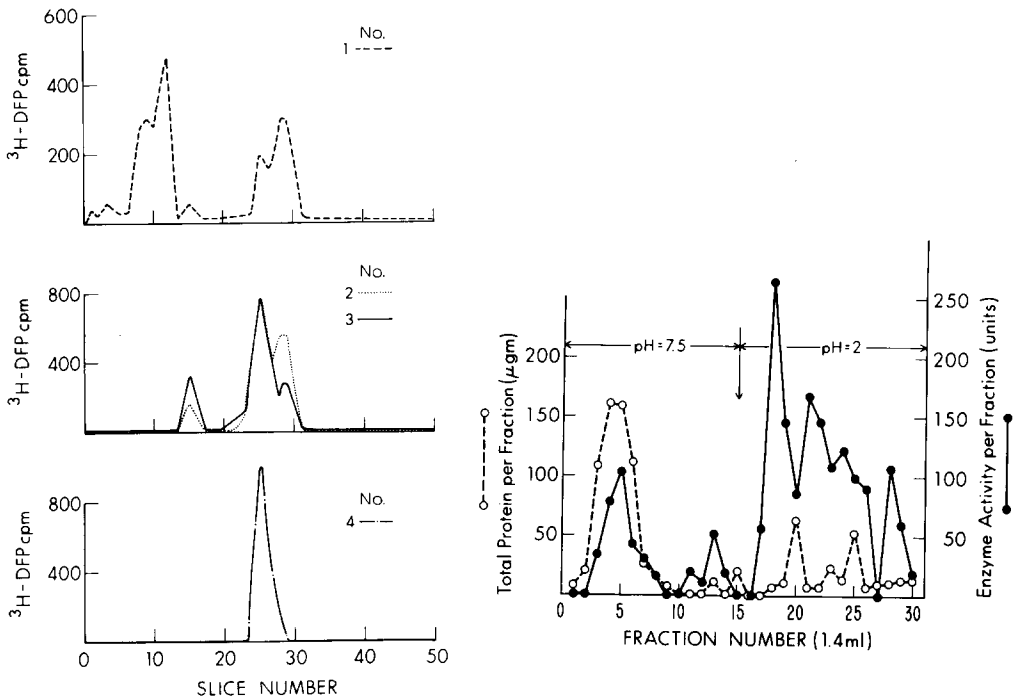

Fig. 2. SDS-polyacrylamide gel electrophoresis of [^{3}H]DFP-labelled components of Fractions I, II, III and IV from Sephadex G-75 chromatography.

Fig. 3. Affinity chromatography of an aliquot of Sephadex G-75 Fraction IV on soybean trypsin inhibitor-Sepharose 4B (1 X 2.5 cm, 4°C, 20 ml/h). Neutral proteinase activity using [^{3}H] acetyl casein (●———●) and protein (●- - - - - -●) per 10 μl are presented. The buffers used for the elution of the proteinase are described in the text.

SDS at pH 8.9 also demonstrated a single band. Injection of the purified material into rabbits resulted in a monospecific antiserum which demonstrated a single precipitin line against purified enzyme and a single line against crude skin homogenate in Ouchterlony double diffusion plates (Yost, F.J., Thomas, C. and Lazarus, G.S., unpublished).

Amino acid analysis. The amino acid analyses rounded to an integral number of residues per 100 residues of the human skin enzyme is shown in Table II.

Inhibition studies. Results of inhibition studies with the purified proteinase are shown in Table III. Complete inhibition of the enzyme was obtained with α_2-macroglobulin, α_1-antitrypsin, $C\bar{1}$ inactivator, soybean trypsin inhibitor and DFP. Partial inhibition was obtained with TosLysCH_2Cl at a concentration of 5 mM. The other inhibitors examined had no effect on enzymatic activity.

