SKIN PROTEOLYTIC ENZYMES IN NORMAL RATS AND RATS SUBMITTED TO PASSIVE CUTANEOUS ANAPHYLAXIS*

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One of the earliest suggested explanations of allergic manifestations, especially of anaphylactic shock, is an activation of proteolytic enzymes by the antigen-antibody interaction (1). The activation of such enzymes would represent the basic step in a series of reactions leading up to the clinical symptoms and to the release of biochemical substances, such as histamine. There is considerable evidence that proteolytic enzymes are involved in systemic anaphylaxis (2); but there is as yet no evidence that such enzymes play part in the anaphylactic reactions of the skin.

The present study was undertaken in order to determine whether or not proteolytic enzymes extractable from rat skin participate in a passively induced cutaneous anaphylactic response. The isolation and the characterization of two proteolytic enzyme systems obtained both from normal rat skin and from rat skin submitted to a passive anaphylactic reaction will be described. A lack of relationship of one of these two enzyme systems to passive cutaneous anaphylaxis will be demonstrated.

MATERIALS AND METHODS

1. Animals: White male rats (Wistar strain) weighing about 100 g were used. The hair was removed from the abdomen with electric clippers.

2. Induction of Passive Cutaneous Anaphylaxis: For each experiment groups of 10 to 20 rats were used. Skin sensitivity was induced by the intradermal injection of an anti-human-albumin rabbit immune serum diluted with an equal amount of 0.25 M saline solution. No determination of the antibody nitrogen content in the immune serum was performed. Each animal received a set of six intradermal injections of 0.05 ml of immune serum into the abdominal skin, in such a fashion that an area of approximately 2 to 3 cm² was covered at each preparation. Four and one half hours later the antigen (0.5 ml of a 1% solution of crystallized human albumin dissolved in an equal amount of 0.25 M saline) was injected into a tail vein. After this challenge edema developed at the 6 sites of antibody injection. The individual 6 areas of edema merged into one large edematous region which reached its maximum within 40 to 60 minutes after the challenge and subsided completely within 24 hours. The animals were killed and bled by decapitation in subsequent intervals after the challenge. The skin from the combined site of the anaphylactic reaction was removed and pooled.

3. Extraction of Proteolytic Enzymes from the Skin: The samples of skin were cut into small pieces and immediately immersed into ice cold acetone for dehydration. They were freed from adherent subcutaneous tissue and dried under vacuum at room temperature. The dried skin was passed through a Wiley mill, yielding a fine powder, which then was suspended in ice cold acetone. The material was allowed to precipitate. The acetone was then carefully decanted and the skin powder dried under vacuum.

The two proteolytic enzymes of the skin were extracted as follows (Table 1): A 0.06 M phosphate buffer containing KCl of ionic strength was used for extraction according the method described by Beloff and Peters (3). 25 ml of phosphate-KCl-buffer solution was added for each gram of dried skin powder. This mixture was shaken for 40 minutes in a 38° water bath. Thereafter the mixture was centrifuged at 5° at 2,000 rpm for 20 minutes. The supernatant solution was filtered through a Whatman filter No 43 and dialysed overnight against 0.25 M NaCl at 4°. The dialyzed solution was designated Fraction A.

For further purification and separation of the two proteolytic enzyme systems present in Fraction A our recently published method (4) was modified as follows: Fraction A was diluted with an equal volume of 0.25 M saline solution and adjusted to a pH of 3.5 by the addition of glacial acetic acid. Through acidification a precipitate was formed, which contained one of the proteolytic enzyme systems. The other enzyme system was present in the supernatant solution.

The precipitate was centrifuged for 30 minutes at 2,000 rpm at 5° and washed once with a 0.25 M NaCl solution adjusted to a pH of 3.5 with acetic acid. The washed precipitate was resuspended in 0.25 M NaCl and then 0.2 N NaOH was added slowly until the precipitate dissolved. The solution was dialyzed for 48 hours at 4° against frequently exchanged distilled water, whose pH was adjusted to 8.4 with NaOH (The alkaline pH

