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HUMAN SKIN PROTEASES: EFFECT OF SEPARATED PROTEASES ON VASCULAR PERMEABILITY AND LEUKOCYTE EMIGRATION IN SKIN

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Abstract. The effect of human skin proteases on vascular permeability and leukocyte emigration in rabbit skin was investigated. The alkaline protease of human skin capable of hydrolysing trypsin substrate effectively increased vascular permeability. This effect was not inhibited by antihistamine, but almost totally so by Trasylol®. The reaction was protracted. Leukocyte emigration in skin, primarily of PMN-cells at 12 hrs, and later a migration of mononuclear cells, also resulted. Swelling of the dermal fibres was noted. The alkaline protease of human skin capable of hydrolysing chymotrypsin substrate also increased vascular permeability, but this phenomenon was effectively inhibited by antihistamine and the reaction was of brief duration. The leukocyte emigration caused by this enzyme was remarkable. The acid proteases of human skin resembling cathepsin B1 and D also caused brief increased vascular permeability, which was effectively inhibited by antihistamine. The cellular reactions to these acid proteases were mild. The role of protease inhibitors in skin in the enzyme reactions is discussed.

Key words: Proteases; Inflammation; Vascular permeability; Leukocyte emigration

Vascular permeability increasing factors of large molecular size have been reported in human skin (4, 10). Chromatography shows that they eluate very similarly to proteases (4). Proteases of exogenous origin have also been known to cause a marked increase in vascular permeability when injected intradermally (1, 9, 11, 16, 17). Using healthy rabbit skin, Lazarus & Barrett (13) partially purified a neutral protease which caused whealing and leukocyte infiltration in rabbit skin. Lykke et al. (14) have demonstrated in rat skin a cortisol-released protease preparation that increases vascular permeability. Neutral SH-proteases capable of causing increased vascular permeability and leukocyte emigration in skin have been purified from the Arthus skin site in the rabbit (12) (for review see Hayashi, 8) and from guinea pig skin (15).

Several proteolytic enzymes have recently been separated from human skin and characterized (3, 5, 7). These include two alkaline proteases, the one hydrolysing trypsin substrates and the other hydrolysing chymotrypsin substrates. The latter is inhibited by α_1-antitrypsin, while the former is resistant to natural protease inhibitors (6). Among the acid proteases separated from human skin, one resembled cathepsin B1 and the other resembled cathepsin D. The former is inhibited by an inhibitor present in human skin (3).

The four human skin proteases separated were tested in rabbit and human skin as to their ability to increase vascular permeability. The ability of the proteases to cause leukocyte emigration in rabbit skin was studied histologically.

MATERIAL AND METHODS

Protease preparations

Protease preparations were separated from healthy human skin as described earlier (3, 7). The alkaline chymotrypsin substrate (acetyl tyrosine ethyl ester=ATEE) capable of hydrolysing protease was purified about 250-fold (with a specific activity of 95000 U/g) and the alkaline trypsin substrate (benzoyl arginine ethyl ester=BAEE) hydrolysing protease, about 29-fold (specific activity 5500 U/g) from salt extract of human homogenate made after a prior extraction in buffer (5, 7). The activity of the former enzyme was 2000 U/l and that of the latter, 510 U/l. Protein concentrations of the former averaged 0.021 g/l and of the latter, 0.093 g/l. The acid protease resembling cathepsin D (hydrolysing hemoglobin (Hb) at pH 3.5) was purified about 190-fold (specific activity 320 U/g), with a final activity of 5.3 U/l and protein concentration 0.017 g/l (3). The acid protease resembling cathepsin B1 (hydro-

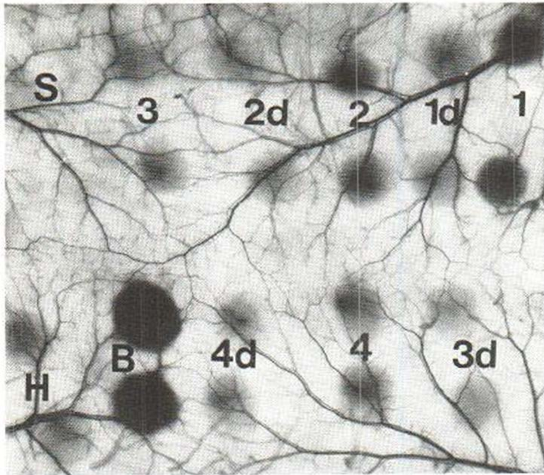


Fig. 1. Effect of intradermal injection (100 μl) of separated human skin proteases on vascular permeability of rabbit skin. 1=BAEE-hydrolase, 2=ATEE-hydrolase, 3=Hb-hydrolase, 4=BANA-hydrolase. *d*=denatured enzyme (100°C for 10 min), *H*=histamine (10 μg), *B*=bradykinin (10 μg), *S*=saline.

