

THE PURIFICATION OF ACID PHOSPHATASE FROM HONEY BEE VENOM (*APIS MELLIFICA*)

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E. BARBONI, D. M. KEMENY, S. CAMPOS and C. A. VERNON. The purification of acid phosphatase from honey bee venom (*Apis mellifica*). *Toxicon* 25, 1097-1103, 1987. — Acid phosphatase from bee venom was purified by a combination of saturated ammonium sulphate precipitation, gel filtration and ion exchange chromatography. The final product which is a glycoprotein contained less than 0.1% phospholipase A₂ or hyaluronidase activity and existed in two molecular weight (96,000 and 45,000) forms. Acid phosphatase is a potent allergen, in bee venom allergic patients, which is capable of releasing histamine from sensitized human basophils and of inducing a wheal and flare reactions in sensitized human skin.

INTRODUCTION

BEE VENOM contains a number of enzymes, peptides and vasoactive amines (HABERMANN, 1972). The function of these enzymes is to break down cell membranes and so increase the penetration of the toxic venom constituents. While these enzymes are relatively harmless in themselves, they can induce an abnormal immunological response which results in the formation of IgE antibodies and the development, in some, of type I hypersensitivity (FRANKLAND, 1981; AMERICAN ACADEMY OF ALLERGY COMMITTEE ON INSECTS, 1981). Because the quantity of these enzymes that are injected following a bee sting is high (approx. 20 µg per sting) compared with the more usual exposure to inhaled allergens (approx. 1 ng of ragweed antigen E per summer), the allergic reaction can be much more severe and in extreme cases fatal (BARNARD, 1973, BROWN and BERNTON, 1970). Previous studies have identified phospholipase A₂, the most abundant of these enzymes, as the major allergen (SOBOTKA *et al.*, 1974, 1976) and this has been confirmed in subsequent studies (KEMENY *et al.*, 1983a). Other enzymes in bee venom reported to provoke an allergic response include hyaluronidase (HOFFMAN and SHIPMAN, 1976; KING *et al.*, 1976; SHEPHERD *et al.*, 1974; KEMENY *et al.*, 1983a) and acid phosphatase (HOFFMAN, 1977). Of the peptides, only mellitin is allergenic and then only in a minority of patients (PAULL *et al.*, 1977).

Phospholipase A₂ was purified by SHIPOLINI *et al.* in 1971. Purification of hyaluronidase, which is present in much smaller amounts, proved more difficult and was achieved by KEMENY *et al.* (1981, 1984) using affinity chromatography with Sepharose-bound antibodies to phospholipase A₂ in the final stages of purification. Despite a

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number of previous attempts (SHKENDEROV *et al.*, 1979; HOFFMAN, 1977) which resulted in the isolation of acid phosphatase, its complete purification has yet to be accomplished; and this was the purpose of the work described here.

MATERIALS AND METHODS

Bee (*Apis mellifica*) venom was purchased from Nectarcop, Sofia, Bulgaria. Materials for gel filtration and ion exchange chromatography were purchased from Pharmacia (U.K.) plc. Fast Garnet salt, α -naphthyl phosphate, and *p*-nitrophenyl phosphate and the acid phosphatase assay kit were purchased from Sigma Ltd, Dorset, U.K. All other reagents were purchased from BDH Ltd. All chromatographic procedures were carried out using LKB equipment with continuous monitoring at 280 nm.

Acid phosphatase activity was assayed using the method described in the Sigma technical bulletin (104). Briefly, samples (0.2 ml) containing 1–2 ng of protein were added to *p*-nitrophenyl phosphate (0.4 mg/ml) in citrate buffer (0.5 ml, 0.09 M, pH 4.8) at 37°C. After 10 min the reaction was stopped by the addition of NaOH (5 ml, 0.1 M) and the absorbance at 410 nm measured using a Perkin–Elmer spectrophotometer. Specific activity is defined as the amount of *p*-nitrophenyl (in μ g) liberated in 1 min per mg of protein. One enzyme unit (EU) is defined as the amount of protein (in μ g) required to produce 1 μ mole of *p*-nitrophenyl in 1 min. Hyaluronidase activity was measured by the method of GRIEF (1952) as described in detail previously (KEMENY *et al.*, 1984). Phospholipase A_2 was measured using a conductimetric assay (LAWRENCE *et al.*, 1974). Electrophoresis in polyacrylamide gels (10%) was carried out at pH 4.3 as described elsewhere (BENTON, 1967). Protein bands were located by staining with Coomassie Brilliant Blue and bands containing phosphatase activity identified by treatment with α -naphthyl phosphate and fast Garnet salt as described elsewhere (BENTON, 1967). Electrophoresis in polyacrylamide (12%) in the presence of sodium dodecyl sulphate (SDS) was carried out using the method of LAEMMLI (1970) at pH 8.9. The molecular weights of the protein staining bands were determined by reference to molecular weight marker proteins (Sigma, U.K. Ltd). The presence of carbohydrate in the protein staining bands was determined by periodic acid schiff staining (PAS).

