

Crossed Immunoelectrophoresis Analysis of Bee Venom

Enzymatic Identification of Antigens

R. EINARSSON, A. ANNERHED, R. KARLSSON, B. OLSSON and B. RENCK

Allergy Research, Pharmacia Diagnostics AB, Uppsala, Sweden

Bee venom obtained by electrical stimulation has been analysed by crossed immunoelectrophoresis against high-titer rabbit antibodies. The antigenic analysis of bee venom revealed that the extract contained 17 antigens, which were detected in the crossed immunoelectrophoresis pattern after staining with Coomassie Brilliant Blue. The crossed immunoelectrophoresis pattern indicated heterogeneity in several of the antigens, as most of the precipitates (exhibiting enzyme and hemolytic activities) represented multiple forms of phospholipase A, hyaluronidase, acid phosphatase and melittin.

Key words: antigenic composition; bee venom; crossed immunoelectrophoresis.

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Bee venom is composed of enzymes (proteins), peptides and different low molecular components, which are capable of producing enzymatical and pharmacological effects (4). The use of modern biochemical procedures has rendered it possible to isolate and characterise two basic enzymes, phospholipase A and hyaluronidase, and various peptides (melittin, apamin, MCD-peptide). Phospholipase A, the most abundant enzyme, and hyaluronidase have been considered as major allergens, while melittin has been suggested to be a minor allergen (9, 12, 14, 18). Furthermore, Hoffman (8) has identified another allergen possessing acid phosphatase enzyme activity.

Crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE) has been successfully applied in allergology for analysis of allergen extracts, as this method may give qualitative as well as quantitative information of individual components of multi-component antigenic systems (1, 13). The usefulness of CIE techniques in allergy research has been demonstrated because of the inherent

high resolving capacity. However, it must be pointed out that these methods have to be combined with various physiochemical methods, not depending on the ability of the antigens to induce antibody formation in animals to get a more complete information on the molecular identities.

The purpose of this study was to identify the individual antigens/allergens of bee venom allergen extract by means of crossed immunoelectrophoresis. The individual antigen components in the CIE-pattern were identified by examination of enzyme activity and hemolytic activity. The role of the antigens identified as allergens in a group of bee venom-sensitive individuals determined by crossed radioimmunoelectrophoresis is presented in an accompanying paper.

MATERIAL AND METHODS

Honey bee venom (*Apis mellifera*, lots 91232 and 00500) obtained by electrical stimulation

was bought from Mr C. Mraz, Champlain Valley, Middlebury, Vermont.

Antisera

Antisera to bee venom proteins were raised in rabbits by bi-weekly injections of 300 µg bee venom intracutaneously in Freund's complete adjuvant, essentially according to Harboe & Ingild (7).

Enzyme assays

Acid phosphatase activity was assayed by hydrolysis of the chromogenic substrate *p*-nitrophenyl-phosphate (8). Phospholipase A activity was measured by using agarose diffusion plates with lecithin as substrate, essentially in accordance with the method outlined by Habermann & Hardt (5). Hyaluronidase was determined by using hyaluronic acid (Healon®) dispersed in agarose as substrate essentially in accordance with the procedure recently described by Richman & Baer (16). For identification of the individual precipitates in the CIE-plate, the various enzyme substrates were incorporated in 1% agarose gel matrix. The agarose gel containing the substrate was then layered on the CIE-plate, and the hydrolysis was visualized as stained or cleared regions.

Measurement of melittin

Melittin was measured by its direct hemolytic activity, using washed sheep erythrocytes in phosphate-buffered saline, pH 7.4 (5). For identification of the immunoprecipitates in the CIE-plate caused by melittin, sheep erythrocytes (1.5%) were incorporated in 1% agarose gel matrix at 37°C. The gel was then layered on the CIE-plate and the zones undergoing lysis at 37°C were identified.

Bee venom components

Acid phosphatase, hyaluronidase, phospholipase A and melittin were prepared by a combination of gel filtration on Sephadex G-75(12) and by low pressure gel permeation chromatography on Shodex OH pak/B804S (15). The

isolated components were identified by assaying their enzyme activity and hemolytic activity. The homogeneity of the isolated fractions was studied by isoelectric focusing, pH 3 – 10 Pharmalyte® (Pharmacia Fine Chemicals, Uppsala, Sweden), sodium dodecylsulfate (SDS) – polyacrylamide gradient gel electrophoresis (Pharmacia Fine Chemicals) and isotachopheresis (3).

