

Immunologic and biochemical evaluation of the potency of whole insect body extracts

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Recent studies have indicated that currently available whole body extracts have little potency and are ineffective for diagnosis and treatment of stinging insect allergy. Pure venom is a potent effective allergen but is difficult to obtain in sufficient quantities from all Hymenoptera species. In these studies, an attempt was made to prepare a potent whole body extract. Whole bee body extracts were prepared with different extraction periods and at cold and room temperatures. Potency was examined biochemically by measurements of phospholipase A (PLA) activity and immunologically by PLA and bee venom radioallergosorbent test (RAST) inhibition experiments and gel diffusion studies with the use of rabbit antisera. All extracts prepared in the laboratory had some potency, indicating that it is possible to make a whole body extract containing small quantities of PLA or bee venom. However, the potency of these extracts was minimal as compared with bee venom. Three commercial extracts were almost devoid of detectable immunologic activity. While further attempts may be made to prepare a potent whole body insect extract, these results suggest that it is necessary to obtain venom in relatively pure form for the diagnosis and treatment of stinging insect allergy.

Whole body insect extracts (WBE) have been used for many years for the diagnosis and treatment of insect-sensitive individuals. These extracts are prepared by mashing or grinding of the whole insect bodies. Recent immunologic and clinical studies have suggested that the presently available commercial whole body extracts are ineffective for both diagnosis and therapy, probably because they lack sufficient content of venom.¹⁻³ Venoms appear to be the appropriate potent diagnostic and therapeutic allergen.³⁻⁵

It is relatively easy to collect large quantities of bee venom.⁶ Other insect venoms (yellow jacket, hornet, and wasp) are more difficult to obtain in sufficient amounts. Because these insects are responsible for a

large number of allergic reactions and because individual sensitivity may be restricted to only one insect,⁴ the availability of all these venoms is imperative. Collection of adequate quantities of vespid venoms will require considerable expenditure of time and money. It therefore seemed appropriate to re-evaluate the potency of WBE in the attempt to find if there was an easier and more economical method of producing a potent antigen. These studies were further prompted by the report of at least one WBE which appeared to be potent in immunologic studies.⁷

METHODS AND MATERIALS

Antigens

Preparation of whole bee body extract (WBE). Frozen bee bodies (10 gm; -20° C) were weighed into a dry, previously weighed Sorvall homogenizer cup. The bodies were rinsed three times with cold phosphate-buffered saline (PBS). After decanting the final rinse, the homogenizer cup was weighed again, and PBS (approximately 100 ml) was added, making a 10% solution. Homogenization was conducted for three 1-min periods at 1-min intervals. Two separate homogenates were prepared. One was maintained at the temperature of melting ice during homogenization and at 4° C for all further processing. The other

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TABLE I. Biochemical assay of phospholipase A: Specific phospholipase activity of six whole bee body extracts and one bee venom preparation

Extracts	Units of PLA activity* per ml	Total protein (mg/ml)	Specific activity (units/mg protein)
Whole body extracts (10%)			
15 min cold	8.40	2.04	4.1
24 hr cold	8.75	2.63	3.3
48 hr cold	8.75	2.68	3.3
15 min RT†	6.67	2.37	2.8
24 hr RT	32.25	6.81	4.7
48 hr RT	13.51	8.44	1.6
Bee venom‡	8400.0	24.70	340.1

*PLA activity: 1 unit equals 1.0 micromole fatty acid liberated/ml/min at 37° C, pH 8.0.

†Room temperature.

‡One bee venom preparation was used as a reference for comparison (24.70 mg dry weight/ml).

TABLE II. Immunologic assay of phospholipase A: Volumes of extracts and reference solutions required for 50% inhibition of PLA and bee venom (BV) RAST

Extracts	50% inhibition	
	PLA RAST (μl)	BV RAST (μl)
Whole body extracts (10%)		
15 min cold	10.93	11.28
24 hr cold	9.63	8.21
48 hr cold	11.03	10.17
15 min RT*	9.93	9.82
24 hr RT	3.10	6.84
48 hr RT	4.82	7.53
Homologous reference solutions		
Sigma PLA 1 mg/ml†	0.037	0.47
Sigma BV 1 mg/ml†	0.270	0.76

None of the three commercial whole bee extracts (20,000 PNU/ml) could inhibit bee venom or phospholipase A RAST to even 40% when used undiluted.