Morphological evaluation of rabbit skin after injection of serine proteinase. The histological examination of rabbit skin after injection of the purified enzyme is shown in Fig. 4. Fig. 4A demonstrates the results of an injection of 1 ng of the active enzyme preparation. Significant infiltration of polymorphonuclear leukocytes into the injection sites is noted. There is also separation of the epidermis from the dermis at the level of the basement membrane. No polymorphonuclear leukocyte infiltration or dermal/epidermal separation

TABLE II

AMINO ACID COMPOSITION OF THE HUMAN SKIN ENZYME, HUMAN GRANULOCYTE, CHYMOTRYPSIN-LIKE ENZYME AND HUMAN GRANULOCYTE ELASTASE

Results are expressed in mol/100 mol amino acid.

Amino acid	Skin enzyme	Granulocyte chymotrypsin-like enzyme *	Granulocyte elastase *
Lys	5	3	1
His	1	3	3
Arg	4	9	9
Asp	9	10	10
Thr	6	7	3
Ser	11	9	6
Glu	12	12	7
Pro	5	4	3
Gly	12	11	10
Cys (half)	n.d.	2	3
Val	4	6	7
Met	n.d.	1	3
Ile	11	5	6
Leu	10	7	9
Tyr	2	3	4
Phe	2	3	6

* Data from Feinstein and Janoff [24,25].
n.d., non-detectable.

could be observed in the preparation inhibited by DFP (Fig. 4B). Identical results were obtained at all sites tested.

Results of cytotoxicity experiments. Fibroblast cultures exposed to 0.2 μg/ml of skin enzyme developed granular cytoplasmic changes within 15 min at 37°C; coincident with the granular changes all the cells lost their ability to exclude eosin Y. At higher concentrations the cells were completely disrupted within 15 min. When fibroblasts were exposed to DFP-inactivated skin enzyme in concentrations of 1.0 μg/ml there was no change in morphology and

TABLE III

INHIBITION STUDIES

Inhibitor	Final concentration	Inhibition (%)
α_2-Macroglobulin	1 μM	100
α_1-Antitrypsin	13 μM	100
$C\bar{1}$ inactivator	8 μM	100
DFP	5 μM	100
Soybean trypsin inhibitor	9 μM	100
TosLysCH_2Cl	5 mM	47
TosPheCH_2Cl	5 μM	0
Kunitz pancreatic trypsin inhibitor	30 μM	0
ε-Amino caproic acid	1.5 μM	0
Trasyol	1000 KI units	0
Ac-$(Ala)_3$-Pro-CH_2Cl	0.3 mM	0
Ac-$(Ala)_4$-CH_2Cl	0.3 mM	0
NEM	2 mM	0
EDTA	2 mM	0
Pepstatin	50 μg	0