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TABLE 1

Extraction for Skin Proteolytic Enzymes

<table>
<thead>
<tr>
<th>Acetone dried skin powder</th>
<th>Extract with KCl phosphate buffer 0.8 ionic strength (25 ml/g skin powder) 40 min. at 58°</th>
<th>Centrifuge at 5°, 2000 rpm, 20 minutes</th>
<th>Dialyze against 0.25 M NaCl overnight at 4°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction A</td>
<td>add equal vol. of 0.25 M NaCl and acidify with glacial acetic acid to pH 3.5</td>
<td></td>
<td></td>
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<tr>
<td>Precipitate</td>
<td>wash once with 0.25 M NaCl of pH 3.5</td>
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<tr>
<td></td>
<td>Disperse sediment in 0.25 M NaCl; add NaOH until sediment has dissolved</td>
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<td></td>
<td>Dialyze 48 hours against dist. water (pH 7.4)</td>
<td></td>
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<tr>
<td></td>
<td>Lyophylize</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Fraction C</td>
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</table>

Prevents precipitation of the dissolved material which easily occurs under acid conditions). The dialyzed material was then lyophylized. The white powder thus obtained was designated Fraction B.

The supernatant solution which had a pH of 3.5 was dialyzed for 48 hours against distilled water at 4°. In the course of dialysis a precipitate usually formed which was free of proteolytic activity when casein was used as substrate. It was therefore removed by centrifugation at 4°. The remaining supernatant was then lyophylized, yielding a white powder which was designated Fraction C.

4. Estimation of Proteolytic Activity: The proteolytic activity of the extracts was determined by using the casein (casein Hammarstein) digestion method of Kunitz (5). Measured amounts of Fractions B and C were dissolved in 0.25 M NaCl solution (adjusted with either diluted NaOH or HCl to the desired pH of 8.4 for Fraction B and pH of 6.0 for Fraction C) and brought to a volume of 2 ml. This was mixed with an equal volume of a 1% casein solution of the same pH. (The casein solution was prepared by dissolving the casein in 0.1 M Sørensen phosphate buffer.) The enzyme-casein mixtures were incubated in a water bath at 38°. At zero time and at intervals of 30 and 120 minutes 1 ml of the mixture was removed. To these aliquots 3 ml of a 5% trichloroacetic acid solution was added. The mixtures were allowed to stand at room temperature for 20 minutes. A precipitate formed and was separated from the supernatant by centrifugation. The supernatant, freed from the trichloroacetic acid precipitated material, was used for measurements of increased absorption at 280 mμ in a Beckman DU spectrophotometer. The increased absorption at 280 mμ served as an index for proteolytic activity.

RESULTS

I. Proteolytic Activity of the Enzyme Preparations Fractions A, B and C obtained from Normal Skin

Fraction A: Fraction A, which contains two enzyme systems, was lyophylized in order to compare its proteolytic activity with that of the other fractions on a dry weight basis (mg). The proteolytic activity of Fraction A is shown in Fig. 1.

Fraction B: The proteolytic activity of Fraction B is also shown in Fig. 1. The lyophylized material was dissolved in 0.25 M saline. Its activity was

![Graph](image-url)
approximately 10 times greater than that of Fraction A as calculated on the basis of dry weight. It has to be noted however, that Fraction A contained salts, whereas Fraction B was free of salts. The proteolytic activity of Fraction B was proportional to the amount of dry weight used. The average yield of Fraction B was 35 ± 7 mg per gram dried skin powder.

Fraction C: The proteolytic activity of Fraction C was 16 to 20 times less than that of Fraction B (Fig. 1). The proteolytic enzyme activity of Fraction C was optimal at a pH of 5.8 to 6.2. No further studies with Fraction C have been done so far.

II. Studies with Fraction B obtained from Normal Skin

Influence of pH: The enzyme activity of Fraction B was optimal at pH 7.4 to 8.4 (Fig. 2). The pH activity curve was obtained by dissolving 1.25 mg of Fraction B per ml (2 ml) of 0.25 M saline, adjusted to the desired pH with alkali or acid. This solution was added to 2 ml of a 1% casein solution in Sorensen phosphate buffer of the desired pH. The mixture was incubated at 38° for 120 minutes and the increase in absorption of the supernatant after trichloroacetic acid precipitation was determined at 280 mµ. The enzyme solution was found to be clear at a slightly alkaline pH and somewhat turbid at an acid pH.