RESULTS

Purification of acid phosphatase

Step 1: Ammonium sulphate precipitation. A sample (10 g) of the high molecular weight concentrate of bee venom, obtained by negative pressure dialysis against distilled water followed by lyophilisation, was dissolved in 1 liter of ammonium formate buffer (0.1 M, pH 4.7). Solid ammonium sulphate was added slowly with continuous stirring until a 45% saturation was reached, this concentration having previously been shown to precipitate the majority of the acid phosphatase activity. The precipitate formed (c. 1.0 g) was collected by centrifugation at 2000 *g* for 40 min.

Step 2: Gel filtration with Sephadex G-100. The precipitate from step 1 was redissolved in a minimal amount of Tris–HCl buffer (0.1 M, pH 7.2) and applied to a column (5 × 90 cm) containing Sephadex G-100 superfine equilibrated and eluted with this buffer at a flow rate of 25 ml/hr (Fig. 1). When the concentration of the sample applied to the column was > 70 mg/ml the majority of the acid phosphatase activity was in the first peak (Fig. 1a). At a lower protein concentration, < 50 mg/ml, the greatest activity was found in latter fractions (Fig. 1b). This phenomenon, which had been noted by HOFFMAN (1977) suggests that the enzymes exists in various degrees of aggregation.

Step 3: Ion exchange chromatography on CM Sephadex C50. The most active fraction from Step 2 (peak I from Fig. 1a, or peak II from Fig. 1b) was passed through a column (2.5 × 21 cm) containing CM Sephadex C50 in Tris–HCl buffer at pH 7.2, 0.1 M. The acid phosphatase activity was recovered in the void volume and, despite a purification factor of c. 3 fold, did not result in any loss of enzyme (Table 1).

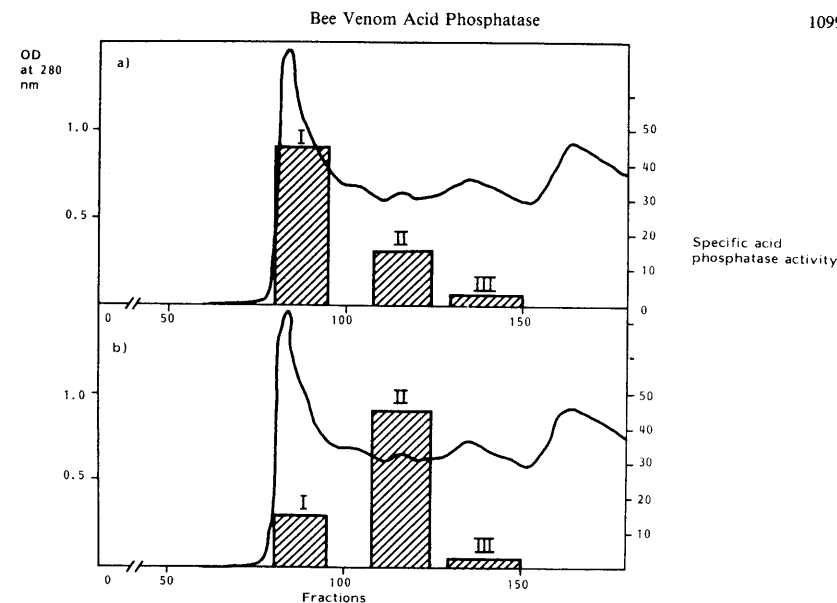


FIG. 1. ELUTION PROFILE SHOWN BY GEL FILTRATION.