lysing benozyl arginine naphthyl-amide=BANA) was purified about 760-fold (specific activity 51 U/g) with final activity 1.1 U/l and protein concentration of 0.022 g/l (3). The purification coefficients of the alkaline chymotrypsin and trypsin substrate hydrolysing proteases were about 2 100-fold and 54-fold, respectively, when calculated from the salt extract of human skin (5, 7).

The alkaline chymotrypsin and trypsin substrate hydrolysing enzymes were dialysed before use against 10 mmol/l phosphate buffer, pH 8.0, containing 0.15 mol/l NaCl. The acid protease resembling cathepsin D was dialysed against 10 mmol/l phosphate buffer, pH 6.0, containing 0.15 mol/l NaCl. The acid protease resembling cathepsin B1 was dialysed against 10 mmol/l phosphate buffer, pH 6.0, containing 10 mmol/l NaCl and 1 mmol/l EDTA (ethylene diamine tetra-acetic acid; E. Merck AG, Darmstadt, West Germany), and DTT (dithiothreitol; CalBiochem, Los Angeles, USA) was added before injections ad 1 mmol/l in enzyme preparation.

In the controls, the enzyme activity was destroyed by heating at 100°C for 10 min. All the enzyme activities were found to be lost.

Test for vascular permeability

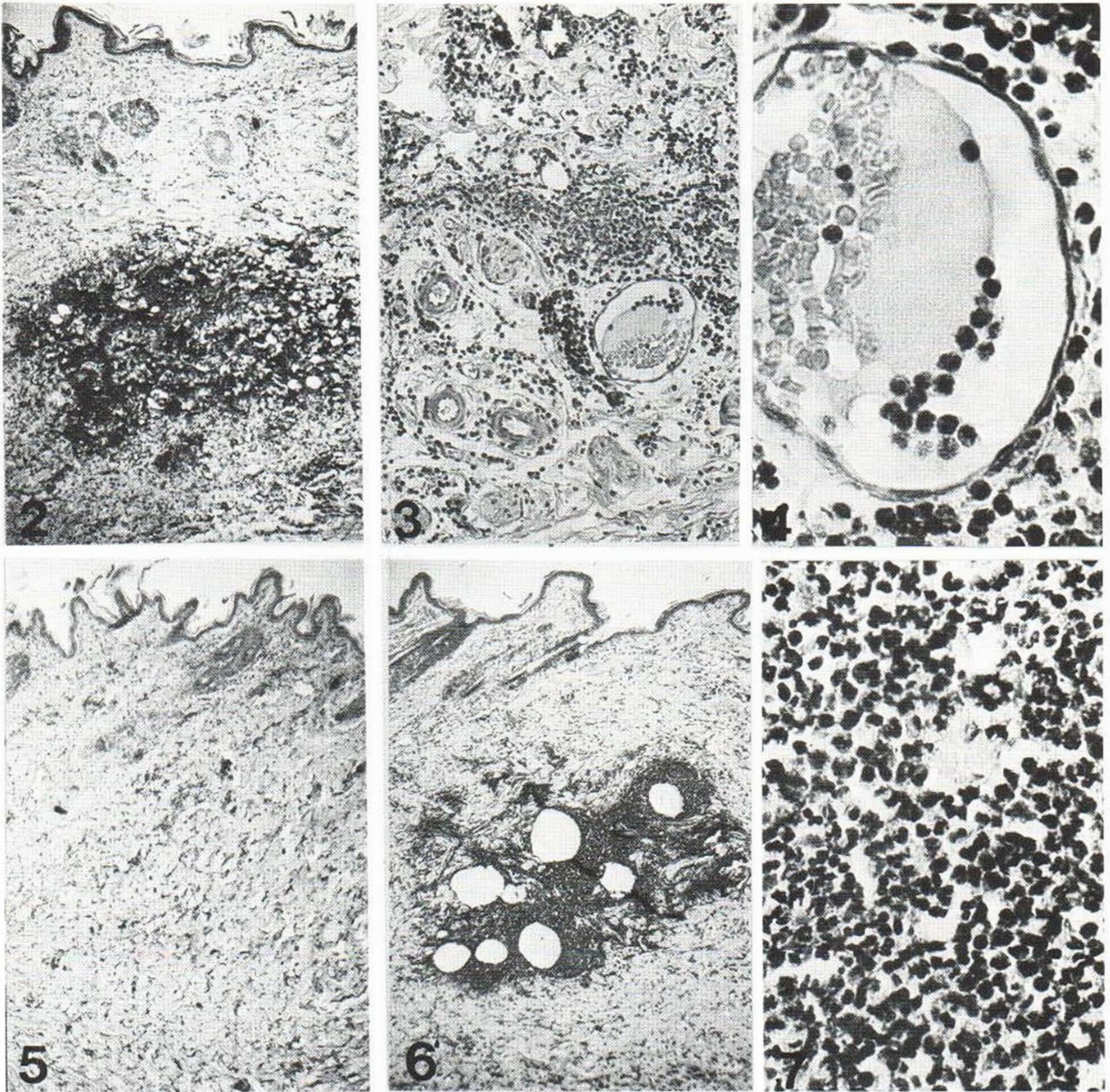
The vascular permeability increasing activity of the protease was tested in rabbit skin (and for comparison, in skin rat) by using Evans blue vital stain as an indicator of extravasation of plasma proteins, according to the principles of Frimmer & Müller (2) with slight modifications. Usually 2 ml of Evans blue (T-1824) (1 ml/kg) (5 g/l Evans blue; Curr Ltd., London, England, in saline) was injected into a rabbit ear vein, or 0.5 ml into a rat tail vein. Usually after 20 min, 100 μl of the test solution was injected intradermally into rabbit skin (50 μl into rat skin). Bradykinin (bradykinin