*Room temperature.

†Dry weight.

homogenate was processed entirely at ambient room temperature. All other treatment was identical.

Immediately after homogenization, one third of a homogenate was centrifuged for 15 min at 15,000 rpm. The supernatant was filtered through Whatman No. 1 filter paper, aliquoted, and stored at -70° C for further studies. The remaining two thirds of each homogenate was stirred in an Erlenmeyer flask with the use of a magnetic stirrer. After 24 hr, one half

of this homogenate was removed and the remainder stirred for an additional 24 hr (total, 48 hr). These were processed as indicated above.

Thus, 6 different extracts were prepared. One set of 3 extracts was prepared at cold temperature and one set at room temperature. Each set contained 3 extracts which had been extracted for periods of 15 min, 24 hr, and 48 hr, respectively.

The protein content of each of the 6 different extracts was determined by the biuret method, compensating for the presence of pigment as previously described.^{8, 9}

Commercial whole body extracts. Three different whole bee body extracts (20,000 protein nitrogen units [PNU]/ml) were obtained from commercial suppliers.*

Venom and phospholipase A. Bee venom and phospholipase A for these studies were obtained from the Sigma Chemical Company, St. Louis, Mo.

Biochemical assay of phospholipase activity

Phospholipase activity (PLA) of the extracts and bee venom was assayed by the titration of the free fatty acids released from phospholipids present in crude egg yolk.¹⁰ One unit of activity releases one micromole of fatty acid per minute at 37° C, pH 8.0. Free fatty acid was titrated with 0.007 N NaOH with the use of a pH stat and microreaction vessel (Radiometer model TT11/SBR2/ABU12/TTA31, Copenhagen, Denmark).

Goat antiserum to PLA (5 to 25 μl) was found to inhibit hydrolysis, while normal goat serum in equal volumes decreased activity by less than 10% as previously reported.¹⁰

A bee venom preparation (24.7 mg dry weight/ml) was used as a reference solution for the titration.

Immunologic assay—radioallergosorbent test (RAST) inhibition

The immunologic activity of the 6 whole body extracts and of the 3 commercial whole bee body extracts (20,000 PNU/ml) was evaluated by the RAST inhibition technique. The methodology has been previously described.¹¹ Briefly, cellulose discs were coupled with either 20 mg of bee venom or 2 mg of phospholipase A per 200 discs. A reference serum pool diluted 1:1 with pooled normal human serum was prepared by using serum of 4 bee-sensitive patients, all known to have high levels of bee venom—

*Center Laboratories, Inc., Port Washington, N. Y. 11050, No.7748279; Greer Laboratories, Inc., Lenoir, N. C. 28645, No.B2-301-1F; Hollister-Stier Laboratories, Inc., Yeadon, Pa. 19050, No.0052404.

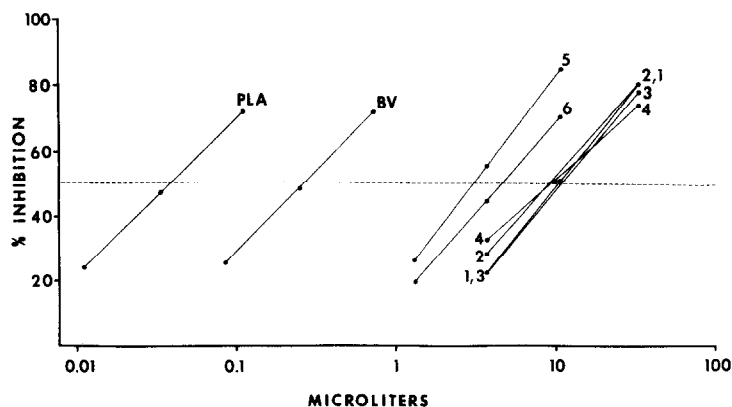


FIG. 1. Phospholipase disc RAST inhibition. Least-squares plots of percent inhibition vs log microliters of test solutions. BV and PLA each 1 mg dry weight/ml 2% human serum albumin. 10% whole bee body cold extracts; 1, 15 min; 2, 24 hr; 3, 48 hr. 10% whole bee body room temperature extracts: 4, 15 min; 5, 24 hr; 6, 48 hr. Positive human anti-bee venom serum pool used at 1:1 dilution in normal human serum.