Effect of Temperature: Pre-incubation of Fraction B at 56° for 30 minutes destroyed approximately 50% of the enzyme activity, while 70° destroyed 90% of the proteolytic activity (Fig. 3).

Inhibition of Proteolytic Activity by Serum: Beloff (6) had observed in her experiments that the proteinase activity of a KCl extract of acetone dried skin of 0.8 ionic strength (identical with our Fraction A) could be inhibited by 70–80% by incubation at 38° for 30 minutes with rat or human plasma prior to the assay with casein. When Fraction B was dissolved in a 0.25 M saline solution of pH 8.4 to which had been added between 1 to 10% fresh rat, guinea-pig or rabbit serum and was preincubated for 30 minutes at 38°, proteolysis by Fraction B was inhibited proportionally to the amount of serum used (Fig. 4).

Influence of Streptokinase on Fraction B: Streptokinase (Varidase Lederle), 200 to 1,000 to 5,000 units per ml was added to the solutions of Fraction B either alone or together with fresh guinea-pig serum (as a source of complement; see discussion) in a concentration of 1 to 10%.
Fig. 4. Curve 1 demonstrates the proteolytic activity of 2.5 mg/ml solvent (NaCl) of Fraction B at a pH of 8.4. Curve 2: the same preparation containing 1% fresh rat serum. Curve 3: the same preparation containing 10% fresh rat serum.

The mixtures were incubated for 10 minutes at 38° prior to the assay. No increased proteolytic activity of Fraction B was observed. In the mixtures which contained Fraction B, streptokinase and serum complement, the inhibitory effect of serum on the proteolytic activity of Fraction B occurred, as described above.

Vascular Effects of Fraction B: The intradermal injection of 0.1 ml of Fraction B, dissolved in 0.25 M saline, provoked in rats a rapid increase in vascular permeability, leading to edema formation, which in the range studied (0.05 to 0.5 mg of Fraction B) was proportional to the amount of Fraction B used. The diameter of the edematous lesions after the intradermal injection of Fraction B was made visible by the simultaneous intravenous injection into a tail vein of 0.3 ml of a 5% solution of Evans Blue. The animals were killed 40 minutes after the intradermal injection. The diameter of the areas of blueing was measured on the undersurface of the skin. The extent of edema obtained was compared with the vascular action of histamine-dihydrochloride (Fig. 5).

Pre-treatment of animals with an antihistamine (50 mg of Phenergan/kg), intraperitoneally 30 to 45 minutes prior the intradermal injection of Fraction B completely abolished the vascular effects not only of histamine but also of Fraction B (Fig. 6).

Fraction B did not contain histamine in detectable amounts as shown by negative tests when assaying it on the isolated atropinized guinea-pig ileum.

III. Proteolytic Activity of Fraction B from Rat Skin submitted to a Passive Cutaneous Anaphylactic Reaction

The anaphylactically reacting skin areas of 10 to 20 rats were pooled. Fraction B was extracted from the anaphylactically reacting skin 40 to 60 minutes, 2, 6 and 24 hours after the challenge. No alterations in the proteolytic activity of Fraction B obtained from anaphylactically reacting skin was found on comparison with normal skin. The average yield of Fraction B per g. dried skin powder did not significantly differ from that obtained from normal skin. No differences in the vascular permeability increasing effect was found between Fraction B obtained from normal and obtained from anaphylactic challenged rat skin.
Discussion

A typical example of an experimental evanescent type of a cutaneous anaphylactic response (urticarial wheal) is the passive cutaneous anaphylaxis in laboratory animals. It is not abolished by antihistamine drugs (7), and occurs in rat skin previously depleted of its histamine in the same size and the same intensity as in normal rat skin (8). Passive cutaneous anaphylaxis in rats furthermore releases histamine not only at the site of the antigen-antibody interaction, but also in the adjoining control area where no edema occurs (9). All these findings suggested that histamine release is not the immediate consequence of the local antigen-antibody interaction, nor the cause of the vascular disturbances (edema) seen in this type of cutaneous anaphylaxis.

