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SEPARATION, PURIFICATION AND PROPERTIES OF TWO PROTEINS FROM RAT SERUM WHICH ARE ESSENTIAL FOR ANAPHYLATOXIN FORMATION

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Abstract—The anaphylatoxinogen containing fraction obtained by gel filtration from rat serum has been shown to contain two different active components. Both of them are essential for anaphylatoxin formation by incubation with the specific enzyme of cobra venom. One of these factors, P 2, is the anaphylatoxinogen proper, the other one, P 1, acts as a co-factor of the cobra enzyme. P 1 is not an activatable enzyme itself, it cannot be identified with the endogenous anaphylatoxin-forming enzyme of rat serum.

WE HAVE previously found that anaphylatoxin can be formed in rat plasma by a specific enzyme of cobra venom which acts on a specific substrate, anaphylatoxinogen.[1–4] It has now been shown that the substrate fraction, as obtained by sephadex gel filtration,[5] can be separated further into two fractions both of which are essential for anaphylatoxin formation. A preliminary note on some of the results has appeared before.[6]

PREPARATIONS AND METHODS

The anaphylatoxin-forming enzyme of cobra venom was prepared from lyophilized *Naja naja* venom as described earlier.[2] It was used in a final concentration of 7 μg/ml which corresponded to 100 μg/ml of crude venom. Polyvalent horse anti-snake venom (Behringwerke Marburg) was used as antibody against the cobra enzyme.

Rat plasma was obtained by centrifugation of heparinized blood (0·1 mg heparin/ml whole blood); serum, after spontaneous clotting of normal blood.

Chromatography. Sephadex G 200 was used as supplied, after swelling in 0·15 M NaCl solution containing 4 mM Na-azide. The DEAE cellulose preparation used was DE 32 (Whatman and Co.). It was washed with saline, 0·5 N HCl, water, 0·5 N NaOH and water in that order after each run, and was then equilibrated with 0·04 M phosphate buffer pH 8·0. Samples to be fractionated on DEAE cellulose were dialysed against the same buffer first.

Hydroxyl-apatite was prepared according to Tiselius, S. Hjertén and Ö. Levin.[7] All chromatographic separations were performed at +4°. Fractions were concentrated by ultrafiltration through collodium tubes or through LSG 60 membranes (Membran-Filter-Gesellschaft, Göttingen). Protein content was determined by a modified Folin–Lowry reaction.[8] When given in g wt./ml the protein content was calculated according to Waddell.[9] Nitrogen was estimated by Nesslerization.[10]

Biological assay. Fractions to be assayed for the whole substrate-cofactor system were incubated with the cobra enzyme for 30 min at 37°. They were then assayed as

described earlier on strips of isolated guinea pig ileum.[4] For assay of P 1 activity 0·1 ml P 2 solution was added in addition to the cobra enzyme and incubated for 30 min at 37°. *Vice versa* samples to be tested for P 2 activity were supplied with 0·1 ml P 1 solution and cobra enzyme. (For preparation and properties of these solutions, see Results.)

Whole rat serum or plasma was activated to form anaphylatoxin by adding 10 mg Sephadex G 75/ml and incubating for 30 min at 37°.

RESULTS

Gel filtration on Sephadex G 200

Batches of 50 ml rat serum were passed through a column of Sephadex G 200 (5 × 100 cm)in saline solution. Fig. 1 shows the distribution of proteins in the fractions

FIG. 1. Gel filtration of rat serum on Sephadex G 200. Ordinate: extinction at 570 mμ after Folin reaction. |——| Region which formed anaphylatoxin after incubation with the cobra enzyme.

obtained. When samples of the eluted fractions were incubated with the cobra enzyme, anaphylatoxin formed in the region indicated in Fig. 1. These fractions, however, did not produce anaphylatoxin on incubation with Sephadex G 75, i.e. they lacked the activatable anaphylatoxin-forming enzyme of rat serum but contained substrate only.[3, 4, 5]