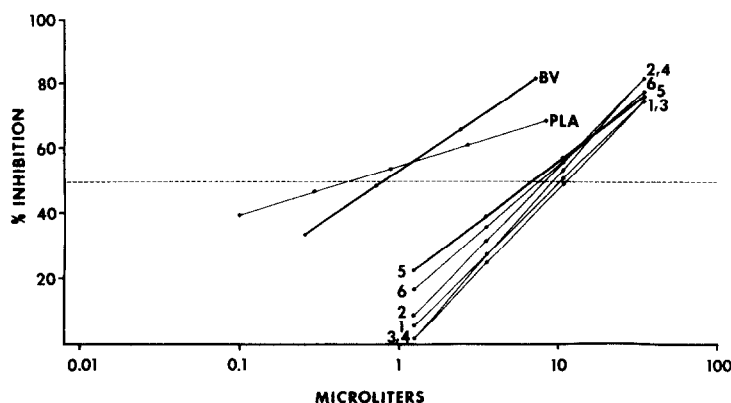


FIG. 2. Bee venom disc RAST inhibition. Least-squares plots of percent inhibition vs log microliters of test solutions. 10% whole bee body cold extracts: 1, 15 min; 2, 24 hr; 3, 48 hr. 10% whole bee body room temperature extracts: 4, 15 min; 5, 24 hr; 6, 48 hr. Positive human anti-bee venom serum pool used at 1:1 dilution in normal human serum.

specific IgE and phospholipase-specific IgE. None of the patients had received specific immunotherapy. Homologous bee venom and phospholipase A were used as reference standards. These were each prepared as 1 mg/ml dry weight solution in 2% human serum albumin.

The inhibition studies were performed in duplicate. Threefold serial dilutions in 100- μ l amounts of each of the whole body extracts and reference solutions in 2% human serum albumin were incubated with 50 μ l of the reference serum pool for 24 hr at 4° C. The appropriate discs were then added to each tube and incubated at room temperature for 3 hr. Following three washings with PBS, 50 μ l of I¹²⁵-labeled goat anti IgE (50 ng antibody) was added to each tube and incubated for 24 hr at room temperature. After washing the discs 3 times with PBS, residual radioactivity was counted in a Packard Tandem Scintillation Counter. Preliminary data for duplicates were aver-

aged and the inhibition curves plotted. The log-linear straight portion around the 50% inhibition region of each curve was determined. The least-squares 50% inhibition doses or dilutions of the reference antigens and test solutions were then determined with all the selected individual data points (6 to 12 points). These results were reproducible within 5% to 10%.

Controls for these experiments consisted of tubes without any inhibiting antigen and tubes with normal serum plus inhibiting antigen.

Radial immunodiffusion studies

Rabbit antisera to bee venom, phospholipase A, and whole body extracts* were raised in 6-to 8-pound New Zealand White rabbits. The rabbits were injected

*The whole body extract prepared by extraction for 15 min at cold temperature was empirically chosen for immunization.