In earlier studies we had observed that the intradermal injection of proteolytic enzymes provoked tissue alterations comparable to those seen in passive cutaneous anaphylaxis in rats (10). We were especially interested in the observation that streptokinase which indirectly activates plasminogen to the proteolytic enzyme plasmin, had similar effects. As in the passive cutaneous anaphylactic reaction streptokinase released histamine in the skin not only at the site of the injection, but also in the adjoining control area of skin, where no edema occurred. The edematous reaction following intradermal injection of streptokinase and the edematous reaction that follows passive cutaneous anaphylaxis depended both upon the availability of serum complement. In other experiments we had observed that fresh rat skin previously submitted to passive cutaneous anaphylaxis showed a decreased proteolytic activity in vitro when compared with normal skin (6). This in vitro decrease of proteolytic activity after a cutaneous anaphylactic reaction prompted us to investigate as to whether or not proteolytic enzymes extractable from rat skin participate in vivo in this type of a cutaneous allergic manifestation. Fraction B, representing one of two enzyme systems, extractable from rat skin was studied in this respect. The proteolytic enzyme system contained in this Fraction

Fig. 6. The edematous lesions on the left side were produced by the intradermal injection of 0.5, 0.25 and 0.125 mg of Fraction B. To the right 0.5 and 0.25 mg were injected into an animal pretreated with 50 mg.kg of phenergan.
B was fairly heat stable. It digested casein optimally at a slight alkaline pH. The proteolytic activity of Fraction B was not influenced by streptokinase, regardless whether complement was present or not. Yamura and Cormia (1961) (11) had reported that extracts of human skin contained a proteolytic active component, whose activity could be increased through the addition of streptokinase. They assumed however that this proteolytic enzyme originated from the interstitial tissue fluids rather than from the skin tissue per se. The proteolytic activity of our Fraction B was strongly inhibited by fresh serum. This inhibitory effect of serum upon skin enzymes of proteolytic nature was first observed by A. Beloff (8).

Fraction B, when injected intradermally in rats increased the vascular permeability thus leading to edema formation. It seems that this increase in vascular permeability is due to the release of histamine since it can be prevented by pretreatment of the animals with antihistamines. No alterations in the activity of the enzyme Fraction B was observed in preparations obtained from anaphylactically challenged skin in comparison to preparations obtained from normal skin. There was also no significant alteration in the average yield of the enzyme preparation per gram dried powder obtained from anaphylactic reacting skin as compared with that obtained from normal skin.

SUMMARY

A proteolytic enzyme system (Fraction B) was extracted from normal rat skin submitted to passive cutaneous anaphylaxis. The proteolytic activity of Fraction B obtained from anaphylactically reacting skin was not different from proteolytic activity obtained from normal skin.

The proteolytic component of Fraction B was found to hydrolyze casein optimally at a slight alkaline pH. It was moderately heat-stable. The proteolytic activity of Fraction B was not influenced by streptokinase; it was however strongly inhibited by fresh serum from rats, guinea-pigs or rabbits.

Although Fraction B, when injected intradermally mimics the vascular disturbances seen in passive cutaneous anaphylaxis, our data obtained in these studies seem to indicate that the proteolytic enzyme system contained in Fraction B is apparently not responsible for edema formation observed in the wake of passive cutaneous anaphylaxis in rats.

REFERENCES


DISCUSSION

Dr. Peter Flesch, Philadelphia, Pa.: I wonder if Dr. Inderbitzin used some other animals, because the rat is not the best animal for studies of histamine effects.

Dr. Andrew E. Lorincz, Gainesville, Florida: I wonder if Dr. Inderbitzin would be permitted to have the time to give the biochemical data that characterize this enzyme?

Dr. A. J. Reiches, St. Louis, Mo.: We have been working for years at the County Hospital in St. Louis with enzymes. We have found numerous enzymes in epidermis. We find the two trans-
aminases, GOT and GPT, proteolytic type of things. We find arginase. May I ask—Is it possible for the presenter to be a little more specific.

Dr. Theodor M. Inderbitzin, (in closing):
In answer to Dr. Flesch's question, we have not been able to obtain a good active enzyme preparation from guinea-pig skin using the same method as for rat skin.

In regard to the characterization of the enzyme, I can say that our enzyme preparation contains proteolytic activity on the basis of chemical and kinetic analysis of the reaction. The enzyme preparation may contain other enzymes, such as transaminases, in addition to proteolytic enzymes, but we are only interested in the proteolytic activity of these preparations.