triacetate; Sigma Chem. Comp., St. Louis, Mo., USA) and histamine (histamine dihydrochloride; Fluka AG, Switzerland) 0.1 g/l in 10 mmol/l phosphate buffer, pH 7.4, containing 0.15 mol/l NaCl as well as this buffer solution alone were used as reference solutions. About 30 min after injections the test animal was killed by i.v. injection of pentobarbitone (Nembutal®; Abbott Ltd, Kent, England), skin was removed and the spots were measured (two diameters at right angles) or photographed. Thereafter, the blue spots at the injection sites were clipped out, minced, and placed in 4 ml formamide (E.

Table I. *Effect of separated human skin proteases on vascular permeability in rabbit skin*

Activity is expressed as μg dye extravasated by 100 μl of solution injected into skin. Values corrected with saline-induced reactions represent means of three separate determinations

	Intact animal	Antihistamine treated animal	Prolonged[a] reaction
Enzyme			
1. BAEE-hydrolase	2.0	1.3	2.2
BAEE-hydrol. den.[b]	0.10	0.04	0.14
2. ATEE-hydrolase	1.4	0.40	0.40
ATEE-hydrol. den.[b]	0.26	0.12	0
3. Hb-hydrolase	0.66	0	0
Hb-hydrol. den.[b]	0.0	0	0.24
4. BANA-hydrolase	1.4	0	0.24
BANA-hydrol. den.[b]	0.60	0	0
Controls			
Bradykinin, 10 μg	8.5	4.9	0.16
Histamine, 10 μg	2.8	0.24	0.08

[a] Dye was injected i.v. 60 min after injection of test solution into rabbit skin.
[b] Denatured at 100°C for 10 min.



Figs. 2–5. Effect of intradermal injection (100 μl) of separated BAEE-hydrolysing protease on the histological appearance of rabbit skin at 12 hrs. Fig. 2. 38×, Fig. 3. 120×, Fig. 4. 480×, and Fig. 5. denatured enzyme (100°C for 10 min) 38×.

Figs. 6–7. Effect of intradermal injection (100 μl) of separated ATEE-hydrolysing protease on the histological appearance of rabbit skin at 12 hrs. Fig. 6. 38×, Fig. 7. 480×.