5 ml of (a) a high (>70 mg/ml) or (b) a low (<50 mg/ml) concentration of the 45% ammonium sulphate precipitate through a column (5 × 90 cm) containing Sephadex G 100 superfine equilibrated with Tris–HCl buffer (0.1 M, pH 7.2) at a flow rate of 25 ml/hr. Fractions (15 min) were collected and acid phosphatase activity tested using *p*-nitrophenyl phosphate and expressed as units/ml where one unit is defined as the amount of protein (in μ g) required to produce one μ mole of *p*-nitrophenyl in 1 min at 37°C, pH 4.8.

TABLE 1. PURIFICATION OF BEE VENOM ACID PHOSPHATASE

Separation method	Protein (mg)	Specific activity*	Total activity†	Yield %	Purification factor
Negative pressure dialysis	10,000	0.69	6900	100	1
Ammonium sulphate (45%) precipitation	884	9.7	8504	123	14
Gel filtration (Sephadex G100)	40	46.8	1867	27	68
Ion exchange chromatography (CM Sephadex C50)	14	135.0	1890	27	196

*Specific activity in μ g of *p*-nitrophenol liberated in 1 min, at 37°C per mg protein.

†Total activity = specific activity × yield.

Characteristics of the purified enzyme

The purified enzyme contained less than 0.1% phospholipase A_2 and hyaluronidase activity (determined by competitive radioimmunoassay). When reduced with 2 mercaptoethanol two protein staining bands could be seen on SDS-PAGE (Fig. 2). Their molecular weights were determined by reference to marker proteins and were 45,000 and 96,000 respectively. Both of these bands produced a pink colour when stained with PAS indicating that they were glycoproteins. The enzyme gave two protein staining bands when electrophoresed at pH 4.3 which both stained for acid phosphatase activity.

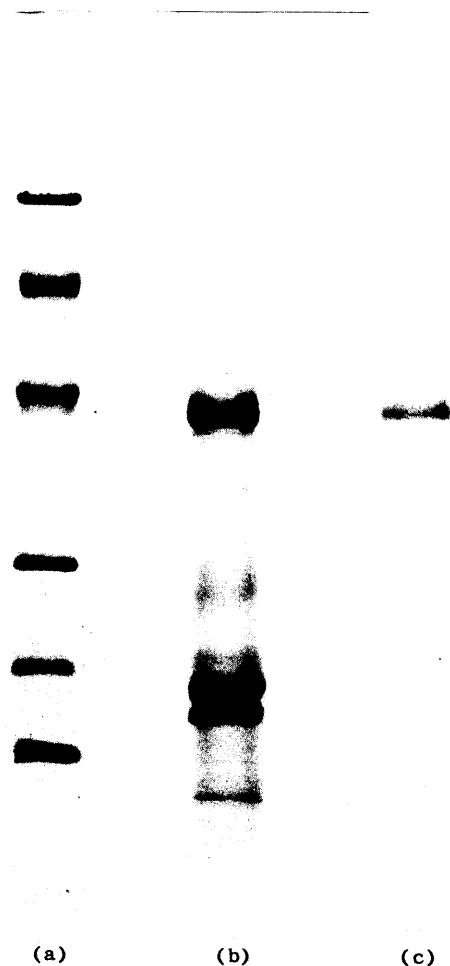


FIG. 2. SDS-PAGE SUMMARISING THE MAIN STEPS TAKEN IN THE PURIFICATION OF BEE VENOM ACID PHOSPHATASE.

The top of the gel is the cathode and the bottom the anode. A 12% acrylamide concentration was used at pH 8.9. Track (a) contains the molecular weight markers which are, from top to bottom, Phosphorylase b 97,400, bovine serum albumin 66,000, ovalbumin 45,000, carbonic anhydrase 29,000, soy bean trypsin inhibitor 20,100, α -lactalbumin 14,200. Track (b) contains c. 20 μ g of peak 1 obtained by gel filtration over Sephadex G 100; track (c) 5 μ g purified acid phosphatase.

The optimum pH for the enzyme was determined in citrate buffer at different pH values and was found to be 4.4 (Fig. 3). The Michaelis-Menten constant (K_m) was determined under optimum conditions of initial velocity (V_0) and incubation time (30 min, 37°C) using *p*-nitrophenyl phosphate and was found to be 4.16×10^{-4} M (Fig. 4).