Chromatography on DEAE-cellulose

The anaphylatoxinogen fractions obtained by gel filtration of 4 batches of rat serum were collected, concentrated by ultrafiltration, and transferred to 100 ml of 0·04 M phosphate buffer pH 8·0. The solution was placed on top of a column of DEAE-cellulose (4 × 20 cm). After running in 450 ml each of the following buffer solutions were passed through: 0·04 M phosphate buffer (p.b.s.) pH 8·0; 0·04 M p.b.s. pH 7·3; 0·04 M p.b.s. pH 6·5. The eluates were collected in fractions of 10 ml and were assayed for anaphylatoxinogen. No single fraction nor combined contents of 5–10 successive tubes did form any anaphylatoxin activity when incubated with cobra venom. When aliquots from all eluted fractions were combined and concentrated adequately, by ultrafiltration, anaphylatoxin activity did develop on incubation with the cobra enzyme. Apparently the preparation of anaphylatoxinogen had been split into two essential fractions which were placed well apart in the elution series. To find the position of the essential eluates, aliquots of all fractions were combined with

only one series of 5–10 successive tubes being omitted. All samples prepared in this manner developed anaphylatoxin after incubation with the cobra enzyme except those which lacked the tubes nos. 6–15 or 46–55. These two regions coincided with two peaks of protein (Fig. 2). That they contained an essential fraction each, called P 1 and P 2 is shown by direct assay in Fig. 3. Only an incubated solution which contained the free factors P 1, P 2 and cobra enzyme contracted the guinea pig ileum. All other samples which lacked one or the other component were inert.

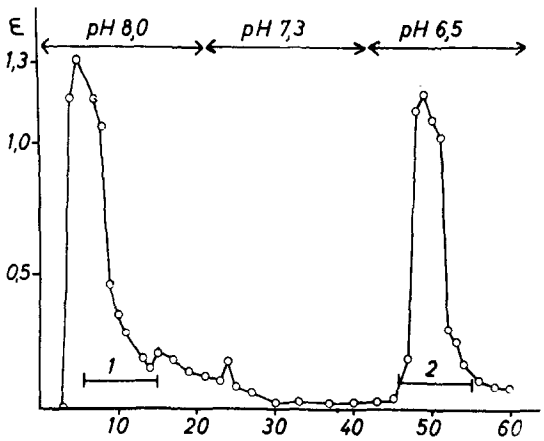

FIG. 2. Chromatography on DEAE cellulose of the anaphylatoxin-forming fractions obtained after gel filtration of rat serum. Ordinate as in Fig. 1. Abscissa: tube number (fractions of 10 ml each). |——| Regions containing P 1 or P 2 activity, respectively.

The fractions containing P 1 and P 2 activity, respectively, were combined, concentrated and washed with saline by ultrafiltration and were stored in saline solution (half the original volume of serum) at −20°. These stock solutions usually contained about 40 mg of protein/ml P 1, and 12 mg/ml P 2. The nitrogen content of the proteins was 15 per cent.

Properties of P 1 and P 2

Both factors are proteins. They are not dialyzable, and thermo-labile. Heating of rat plasma to 56° for 30 min destroys all P 2 activity and much of P 1. Addition of 2·2 g ammonium sulphate to 10 ml of a neutral saline solution containing both factors (eluate of rat serum from Sephadex G 200) precipitates most of P 2 but not P 1. After precipitation of euglobulins by adding 10 vol. of distilled water to rat serum and adjusting the pH to 5·2 both factors are recovered partly from the precipitate and partly from the supernatant.

Function of P 1 and P 2 in the anaphylatoxin-forming system

The question arose how P 1 and P 2 interact during formation of anaphylatoxin with the cobra enzyme. The following possibilities were envisaged: (1) One of the two factors might be a pre-enzyme, which after activation by the cobra enzyme would act on the other factor. (2) Both factors might react with each other in a stoichiometric reaction, catalyzed by the venom. (3) One of the two factors might be a co-factor essential for the cobra enzyme to act on the other factor.

To test these possibilities the following experiments were performed. 0·4 ml of P 1 was incubated with 2·8 μg of cobra enzyme in 0·04 ml saline for 30 min at 37°. After this the cobra factor was inactivated by addition of 0·04 ml of a dilution of 1/10 horse anti-cobra serum and then 0·5 ml P 2 and 0·32 ml saline were added followed by incubation for another 30 min at 37°. Anaphylatoxin activity did not develop.