Merck, Darmstadt, West Germany) in airproof tubes and incubated at 37°C for 3 days. The solution was filtered to obtain a clear solution and the optical density was measured by a spectrophotometer at 620 nm. Various concentrations of Evans blue in formamide were used as standards.

Promethazin (Phenergan®; May & Baker Ltd, Dagenham, England) was injected with Evans blue solution (1 mg/kg) to eliminate the histamine effect on vascular permeability.

Trasylol® (Bayer AG, Leverkusen, West Germany), an inhibitor of alkaline trypsin substrate hydrolysing human skin protease (7) was incubated for 15 min at 37°C with this enzyme preparation at a concentration of 330 IU/ml in order to inhibit this enzyme.

To test protease preparations in human skin, 100 μl of solution was injected intradermally in the dorsal skin of a healthy young man. The reactions were noted by measuring the diameter of the reaction area and consistency of the dermal induration. The skin site was photographed.

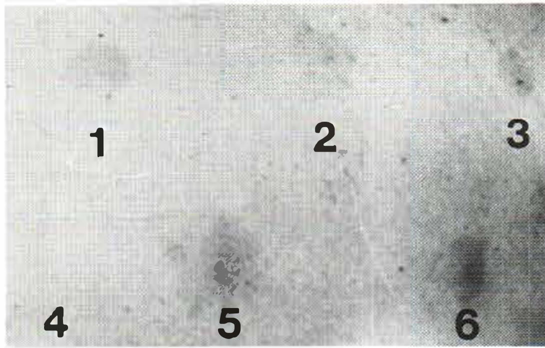


Fig. 8. Effect of intradermal injection (100 μl) of separated human skin protease on human skin at 60 min. 1=ATEE-hydrolase, 2=BAEE-hydrolase, 3=BAEE-hydrolase preincubated with Trasylol® (330 IU/ml), 4=saline, 5=Hb-hydrolase, 6=BANA-hydrolase.

Histological means

Skin samples obtained 12 and 24 hours after intradermal injections were fixed in 40 g/l formaldehyde for 24 hrs at room temperature and embedded in paraffin. In most cases 5 μm sections were cut throughout the sample and stained routinely with hematoxylin and eosin.

RESULTS

Effect of protease on vascular permeability

All the native protease preparations caused some increase in vascular permeability, but the alkaline BAEE- and ATEE-hydrolysing enzymes were the most active, as seen in Fig. 1 and Table I. The denatured enzyme preparations without enzymic activity caused only a slight increase in vascular permeability. The effects of BAEE-, ATEE- and Hb-hydrolysing proteases were particularly diminished after denaturation, but that of the BANA-hydrolysing enzyme preparation was not diminished to the same extent (Fig. 1 and Table I).

The antihistamine, promethazine, injected 20 min before intradermal injection of the test solutions, caused the greatest inhibition of the vascular permeability increasing activity of ATEE-, Hb- and BANA-hydrolysing enzymes, while the activity of BAEE-hydrolysing enzyme was reduced only to one-half, as was the effect of bradykinin (Table I). The effect of histamine was almost totally abolished by antihistamine (Table I).

The prolonged reaction of proteases was studied by injecting the test solutions into rabbit skin 60 min before intravenous injection of Evans blue. The results are seen in Table I. Only the BAEE-hydrolysing human skin protease showed a definite prolonged effect on vascular permeability. The ATEE- and BANA-hydrolysing enzymes increased permeability by only about one-fourth that of the immediate reaction. Bradykinin and histamine caused only a minor prolonged vascular permeability increase, as seen in Table I.

The BAEE-hydrolysing enzyme preincubated 15 min at 37°C with Trasylol® (330 U/ml) caused a vascular permeability increase of about 23 % of that with the intact enzyme solution.

Very similar results were obtained with the rat as test animal, though the deviations were greater.

Effects on histology

The effect of proteases on the histological picture was studied by taking skin samples from the injection site 12 hrs and 24 hrs after injection of separated human skin protease preparations into rabbit skin.

After 12 hrs the BAEE-hydrolysing enzyme caused a marked leukocyte infiltration at the injection site (Fig. 2), dilatation of veins (Fig. 3) and emigration of leukocytes (primarily polymorphonuclear cells, Fig. 4). The denatured enzyme caused only a minor swelling of tissue and mild leukocytosis (Fig. 5). After 24 hrs a considerable increase in mononuclear small cells, primarily lymphocytes, was seen, as well as swelling of dermal fibres with increased eosinophilia.