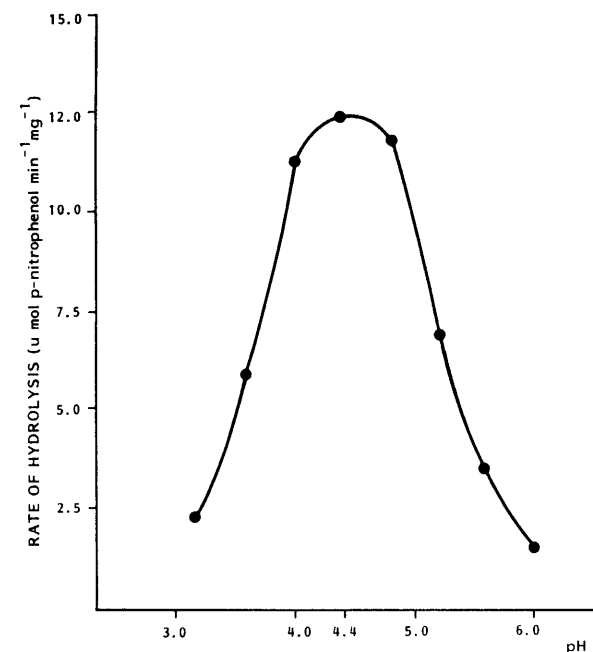


FIG. 3. DETERMINATION OF THE OPTIMUM pH FOR BEE VENOM ACID PHOSPHATASE.

DISCUSSION

The enzymes in bee venom are of considerable interest to allergists because of their ability to provoke severe, life threatening anaphylactic reactions in individuals who are sensitive to them. Allergy to bee stings differs from that to inhalant allergens as its treatment, by administration of increasing doses of bee venom, is almost universally successful (HUNT *et al.*, 1978). Furthermore, studies of the immunological response to purified bee venom allergens has revealed that IgE and IgG antibodies can be regulated independently (KEMENY *et al.*, 1983b). The isolation and characterization of acid phosphatase from bee venom would be of considerable help in understanding more about the role of the different bee venom enzymes in bee sting allergy.

During the purification of acid phosphatase described here, we found that there was a loss of enzyme activity during the gel filtration step either through precipitation, aggregation or inactivation. Despite a number of different chromatographic steps, two bands remained on SDS-PAGE with molecular weights of 96,000 and 45,000.

Bee venom acid phosphatase is a potent releaser of histamine from sensitized human basophils (WAHN *et al.*, 1984), as well as being capable of producing an immediate-type wheal and flare in the skin. Preliminary studies (KEMENY *et al.*, 1983b) suggested that acid phosphatase was the major allergen in 18% of bee sting allergic patients and this has

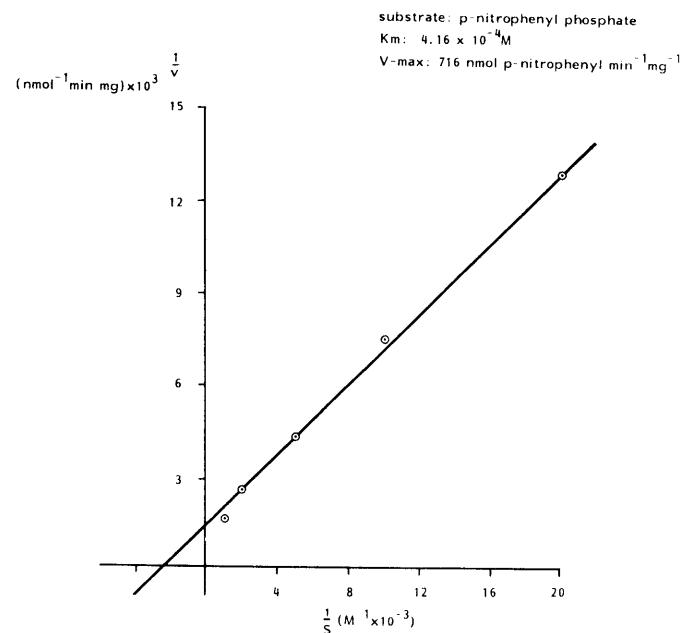


FIG. 4. DETERMINATION OF THE MICHAELIS-MENTON CONSTANT (K_m) FOR BEE VENOM ACID PHOSPHATASE.

subsequently been confirmed using the material prepared here. Our present studies are directed at identifying human B lymphocytes that produce IgE antibody to acid phosphatase and the other bee venom allergens.

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