VENOM IN A STING AND IN SILICO PREDICTION OF ANTIGENIC DETERMINANTS OF VENOM OF APIS DORSATA

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Abstract: The honeybee differs from other flying Hymenoptera from both entomologic and allergic viewpoints. Beekeepers are particularly at risk for honeybee exposure and allergy. Bee venom is a complex mixture of proteins (enzymes and peptides) with unique pharmacological activities. The main enzyme in A. dorsata venom is phospholipase A2. Honeybee venom is complex, and the delivery mechanism provides for a large but often variable amount of injected venom. However, in present study is designed to find out quantity of honeybee venom injected in a single sting by A. dorsata and in silico prediction of antigenic determinants of their proteins.

Key words: Honeybee venom, Antigenic determinants, In silico

INTRODUCTION

Allergic reactions to hymenoptera stings are one of the major reasons for IgE-mediated anaphylaxis. Beekeepers are usually at risk for honeybee exposure and allergy. Phospholipase A2 is the most lethal of the honeybee venom peptides, whereas melittin, which is only slightly less lethal, is the most abundant. Also, bee venom has long been used as one of folk remedies for the arthritis and gout [1,2]. Bee venom, known to be effective on the inflammatory diseases and pains, is composed of complex mixture of various components. Of them, the peptides have anti-inflammatory [3,4], antibacterial [5] and strong analgesic (6) actions. They also contribute to the enhancement of immune responses [7]. Bee venom is an alternative approach to the arthritis drugs containing steroids and immunosuppressant, of which prolonged use causes serious side effects on the patients [10].

Bees have more potent venom during the summer [11,12]. Venom of Apis dorsata contains phospholipase A-2 and melittin. PhospholipaseA-2 hydrolyzes phospholipids to free fatty acids and lypolipids and thus initiates the biosynthesis of eicosanoids and platelet–activating factor, potent mediators of inflammation, allergy, apoptosis, and tumorigenesis. Melittin is the principal component of bee venom. It is 100 times more potent than hydrocortisone [13,14]. Melittin, a major component of the dried bee venom, stimulates the pituitary and adrenal glands to produce catecholamine and cortisone, and stabilize the cell membrane of the lysosome for the anti-inflammatory action [7-9]. The objective of present study is to find out quantity of venom introduce in a single sting by A. dorsata and in silico prediction of antigenic determinants of their proteins.

MATERIALS AND METHODS

Collection of honeybee venom: Honeybees (Apis dorsata) are collected locally from beekeepers in a basket. Each honeybee was stimulated to sting on a clean glass plate. With a sting a little drop of venom came out on the site of sting. The venom drops on the glass plate were allowed to dry. The air dried venom was collected in a clean vial and stored at
low temperature (-40°C). Extracted venom of *Apis dorsata* was used for further experimentation.

**Experimental animal:** Wistar rats (*Rattus norvegicus*) of 120 ± 5 gms, procured from National Institute of Nutrition (NIN) Hyderabad, were used. Animals’ experimentation was conducted according to “INSA-Ethical Committee approval.” The animals were housed in polypropylene cages in the adequately ventilated room. The rats were fed standard feed and water *ad libitum* throughout the course of the study. Experimental rats were administered with 1 mg dose of bee venom. Blood sample were collected after 9 hours of envenomation.

**Collection of blood sample:** The venous blood was obtained from the orbital sinus (retro-orbital vein) of the control and experimental albino rats. The blood was collected into tubes (5 ml) and was kept for separation of serum. Then the separated serum was centrifuged at 4000 rpm in ultra cooling centrifuge (Remi) for 10 minutes. The serum was stored at -20°C.

**Radial immunodiffusion assay:** Single radial immunodiffusion (RID) is used extensively for the quantitative estimation of antigen antibody precipitation. It is made more sensitive by the incorporation of antiserum in the agarose. Antigen is then allowed to diffuse from wells cut in the gel in which the antiserum is uniformly distributed. Initially, as the antigen diffuses out of the well, its concentration is relatively high and soluble antigen-antibody adducts are formed. However as Ag diffuses further from the well, the Ag-Ab complex reacts with more amount of antibody resulting in a lattice that precipitate to form a precipitin ring.