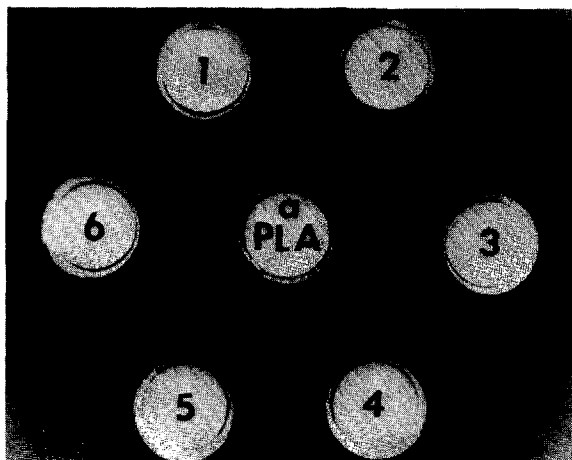


FIG. 3. Immunodiffusion: Reaction of extracts with rabbit antiserum to bee venom phospholipase A (aPLA). 10% whole bee body cold extracts: 1, 15 min; 2, 24 hr; 3, 48 hr. 10% whole bee body room temperature extracts: 4, 15 min; 5, 24 hr; 6, 48 hr.

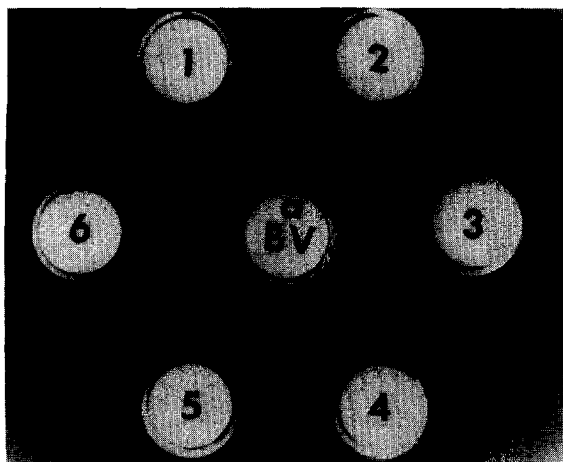


FIG. 4. Immunodiffusion: Reaction of extracts with rabbit antiserum to whole bee body (aBV). 10% whole bee body cold extracts: 1, 15 min; 2, 24 hr; 3, 48 hr. 10% whole bee body room temperature extracts: 4, 15 min; 5, 24 hr; 6, 48 hr.

intradermally at monthly intervals with a mixture containing 100 μ g of antigen in 0.5 ml PBS and 0.5 ml of complete Freund's adjuvant. Periodic trial bleedings were carried out. When an antibody titer of 1:32 was present, as measured in gel diffusion, the antiserum was harvested. Each of the antisera used in the studies represented a serum pool from 2 to 4 rabbits injected with the same antigen.

Radial immunodiffusion studies were performed with the various extracts and reference antigens in disposable 7 $\times$ 50 mm covered plates containing 3 ml of 1% agarose, pH 8.0, in borate-buffered saline. Wells, 5 mm in diameter, spaced 6 mm apart, each had a capacity of 25 μ l of antigen or antiserum.

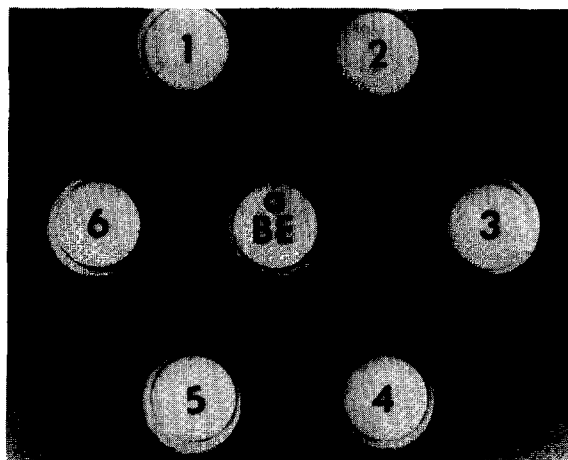


FIG. 5. Immunodiffusion: Reaction of extracts with rabbit antiserum to whole bee body extract (aBE). 10% whole bee body cold extracts: 1, 15 min; 2, 24 hr; 3, 48 hr. 10% whole bee body room temperature extracts: 4, 15 min; 5, 24 hr; 6, 48 hr.