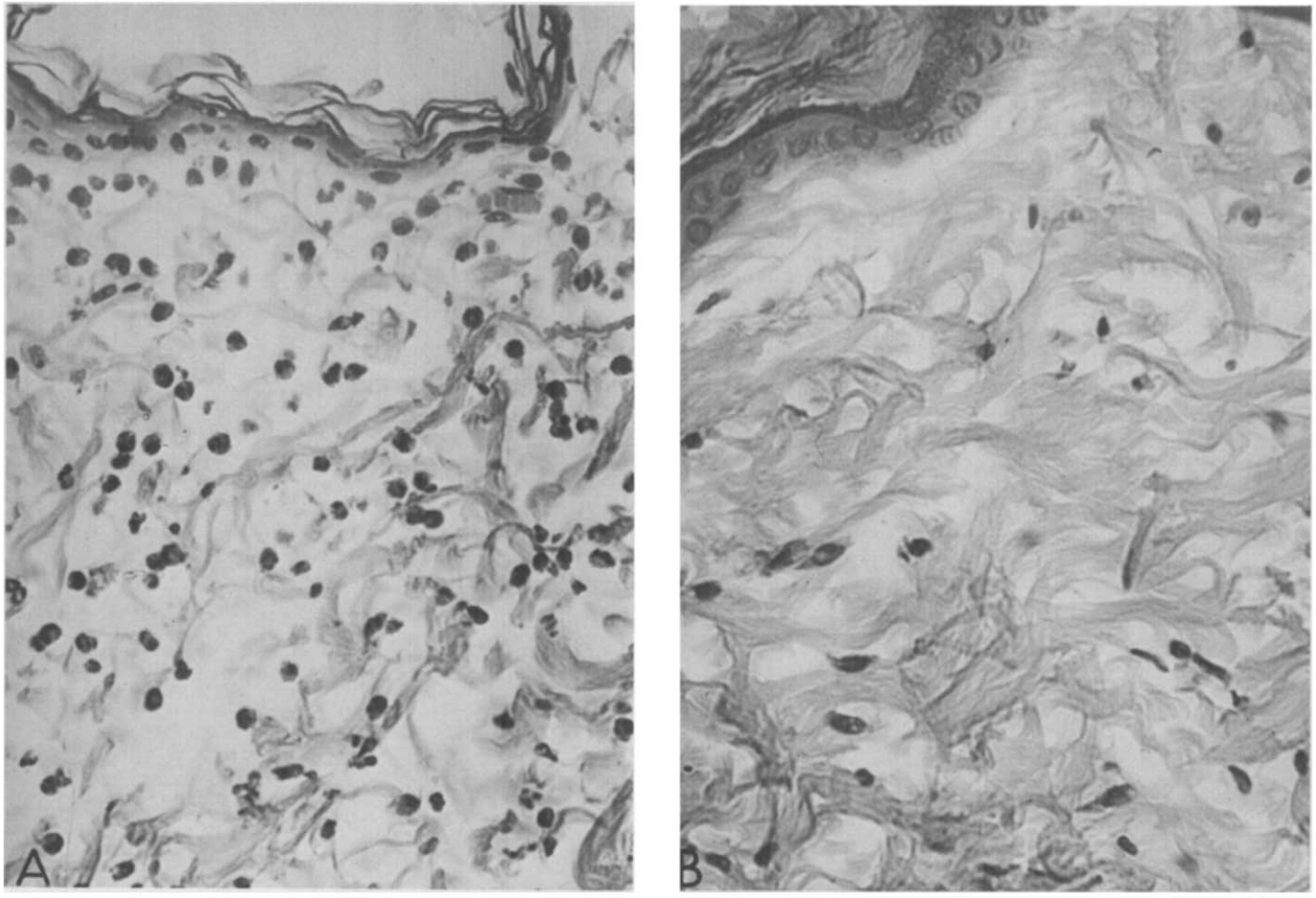

Fig. 4. Photomicrographs of inflammatory response obtained after injection of soybean trypsin inhibitor-Sepharose 4B-purified skin proteinase into rabbit skin. (a) Skin biopsy section 6 h after intradermal injection of proteinase. (b) Skin biopsy section 6 h after intradermal injection of DFP-inactivated proteinase. Hematoxylin and eosin. X472.5.

TABLE IV

INCORPORATION OF [^{3}H]LEUCINE INTO TRICHLOROACETIC ACID-PRECIPITABLE PROTEIN BY FIBROBLAST CULTURES AFTER TREATMENT WITH SKIN PROTEINASE OR TRYPSIN

	cpm
Skin proteinase concentration	
No enzyme	11 239
0.1 μg/ml	8 351
0.2 μg/ml	366
0.5 μg/ml	299
1.0 μg/ml	387
1.0 μg/ml DFP-inactivated enzyme	12 201
Trypsin concentration	
No enzyme	9 702
0.1 μg/ml	10 118
1.0 μg/ml	14 627
100 μg/ml	10 188
10 mg/ml	7 318

all the cells excluded eosin Y. Trypsin in concentrations above 100 μg/ml caused the cells to retract and round up but did not effect eosin Y exclusion even at concentrations of 10 mg/ml.