Immunological methods

Crossed immunoelectrophoresis (CIE) was carried out on 8 × 8 cm glass plates essentially in accordance with the previously described procedure (1, 13). Samples of 5 µl bee venom (15 mg/ml) were analysed in 1% agarose gel (Agarose A with medium electroendosmosis (Pharmacia Fine Chemicals) containing 0.073 M Tris, 0.024 M barbital, 0.0006 M calcium lactate (pH 8.6)). Antigen was run in the first dimension for 60 min, 10 V/cm at 15°C. This was followed by a second electrophoresis perpendicular to the first dimension into the rabbit antibody-containing agarose (13 µl antiserum/cm² gel) for 20 h, 2 V/cm at 15°C. Identification of individual components was achieved using tandem CIE in accordance with Axelsen et al. (1). The isolated bee venom components were used as reference in the first well, and the bee venom antigen mixture to be identified was in the second well.

RESULTS

The results of the CIE study of bee venom are demonstrated in Fig. 1a, and in Fig. 1b is shown a schematic drawing of the CIE reference pattern with arbitrarily numbered immunoprecipitates. As can be seen just a few antigens moved towards the anode in the first dimension electrophoresis, while most of the antigens migrated towards the cathode, demonstrating the high content of basic proteins/peptides in bee venoms. Attempts to induce all the antigens to migrate in the same direction by changing the mobility of the antibodies (carbamylation) were not successful. Variations in the

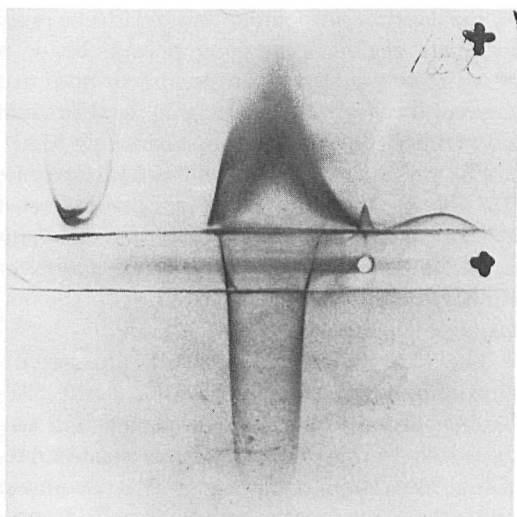


Fig. 1a. Crossed immunoelectrophoresis of bee venom antigen against hyperimmune rabbit antisera. 5 μ l of bee venom raw material (0.15%) analysed in agarose electrophoresis (pH 8.6) with 10 V/cm, 15°C for 60 min followed by electrophoresis perpendicular to the first with 2 V/cm, 15°C for 20 h in agarose containing rabbit anti-bee venom serum (13 μ l antiserum/cm² gel). Anode to the right in first dimension and at top in second dimension.

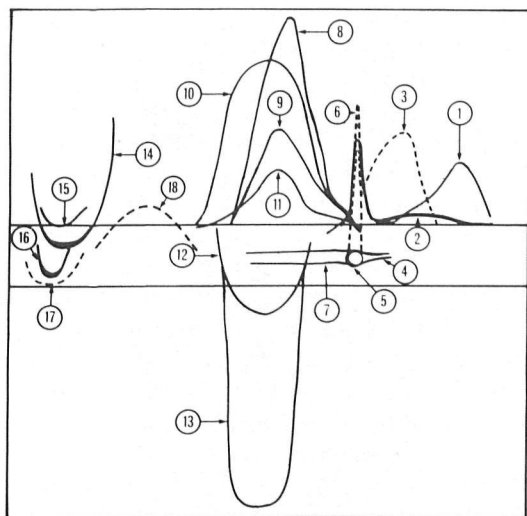


Fig. 1b. Schematic representation of the immune precipitates (Fig. 1a) arbitrarily numbered 1-18.

— : sharp precipitates.
 ---- : fuzzy precipitates.

Table 1

Identification of individual antigens (arbitrarily numbered in accordance with the schematic drawing) in bee venom CIE-pattern by applying enzyme activity of hemolytic activity assays

Antigen No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Acid phosphatase		×		×	×	×												
Hyaluronidase								×	×									
Phospholipase A							×			×	×	×	×					
Melittin					×		×								×	×	×	×

electrophoretic conditions (positions of the application well, time of the first dimension electrophoresis, voltage, etc.) and in antigen or antibody concentration indicated the presence of 17 antigens in the CIE-pattern by this high resolving method (antigen No. 18 was only visible after performance of crossed radioimmuno-electrophoresis). The antigens Nos. 3, 6, 9, 11 and 17 showed fuzzy immunoprecipitates, which might be due to differences in charge

and/or molecular weight of antigenic molecules bearing immunologically identical binding sites. Antigens Nos. 4, 5 and 7 showed fuzzy zones starting from the application well, indicating association with denatured proteins/peptides.