RESULTS

Biochemical assay of phospholipase A activity

The biochemical activity of the extracts, as defined previously, is shown in Table I. The total protein, measured in milligrams per milliliter, is also noted. The three extracts prepared at 4° C and the 15-min extract prepared at room temperature had approximately equal total protein content and phospholipase A activity. The extracts prepared at room temperature following 24- and 48-hr extraction both had a somewhat higher total protein and higher phospholipase A activity. The reference bee venom was found to have a specific phospholipase activity of about 340 units/mg protein. In comparison, only 2 of the WBE exceed 1% of the specific activity of this bee venom.

Immunologic assay—RAST inhibition

The results of the RAST inhibition studies are shown in Table II and Figs. 1 and 2. The activities of the 6 prepared whole body extracts and 3 commercial whole body extracts were compared with a bee venom and phospholipase A preparation.

Figs. 1 and 2 depict graphically the least-squares slopes from which the 50% RAST inhibition data of Table II are calculated. PLA RAST inhibition curves are parallel and the potency of the 6 prepared extracts can be compared directly. The bee venom disc RAST inhibition curves generally do not parallel the bee venom reference curve as closely except for the 24-hr room temperature extract. Of the extracts prepared for these studies, the 24- and 48-hr room temperature extracts were most potent in both the PLA and bee venom RAST inhibition. Relatively large amounts of

these whole body extracts were required for 50% inhibition of RAST when compared with bee venom and phospholipase A. None of the 3 commercial whole body extracts could inhibit the bee venom or PLA RAST even to the extent of 40% when used undiluted.

In Table II RAST inhibition data are expressed in microliters of extract or reference solutions necessary for 50% inhibition. The volumes of the 10% whole body extracts (WBE) ranged from about 11 to 41 times the volume of bee venom and about 84 to 298 times the volume of PLA required for 50% inhibition of PLA RAST. The volumes (extrapolated) of the commercial extracts would be 10-fold to 50-fold larger than those for our prepared WBE.

There was close agreement between the results of the immunologic and biochemical assays of PLA potency in the various extracts.

Antigen content—gel precipitation studies

The results of the radial immunodiffusion studies are shown in Figs. 3 to 7. Figs. 3, 4, and 5 depict the reactions of the various whole body extracts with rabbit sera prepared to PLA, bee venom, and whole body extract, respectively. It would appear that all the whole body extracts contain a small amount of PLA, the largest perhaps being in the 24- and 48-hr room temperature extracts (Fig. 3). Similarly in Fig. 4 all extracts react with antiserum prepared to bee venom, with the 24- and 48-hr room temperature extracts again showing the most activity. Fig. 5 shows the reaction of the extracts with antisera prepared to whole body extract. There are numerous lines of precipitation with all extracts.

The three antisera were tested against bee venom, 100 $\mu\text{g}/\text{ml}$, and phospholipase A, 50 $\mu\text{g}/\text{ml}$, in 2% human serum albumin. These results are shown in Fig. 6. Antiserum to whole body extract did not show precipitation reactions with venom components, indicating that whole body antigens are not detectable in this bee venom. Phospholipase A and bee venom react very well with their homologous and heterologous antisera.

Studies with the 3 commercial whole body extracts are shown in Fig. 7. These extracts do react with antisera prepared to whole bee body extracts. In contrast to the results with the specially prepared WBE, these extracts do not show precipitation reactions with antisera prepared with whole bee venom.

DISCUSSION

The whole body extracts were prepared in the described manner for several reasons. Concern had been raised that in the traditional preparation of these ma-