Leucine incorporation (Table IV) was profoundly decreased in fibroblast cultures exposed to 0.2 μg/ml or greater of active skin enzyme. DFP-inactivated skin enzyme had no effect on leucine incorporation. Trypsin at concentrations of 100 μg/ml had no effect on protein synthesis. Incubation of cells with 10 mg/ml of trypsin caused a slight depression in leucine incorporation into trichloroacetic acid-precipitable protein.

Discussion

We have isolated a proteinase from human skin and purified it 254-fold by gel filtration on Sephadex G-75 and affinity chromatography on soybean trypsin inhibitor-Sepharose 4B. Polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis of our preparation demonstrated a single protein component which labels with [^{3}H]DFP. This data coupled with our immunological observation suggests that we have isolated a single proteinase; it seems very unlikely, but the possibility does exist, that a second proteinase with an identical molecular weight and inhibitor profile is present in our preparation. The molecular weight of the enzyme as evaluated by SDS-polyacrylamide gel electrophoresis, is approx. 28 000 which is similar to the human granulocyte chymotrypsin-like enzyme, cathepsin G [23]. The amino acid analysis of the enzyme reveals that the enzyme has a high percentage of hydrophobic amino acids and a low percentage of basic amino acids. In this regard the molecule is quite different from the granulocyte elastase or the granulocyte chymotrypsin-like enzyme [24,25].

The skin enzyme is completely inhibited by α_2-macroglobulin, α_1-antitrypsin and $C\bar{1}$ inactivator. These results are not unexpected since these serum inhibitors appear able to inhibit many serine proteinases [23,26,27]. Interesting-

ly, our enzyme was not inhibited by Kunitz pancreatic trypsin inhibitor whereas cathepsin G was partially inhibited by this material.

Lazarus and Barrett [11] extracted a neutral proteinase from rabbit skin capable of degrading skin protein and inducing an inflammatory response. That proteinase was capable of degrading albumin and hemoglobin but not collagen or benzylarginine *p*-nitroanalide. Injection of rabbit proteinase into rabbit skin produced marked edema, polymorphonuclear leukocyte infiltration and dermal/epidermal separation below the basement membrane.

In the present investigation we have injected purified human skin proteinase into rabbit skin and examined the tissue 6 h later. Histologically, there was polymorphonuclear leukocyte infiltration and dermal/epidermal separation. No infiltration was evident in the samples which were inhibited by DFP. The observation that injection of proteinase or other compounds into skin results in polymorphonuclear leukocyte infiltration is not unique to this investigation [28,29]; the isolation of a proteinase from human skin which is capable of inducing leukocyte accumulation in that same tissue is a new observation.

Hill and Ward [30] reported that extracts of rat tissue were chemotactic for polymorphonuclear leukocytes. They suggested that cleavage of C_3 by proteolytic enzymes in their preparation was responsible for chemotactic activity. Leukocyte lysosomes are capable of activating C_5 to chemotactic peptides [31]; injection of our purified enzyme into the peritoneal cavity of normal mice and mice deficient in the fifth component of complement suggests that cleavage of C_5 is important in the chemotactic response [32].

We have found that preparations of purified human epithelium [16] and cell cultures of mouse epithelium [33] contain chemotactic proteinase. Fractionation of human fibroblasts reveal that the enzyme is in a sedimentable particle [34]. These data permit formulation of an hypothesis which could explain how cellular injury could instigate leukocyte infiltration. Cellular damage might release neutral proteinase, along with other enzymes which through proteolysis of serum constituents might generate chemotactic factors which would result in infiltration of the tissue by polymorphonuclear leukocytes.