At 12 hrs after the injection, the ATEE-hydrolysing enzyme caused a marked leukocyte infiltration, with polymorphonuclear and mononuclear cells (Figs. 6 and 7), much greater than in the control site injected with the denatured enzyme. At 24 hrs the reaction was very similar but without fibre swelling.

After 12 hrs the Hb-hydrolysing enzyme caused a less marked leukocyte infiltration and the control (denatured enzyme) also caused some leukocyte infiltration. The reaction was quite similar after 24 hrs.

The BANA-hydrolysing acid protease caused a small perivascular leukocyte infiltration as well as denatured enzyme used as control. The reaction was similar after 24 hrs.

The effect of proteases in human skin

All native protease preparations tested in human skin caused erythema by 10–20 minutes after intradermal injection and slight induration within sev-

eral hours. The BAEE-hydrolysing and BANA-hydrolysing enzymes were the most active in causing erythema and whealing, as seen in Fig. 8. Trasylol® when preincubated with BAEE-hydrolysing enzyme clearly diminished the long-lasting erythema and induration, but this preparation, still caused a brief flush at the injection site.

DISCUSSION

Our findings support the claim that human skin proteases can induce increased vascular permeability in skin (4). Proteases may not be the only mediators that have a large molecular size, but they are of special interest because of their possible activation, release, and inactivation in the course of inflammation (8).

The increased vascular permeability caused by the separated alkaline trypsin substrate (BAEE) hydrolysing human skin protease was only partially inhibited by antihistamine, but effectively so by Trasylol®. The vascular permeability reaction of this protease was protracted. The results support the suggestion that the action of this protease on vascular permeability is attributable to enzymic action. No inhibitors to this enzyme have been found in skin or serum (5, 7) and this may explain the prolonged duration of the effect of this protease.

The increase in skin vascular permeability caused by alkaline chymotrypsin substrate (ATEE) hydrolysing protease was effectively inhibited by antihistamine and it was of shorter duration. Human serum and skin contain an inhibitor to this protease, α_1-antitrypsin (5, 6), which can inhibit the enzyme injected into skin, thus abolishing the enzyme effect.

The Hb- and BANA-hydrolysing enzymes resembling cathepsin D and B1, respectively (3), caused a minor and brief vascular permeability increase that was effectively inhibited by antihistamine. The denatured BANA-hydrolysing enzyme (containing 1 mmol/l DTT) also caused a vascular permeability increase. This could have been due to DTT present in the injected solution. The skin contains an inhibitor against BANA-hydrolysing enzyme (3) and this could inhibit any long-lasting effect of this enzyme. The reaction of the three latter enzymes, ATEE, Hb and BANA hydrolysing enzymes, may be due to histamine release at the injection site, known to be a mechanism of many exogenous proteases and causing increased vascular permeability in skin (9).

The histological findings on the effects of proteases in rabbit skin show that all the proteases caused some leukocyte infiltration at the injection site. This was most pronounced with the BAEE- and ATEE-hydrolysing enzymes. The BAEE-hydrolysing protease caused a definite leukocyte infiltration, first of PMN cells and, at 24 hrs, of mononuclear cells, as well as dilatation of the venules, seen in 12 hrs, and swelling of skin fibres with increased acidophilia. This was also seen by Hayashi et al. (8) after injection of Arthus protease in skin. The ATEE-hydrolysing protease also caused clear leukocyte infiltration but the histological picture was slightly different from that caused by the BAEE-hydrolysing enzyme. The vascular reactions were less prominent with ATEE-hydrolysing protease. The reactions of Hb- and BANA-hydrolysing acid proteases were much smaller.

The findings with human skin corroborate the results obtained with rabbit skin. The effect of BAEE-hydrolysing enzyme was long-lasting and was partially inhibited by Trasylol®. The reaction of ATEE-hydrolysing enzyme was less marked. The acid proteases, especially BANA-hydrolysing, also caused a definite erythema and induration, but that caused by BANA-hydrolysing enzyme was perhaps attributable to the irritating effect of the reducing agent, DTT, in the injection site.

The role of these human skin proteases in vascular as well as cellular reactions in human skin diseases remains to be elucidated. In particular, in such dermatological reactions in which local or serum protease inhibitor levels are low, the released or activated skin proteases may be effective in causing tissue reactions.

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