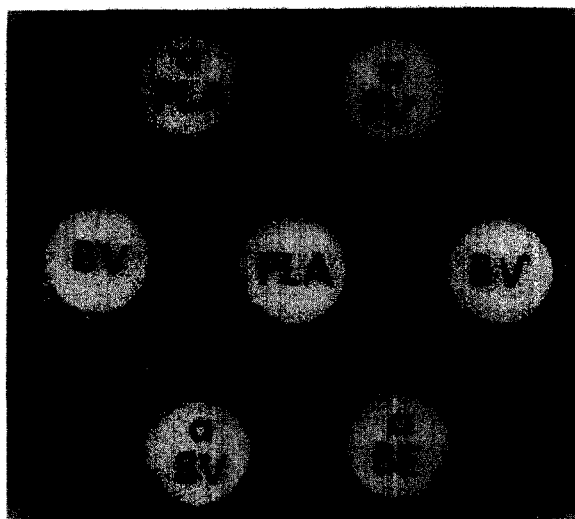


FIG. 6. Immunodiffusion: Reaction of bee venom (100 μg protein/ml) and PLA (50 μg protein/ml) with rabbit antisera to bee venom phospholipase A (*aPLA*); whole bee venom (*aBV*), and whole bee body extract (*aBE*).

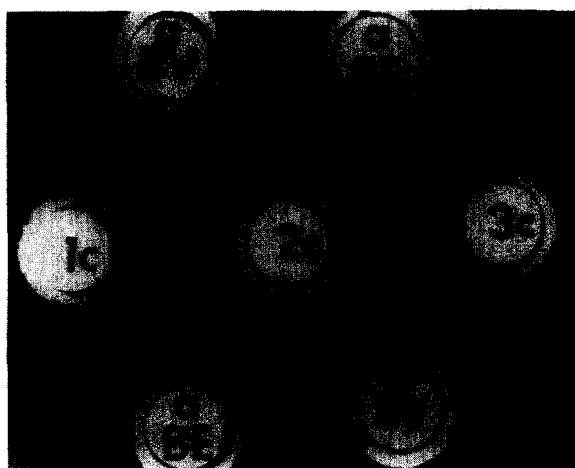


FIG. 7. Immunodiffusion: Reaction of three different commercial whole bee body extracts (1C, 2C, 3C) (20,000 PNU/ml) with rabbit antisera to whole bee venom (*aBV*) and whole bee body extract (*aBE*).

terials, proteolytic enzymes released from tissue may destroy the potent allergens present in venom. We attempted to obviate this potential problem in several ways. First, an extract was prepared after very rapid extraction (15 min). Previous studies have shown this to be very effective in preparation of extracts of cad-dis fly.⁹ Most commercial manufacturers extract from 24 to 48 hr in preparation of their whole body extracts. Secondly, extraction also was done at a cold temperature.

The extracts prepared in the cold along with the extract prepared following rapid extraction at room temperature all appeared to have about the same po-

tency. The two extracts prepared after 24- and 48-hr extraction at room temperature did have somewhat more activity. However, all the extracts as compared with bee venom and phospholipase A were relatively impotent. It should also be noted that the 3 commercial extracts evaluated by the RAST inhibition and gel diffusion studies had even less potency. We have previously reported similar findings in earlier studies of four different commercially available extracts.¹¹

Phospholipase A assays with the use of both a biochemical method and RAST inhibition were used as measures of potency of these extracts. Phospholipase A has been identified as a major allergen in bee venom, although it is now evident that other allergens may be important as well.^{12, 13} However, an extract devoid of phospholipase A activity may be seriously questioned as having substantial diagnostic or therapeutic potency. The results of both phospholipase A assays correlated very well.

In the phospholipase A RAST inhibition experiments, the slopes of inhibition by phospholipase A and bee venom were parallel, suggesting they are measuring the same parameter. In the bee venom RAST inhibition experiments, the slopes for bee venom and phospholipase A inhibition are different. This probably is due to the fact that the reference pool contains significant amounts of antibody to components of bee venom other than phospholipase A. The PLA added in the inhibition experiment is only able to neutralize the anti-PLA antibody. Antibodies directed at other components of bee venom can only be neutralized by whole bee venom. While inhibition by the whole body extracts is relatively weak, their slopes parallel more closely the bee venom inhibition slopes.

These results suggest that it is possible to make a whole bee body extract containing small quantities of phospholipase A or bee venom. Overall, however, it is doubtful that the activity present is sufficient to have demonstrable diagnostic or therapeutic value. While further attempts may be made to prepare a

whole body extract with some potency, the results of these studies suggest that it is necessary to obtain venom in a relatively pure form for use in the diagnosis and treatment of stinging insect allergy.

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