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References

1 Beloff, A. and Peters, R.A. (1945) J. Physiol. Lond. 103, 2P—3P
2 Beloff, A. and Peters, R.A. (1945) J. Physiol. Lond. 103, 461—476
3 Fruton, J.S. (1946) J. Biol. Chem. 116, 721—738
4 Neville-Jones, D. and Peters, R.A. (1948) Biochem. J. 43, 303—308
5 Martin, C.J. and Axelrod, A.E. (1957) Biochim. Biophys. Acta 26, 490—501
6 Martin, C.J. and Axelrod, A.E. (1958) Biochim. Biophys. Acta 30, 79—87
7 Martin, C.J. and Axelrod, A.E. (1958) Biochim. Biophys. Acta 27, 532—538
8 Golubow, J., Martin, C.J. and Axelrod, A.E. (1960) Biochim. Biophys. Acta 100, 142—146
9 Fraki, J.E. and Hopsu-Havu, V.K. (1972) Arch. Dermatol. Forsch. 243, 153—163
10 Lazarus, G.S. and Hatcher, V.B. (1975) in Lysosomes and the Skin (Dingle, J.T. and Dean, R.T., eds.), pp. 111—146, North-Holland, Publ. Co., Amsterdam
11 Lazarus, G.S. and Barrett, A.J. (1974) Biochim. Biophys. Acta 350, 1—12
12 Barrett, A.J. (1972) in Lysosomes: A Laboratory Handbook (Dingle, J.T., ed.), pp. 46—135, North-Holland, Publ. Co., Amsterdam
13 Hille, M.B., Barrett, A.J., Dingle, J.T. and Fell, H.B. (1970) Exp. Cell Res. 61, 470—472
14 Grayzel, A.I., Hatcher, V.B. and Lazarus, G.S. (1975) Cell Immunol. 18, 210—219
15 Hatcher, V.B., Wertheim, M., Rhee, C.Y., Tsien, G. and Burk, P. (1977) Biochim. Biophys. Acta, in the press
16 Levine, M., Hatcher, V.B. and Lazarus, G.S. (1976) Biochim. Biophys. Acta 452, 458—467
17 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
18 Cuatrecasas, P. and Anfinsen, C.B. (1971) Annu. Rev. Biochem. 40, 259—278
19 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
20 Waith, W.I. and Hirschhorn, K. (1971) in In vitro Methods in Cell Mediated Immunity, pp. 455—461
22 Barrett, A.J. and Dingle, J.T. (1972) Biochem. J. 127, 439—441
23 Starkey, P.M. and Barrett, A.J. (1976) Biochem. J. 155, 255—278
24 Feinstein, G. and Janoff, A. (1975) Biochim. Biophys. Acta 403, 477—492
25 Feinstein, G. and Janoff, A. (1975) Biochim. Biophys. Acta 403, 493—505
26 Harpel, P.C. and Mosesson, M.W. (1973) J. Clin. Invest. 52, 2175—2184
27 Harpel, P.C., Mosesson, M.W. and Cooper, N.R. (1975) in Proteases and Biological Control (Reich, E., Rifkin, D.B. and Shaw, E., eds.), Vol. 1, pp. 387—404, Cold Spring Harbor, Cold Spring, N.Y.
28 Lazarus, G.S., Hatcher, V.B. and Levine, N. (1975) J. Invest. Dermatol. 65, 259—371
29 Lazarus, G.S. (1974) J. Invest. Dermatol. 62, 367—371
30 Hill, J.H. and Ward, P.A. (1969) J. Exp. Med. 130, 505—518
31 Goldstein, I.M. and Weissman, G. (1974) J. Immunol. 113, 1583—1588
32 Yost, F., Snyderman, R., Thomas, C., Hatcher, V., Gigli, I. and Lazarus, G. (1977) Clin. Res., in the press
33 Farb, R.M., Fountain, E.A., Yost, F.J. and Lazarus, G.S. (1977) Clin. Res., in the press
34 Lazarus, G.S., Levine, N., Vale, M., Vale, M. and Hatcher, V.B. (1976) in Symposium on Rheumatoid Arthritis: Cellular Pathology and Pharmacology (Hazelton, B. and Gordon, J., eds.), pp. 223—231, Elsevier/North-Holland Publ., Co., Amsterdam