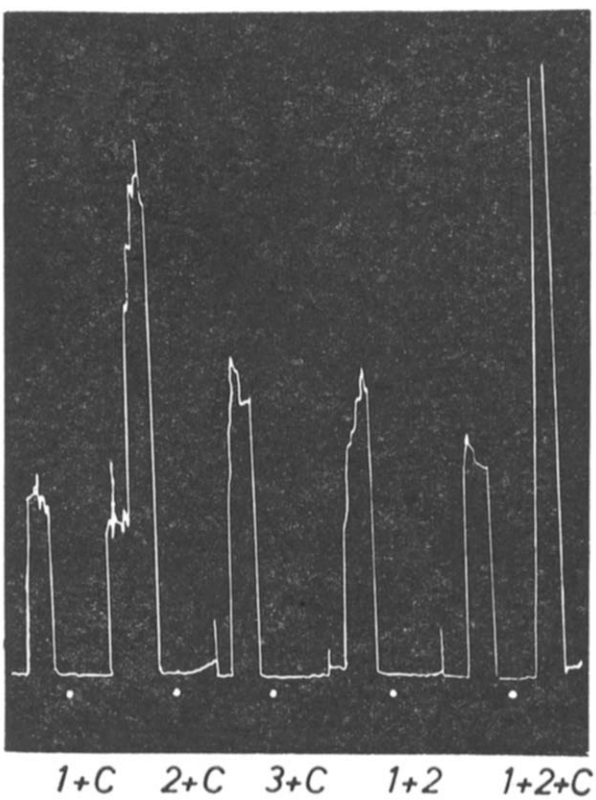

FIG. 3. Guinea pig ileum. Assay of P 1 and P 2. 1,2,3,C: incubated samples containing P1, P2, P3 and/or cobra enzyme, respectively. (P 3 was an inert fraction eluted after P 2 from DEAE cellulose). Only the sample containing P 1, P 2 and cobra enzyme shows anaphylatoxin activity, The five contractions not marked by a dot were induced by acetylcholine.

When the addition of anti-serum was omitted the two-step incubation did lead to anaphylatoxin formation. The same results were obtained when P 2 was preincubated first with cobra venom and P 1 was added in the second step. Thus, no active enzyme was produced from either P 1 or P 2 by the incubation with the cobra enzyme but all three compounds had to be present simultaneously, to generate anaphylatoxin activity.

The second possibility was also excluded. As shown in Table 1, the amount of anaphylatoxin depends on the amount of P 2 present in the reaction system, not on the amounts of P 1 present. In a total volume of 1·1 ml the optimal dose of P 1 was

0·2 ml. When this dose was halved or quartered the amount of anaphylatoxin formed in 30 min decreased (compare no. 1 and no. 3), but the decrease was not proportional to the reduction of P 1 present (compare nos. 2 and 3). On increasing the dose of P 1 above 0·2 ml no further rise in anaphylatoxin formation ensued (compare nos. 4 and 5). In contrast, a correlation was found between the amounts of P 2 added and product

TABLE 1. EFFECT OF VARYING THE AMOUNTS AND PROPORTIONS OF P 1 AND P 2 IN INCUBATES ON ANAPHYLATOXIN FORMATION.

No	P 1	P 2	Saline	Cobra enzyme	Total dose	Dose of P 1	Dose of P 2	Effect
1	0·2	0·2	0·6	0·1	0·04	0·0073	0·0730	7·2
2	0·2	0·2	0·6	0·1	0·02	0·0036	0·0365	0·3
3	0·05	0·2	0·75	0·1	0·04	0·0018	0·0730	5·1
4	0·2	0·2	0·6	0·1	0·1	0·0182	0·0182	2·3
5	0·8	0·2	—	0·1	0·1	0·0728	0·0182	2·3
6	0·2	0·8	—	0·1	0·1	0·0182	0·0728	10·4
7	0·2	0·8	—	0·1	0·025	0·0023	0·0182	2·6

The four vertical columns to the left indicate the composition of the incubation mixture, the four to the right show the dose tested, given as total volume and as dose of P 1 or P 2, respectively, contained in that volume. All amounts are given in ml, the effect in cm contraction height. The first three and the subsequent four assays have been conducted on one strip of guinea-pig ileum each and are comparable among themselves.

The solution of P 1 contained 6·4 mg *N*/ml and 41 mg/ml protein. The corresponding values for P 2 were 2·0 mg/ml; protein 13 mg/ml, and for the cobra enzyme 11·5 μG *N*/ml; 70 μG/ml

formed. When P 2 was increased four times, the resulting anaphylatoxin activity was four times as strong, (compare no. 4 with 6 and 7; also 2 and 3). Thus P 2 only behaves as a substrate determining the amount of anaphylatoxin to be liberated. P 1 then might be a co-factor for the cobra enzyme.