The individual antigenic components in the CIE reference pattern were identified by enzyme analysis, hemolysis and tandem CIE. After electrophoresis, the precipitates were assayed for enzyme activity by placing the agarose gel

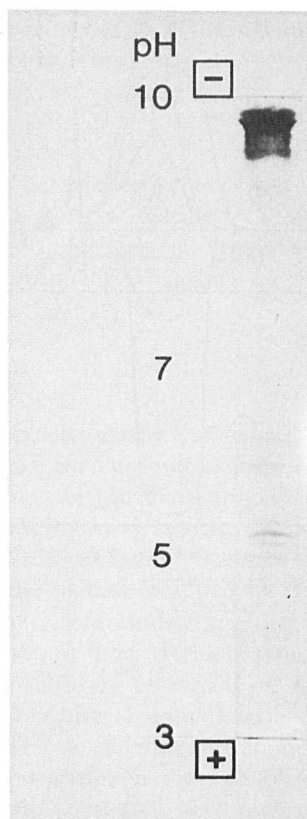


Fig. 2. Isoelectric focusing pattern of bee venom Pharmalyte[®], pH 3–10, 5 μ l of sample (5 mg/ml) was applied with sample adapter. Running time 2 h at 30 W.

containing suitable substrate on the CIE plate. Table 1 demonstrates the identification procedure used for antigens in the bee venom CIE-pattern. In order to have sufficient antigen in the immunoprecipitates, the concentration of bee venom in the CIE-experiments was increased to about 200 μ g. In spite of the high bee venom concentration, antigens Nos. 1 and 3 did not possess measurable enzyme activity or lytic activity. Antigens Nos. 2, 4, 5 and 6 stained for acid phosphatase enzyme activity.

The presence of acidic proteins in bee venom was also evident from the IEF-pattern, which demonstrated several bands positioned between pH 5–6.5 (Fig. 2).

The immunoprecipitates arbitrarily numbered 7, 10–13 (Fig. 1b) indicated the presence of phospholipase A enzyme activity. The clearing

of the lecithin suspension was relatively rapid for these regions containing phospholipase A enzyme activity. It was interesting to note that some of the cleared zones became slightly turbid after extended hydrolysis (approximately 15 h).

The presence of hyaluronidase activity in the CIE-pattern of bee venom was demonstrated by the use of hyaluronic acid (Healon[®]) as substrate. Hydrolysis of the substrate gave clear zones for the regions consisting of the immunoprecipitates numbered 8 and 9.

The identification of acid phosphatase, hyaluronidase and phospholipase A in the CIE-pattern was also checked by performing tandem CIE in the presence of the isolated individual bee venom components. Bee venom was applied in the first well and either acid phosphatase, hyaluronidase or phospholipase A, purified by a combination of Sephadex G-75 gel filtration and low pressure gel permeation chromatography, were applied in the second well. The tandem CIE-experiments showed agreement with the findings achieved with the enzymatic identification.

Analysis by SDS-gradient gel polyacrylamide electrophoresis of an isolated high molecular weight fraction gave a heavily stained band around 50,000 daltons and further bands in the molecular range of 100,000–110,000 daltons. Since the amount of protein applied from the high molecular weight fraction (approximately 200 μ g) was very high, it was also possible to detect very weak stained bands around 200,000 daltons and between 50,000–100,000 daltons. The multiple protein pattern in the IEF-pattern (pH 5–6.5) may be caused by these components. Furthermore, these acid components most probably constitute the anodally migrating proteins seen in the CIE-pattern (Fig. 1a).

The presence of melittin in the CIE-pattern was investigated by examination of direct hemolysis of sheep erythrocytes. Cleared zones demonstrated where the antigens Nos. 5, 7, and 14–17 are located. These findings indicate the occurrence of the basic peptide, melittin. Hemolysis was not visible on the CIE-plate where phospholipase A exhibited enzyme activity.