Thus, by running a range of known antigen concentration on the gel and by measuring the diameter of their precipitin rings, a calibration graph is plotted. Antigen concentrations of unknown samples run on the same gel can be found by measuring the diameter of precipitin ring and extra plotting this value on calibration graph.

**Materials:** Agarose, 10X assay buffer, antigen, antiserum, gel punch with syringe, glass plate, template.

**Methods:** 10 ml of 1.0 % agarose (0.1 g/ 10 ml) was prepared in a buffer by heating solely till agarose dissolves completely. Care was taken to avoid scorch or froth the solution. The molten agarose was allowed to cool to 55°C. 500 µl of antiserum was added to 6 ml of agarose solution mixed thoroughly for uniform distribution of antibody. Agarose solution containing the antiserum was poured on to a grease free glass plate set on a horizontal surface. Left it undisturbed to form a gel. Well was formed with the help of a gel puncher using the template. 20 µl of the antigen was added to the wells. The gel plate was kept in a moist chamber (box containing wet cotton) and incubated overnight at room temperature. The edges of the circle were marked and measured the diameters of the rings. Graph of diameter of ring (on y axis) versus concentration of antigen (on x-axis) on a semi-log graph sheet was plotted. Determined concentration of unknown by reading the concentration against the ring diameter from the graph.

**In silico studies: Database Searching:** Genomic databases are used to store the vast amount of information issuing from the genome projects. There are many different types of databases available, but for routine protein sequence analysis, primary and secondary, Genbank (15), Uniport (16) databases are initially the most important (17). We searched and retrieved genome protein sequences of phospholipase A2 and melittin protein (*A. dorsata*) sequences are downloaded directly in FASTA format (18). For ease of use sequences was retrieved from web sites are as- www.ncbi.nlm.nih.gov.

**Tool for prediction of antigenic peptides:** A program predicts those segments from within phospholipase A2 and melittin protein (*Apis dorsata*) sequence that are likely to be antigenic by eliciting an antibody response. Antigenic peptides are determined using method of Kolaskar and Tongaonkar (19). Predictions are based on a table that reflects the occurrence of amino acid residues in experimentally known segmental epitopes. Segments are only if they have a minimum size of residues (http://www.mifoundation.org).

Antigenic Epitopes of PLA2 and melittin were determined along with their propensities by using Kolaskar- Tongaonkar tools of Antigenicity. www.mifoundation.org/tools. Peptool is a program for the analysis of protein sequences developed by BioTools Inc. the program offers large collection of tools for analysis of protein sequences, including
RESULTS

Immunoelectrophoresis: 9 hours after intraperitoneal administration of 1 mg dose of honeybee venom to the albino rats, blood was removed from the orbital sinus of the envenomated rats, and was allowed to clot to get the serum. 0.5 ml serum was mixed with the gel which was then spread on the clean glass plate (Fig. 4). 5 uniform size wells were made in the gel out of which 4 wells were filled with different concentrations of honeybee venom (antigen) dissolved in glass distilled water. The fifth well was filled with fresh honeybee venom from 5 honeybees. The venom (antigen) got diffused in the gel, reacted with the corresponding antibodies in the serum present in the gel. The antigen–antibody reaction resulted into rings of precipitation (Fig: 4). The diameters of the rings were measured and the results are given in the table 4.5. The diameter of precipitin ring formed from the honeybee venom of 5 honeybees was 13.2 mm. The values were put in the graph (Fig. 3) from which

database similarity searching, pattern/motif searching, and multiple sequence alignment. Here peptool is used for motif searching of PLA2 and melittin
quantity of venom was calculated (1.3 mg). It was a quantity of venom secreted from 5 honeybees. After dividing it by 5, venom quantity from one honeybee is calculated. It is found to be 0.26 mg.