DISCUSSION

It was demonstrated previously that the anaphylatoxin-forming system of rat plasma can be resolved into an endogenous enzyme (which can be replaced by cobra venom) and its substrate which has been called anaphylatoxinogen.[1, 3, 4] Now, a serum co-factor has been separated from the substrate fraction which factor is essential for the cobra enzyme to act on anaphylatoxinogen. In 1956 Giertz, Hahn and Lange[13] obtained two fractions from rat serum both of which were necessary for anaphylatoxin formation by contact agents. These two factors seemed to react stoichiometrically with each other. Therefore they could not be identified with an enzyme and its substrate. In the light of the present findings it seemed at first possible that the two fractions of Giertz *et al.*[13] contained P 1 and P 2, respectively, and in addition the anaphylatoxin-forming enzyme. However, our experiments indicate that P 2 only is a substrate for anaphylatoxin, i.e. anaphylatoxinogen proper. P 1 acts like a co-factor that does not go into the product and bears no relation to the amount of anaphylatoxin formed. It is thus difficult to correlate the fractions of Giertz *et al.* to our terms, i.e. anaphylatoxin-forming enzyme, P 1 and P 2 (anaphylatoxinogen).

The finding that a non-toxic fraction of cobra venom acts on anaphylatoxinogen only when a serum co-factor is present has a striking parallel in the complement system. Nelson[14] as well as Müller-Eberhard *et al.*[15] have found that the complement-inactivating power of cobra venom[16] is due to a non-toxic fraction which in the presence of a serum co-factor acts on a specific component of complement, according to Nelson, C'3c. In fact, Jensen[17] recently described that the same venom fraction also forms anaphylatoxin and this again only in the presence of some other serum constituent. Though the purification methods have been different one may assume that the authors cited above have been dealing with the same venom fraction which has been characterized by us as an anaphylatoxin-forming enzyme. One might conclude, then, that the co-factor necessary for C'3c inactivation is identical with P 1 and that anaphylatoxinogen (P 2) is identical with the complement factor C'3c. However, Jensen described that anaphylatoxin is formed from C'3b, not C'3c, although the latter but not the former looses its haemolytic activity in the complement system after reaction with the venom. To our view, this makes it uncertain whether the co-factors for the action of venom on C'3c and C'3b are identical and whether C'3b can be regarded as anaphylatoxinogen. This the more as other findings seem not to be compatible with a participation of complement (or at least of its components reacting early in the reaction sequence of immune haemolysis) in the formation of anaphylatoxin. Further investigations into this problem are being performed.

As pointed out earlier anaphylatoxin can be formed in rat plasma by an endogenous enzyme which in its functional properties corresponds to the cobra enzyme.[3, 4] The effect of the contact agents known to induce anaphylatoxin formation is to activate this enzyme. Jensen[17] assumed that the serum co-factor, after activation by the cobra venom, might be the anaphylatoxin-forming enzyme of plasma. Our results indicate that no active enzyme is produced from P 1 by the cobra fraction. Rather, P 1 and cobra enzyme have to be present simultaneously to generate anaphylatoxin activity from P 2. This would be in agreement with the scheme drawn by Müller-Eberhard according to which the complement-inactivating component of cobra venom forms a complex with the serum co-factor which complex is the active component proper. The anaphylatoxin-forming enzyme of plasma, then, is different from P 1. Whether it needs P 1 as a co-factor is not yet known. Efforts to obtain enzyme preparations free of P 1 activity have so far failed.

The experiments on stability and solubility of P 1 and P 2 throw also some light on properties of the anaphylatoxin-forming enzyme endogenous to rat plasma. Heating to 56° for 30 min seems to destroy it, for heated rat plasma does not form anaphylatoxin on contact with Sephadex even when fresh P 1 and P 2 are added. The plasma enzyme seems likewise to be destroyed during euglobulin precipitation. In the recombined supernatant and precipitate anaphylatoxin is not formed by contact agents although cobra venom is active, indicating that P 1 and P 2 are present. The anaphylatoxin-forming enzyme of rat plasma thus appears to be rather labile.

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