DISCUSSION

A reference pattern of bee venom in CIE was established. Seventeen precipitates (antigens) were demonstrated by Coomassie Brilliant Blue staining and arbitrarily numbered 1–17. An additional antigen (Ag-18), present in the CIE-pattern, was demonstrated only by autoradiographic development with X-ray film (accompanying paper). It is widely believed that bee venom contains enzymes and peptides acting as both antigens and allergens (9–12, 14, 18). Immunoprecipitates containing hyaluronidase, phospholipase A, acid phosphatase and melittin were identified by means of biochemical methods (zymography) and lytic activity. Our studies based upon electrophoretic separation and subsequent immunological reactions indicate, however, that the antigenic properties of bee venom are more complex than previously assumed. The various enzymes and melittin showed multiple antigenic properties. Each antigen, however (except Ag-1 and Ag-3), should not be regarded as a single protein/peptide, but rather as a group of antigenically (rabbit IgG) and allergenically (human IgE) more or less identical, partially identical or non-identical molecules.

The occurrence of acid phosphatase in bee venom (electrostimulated) has been shown earlier (8), and, according to Benton (2), at least three isozymes of acid phosphatase exist. Tandem CIE indicated partial identity between the purified high molecular weight fraction and antigen No. 1, but not with antigen No. 3. Antigen No. 1 might correspond to that allergenic high molecular weight component (allergen C) previously described (10, 11). This assumption is also supported by the results from the IEF and SDS-gradient gel polyacrylamide electrophoresis of an isolated high molecular weight fraction. However, one cannot exclude the possibility that antigen No. 1 might be a polymer of acid phosphatase, but with masked catalytic activity.

CRIE-studies (accompanying paper) revealed, that antigens Nos. 1, 2, 4, 5 and 6 were all shown to be allergenic for many bee venom-sensitive individuals. This observation is in ex-

cellent agreement with the findings reported by Karpas et al. (11), who found both acid phosphatase enzyme activity and allergenic activity in their HMW-fraction of bee venom.

It is interesting to note that phospholipase A shows quite different behavior during migration in the first and the second dimension electrophoresis. In the first dimension electrophoresis, phospholipase A moves very slowly towards the cathode, which would be expected from its isoelectric points (pI 8.8 and 9.2 (14)). But in the second dimension, phospholipase A migrates towards both the anode and cathode forming immunoprecipitates with IgG rabbit antibodies. It is quite evident that the interaction between phospholipase A molecules and bee venom IgG antibodies causes a significant alteration in accessible charges of the enzyme. Shipolini et al. (17) have suggested that various subforms of phospholipase A exist, which differ only slightly, and, furthermore, these differences may be in the content of carbohydrate or in the degree of amidation of acidic side-chains. This observation might explain the multiple CIE-pattern of phospholipase A. Furthermore, the presence of other types of phospholipase (B or C) cannot be excluded, as the cleared zones turned turbid after extended hydrolysis of lecithin.

Antigens Nos. 8 and 9, associated with hyaluronidase enzyme activity, partly overlapped antigens Nos. 10 and 11 (phospholipase A). Since hyaluronidase is a basic enzyme, (pI above 10.5), the anodal location of the antigens Nos. 8 and 9 appears somewhat puzzling. This effect may be interpreted by the fact that hyaluronidase may undergo a drastic conformational alteration when binding to IgG.

Melittin moves fast toward the cathode (pI above 10 (14)) in the first dimension electrophoresis, which might be expected from its molecular properties (a basic, hydrophobic polypeptide consisting of 26 amino acid residues). In contrast, melittin does not migrate towards the cathode in second dimension electrophoresis (Fig. 1). The formed upside-down multiple immunoprecipitates between melittin and IgG-molecules (antigens Nos. 14–17) are positioned in the anodal region. Melittin ex-

hibits a significant concentration-dependent aggregation in a water-based solvent (12) and exists mainly as a tetramer of identical polypeptide chains in polar solvent (6). The interaction between individual melittin molecules is largely hydrophobic, resulting in positively charged melittin complexes. Furthermore, depending on peptide concentration and ionic strength, melittin might assemble and dissociate into various aggregates (5, 19). There seems to be a dissociation of the melittin complexes in the second dimension electrophoresis when binding to IgG molecules. For that reason, it may be suggested that melittin is bound to IgG as different types of aggregates (tetramers, trimers, dimers and monomers), giving a substantially negative net charge to melittin aggregates at pH 8.6.

Previously it has been proposed that there exist five different allergens in bee venom; phospholipase A, hyaluronidase, acid phosphatase, melittin and allergen C (8). The present study combined with the CRIE data presented in the accompanying paper support that proposal.

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Address:

R. Einarsson, Ph.D.
Pharmacia Diagnostics AB
Box 17
S-751 03 Uppsala
Sweden

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