Results for In silico studies of PLA2 and Melittin from Apis dorsata venom: Through NCBI website amino acid sequences of PLA2 and melittin were searched. PLA2 from Apis dorsata venom is having 134 amino acid and Melittin 26 amino acids. The amino acid sequences are given below-

Sequence of Phospholipase A-2: iiypgtlwcg hgnvssspde lgrfkhtdcrshmpdvm sageskhgl tntashtrsl ccdcdkfydc lknssdtiss yfgemynfni ldtckykehp tvgcgtkrt gccntvdk skpkyqqfwd lrky/

FASTA format of PLA2 is as follows:
>gi|471171012|sp|Q7M415.1|PA2_APIDO
Phospholipase A2 (Phosphatidylcholine 2-acylhydrolase)

Sequence of Melittin: gigailkvlstglpaliswi krkrqe/

FASTA format of Melittin is as follows:
>gi|126955|sp|P01502.1|MEL_APIDO
GIGAILKVLSTGLPALISWIKRKRQE

When any foreign material (here raw honeybee venom) enter in the animal body, it is picked up by macrophages and are processed to form antigenic determinants [20, 21].

Prediction of antigenic epitopes (determinants) of PLA2: The FASTA format sequence for phospholipase A2 was pasted in the window provided on the antigenic epitope search engine page [19] and was then submitted for getting the predicted antigenic determinants (epitopes). The results are shown in figure 1.

The protein phospholipase-A2 from Apis dorsata contains 134 amino acids. It has 6 segments having antigenic determinants from amino acid number 4 to 17; amino acid number 26 to 40, amino acid number 55 to 72; amino acid number 77 to 84; amino acid number 91 to 104 and amino acids number 112 to 128. The highest peak is shown by antigenic determinant from amino acid number 91 to 105, having propensity 1.15. The average antigenic propensity for phospholipase-A2 is 1.0316.

Prediction of antigenic epitope (determinants) of Melittin: The FASTA format sequence for melittin was pasted in the web window provided on the antigenic epitope search engine page and was submitted for getting the predicted antigenic determinant (epitope) and an antigenic plot. The results are shown in figure 2. Protein melittin which contains 26 amino acids has only one segment of antigenic determinant from amino acid number 1 to 18 (IGAILKVLSTGLPALIS) The average antigenic propensity for melittin is 1.0397.

Similarly Melittin processed by macrophages to produce one active antigenic determinant having propensity 1.0397. These results confirm that both PLA-2 and melittin are competent enough to produce allergic response and this is only possible when the macrophages process then to convert them in the active antigenic determinants. The findings are
The process of chemical conjugation is not very reproducible, and uniformity of the peptides density on the carrier protein cannot be ensured. Antigenic epitopes of PLA-2 melittin proteins are important determinants of protection against allergic reactions caused due to sting of honeybee. In silico studies indicated that PLA-2 has more receptor on B cells than on T cells. Motif of PLA-2 has more receptor on B cells than on T cells (Fig. 5). Motif of PLA-2 was searched by using Peptool. These responses are mediated MHC class-I molecule which have a catalytic role in induction of
first immune response and then allergic response by activating B cells. Thus, the in silico studies of PLA-2 and Melittin indicate that the inflammatory responses in rats to honeybee (*Apis dorsata*) venom are through MHC class-I and T-lymphocytes and the allergic responses are mediated through MHC class-II, TH-2 lymphocytes, B-lymphocytes and IgE. Macrophages which are also called as antigen presenting cells can be considered a key link in the development of allergy due to honeybee venom, by exerting a TH-2 proliferation effect on lymphocytes. These predictions are supported by the haematological and biochemical alterations seen in the envenomated rats during actual experiments performed (22).

The findings are supported strongly by the motif (Fig. 5,6) and also the in silico predictions drawn from the antigenic epitopes having propensity higher than 1(Figs. 1,2). The antigenic determinants studies (in silico) showed that both PLA-2 and melittin have antigenic propensity more than 1. PLA-2 has 6 antigenic determinants while melittin has only one antigenic determinant.

It became clear that the whole protein is not necessary for raising the immune response, but small segments of Protein called the Antigenic Determinants or the epitopes are sufficient for eliciting the desired immune response.

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