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BIOCHEMICAL STUDY ON ASIAN BEE VENOM. (APIS CERANA INDICA FABRICIUS)

Rosdiana Natzir[1], Hidetoyo Teranishi[2], Masanobu Kitagawa[1], Minoru Kasuya[2], Departments of Pathology[1] and Public Health[2], Faculty of Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

ABSTRACT

We study on biochemical nature of Asian bee (*Apis Cerana indica Fabricius*) venom to clarify the allergic reaction caused by insect sting. By used SDS-PAGE, the venom was demonstrated to contain several major allergic proteins such as PLA2 and composition of venom was slightly different to compare with another bees. Several venom proteins were demonstrated to have binding capacity to human plasma proteins by immunoblot technique. Human Alpha 1-microglobulin (a1-m or protein HC), a low molecular weight glycoprotein, was isolated from the urine of tubular proteinuria caused by chronic cadmium poisoning using affinity chromatography. Using the purified a1-m as a ligand, we found that the venom proteins were able to bind Alpha 1-microglobulin. On agarose gel electrophoresis, the proteins showed heterogeneous bands between a1 and a2 mobilities (not shown). Using SDS-PAGE, their molecular weights were showed to be about 20 kDa and 50 kDa, respectively. Our results strongly suggest that the bee venom binding proteins with a1-m might have some important roles in sting allergy and / or immuno-pathology.

Key words : Alpha 1-microglobulin, Apis cerana indica Fabricius, Bee, Allergy, Binding protein

INTRODUCTION

Allergic reactions from insect stings are popular in Asian countries especially by Asian bee, hornet and wasps. Many reports have been published on allergic reactions from insect-venom. These are include with stings by honeybee (Apis mellifera), yellow jacket (Vespula spp.), hornet (Dolicho vespula spp. and Vespa spp.), paper wasp (Polistes spp.), bumble bee (Bombus spp.), and fire ant (Solenopsis spp.). However, we could not find any research papers on sting allergies from Asian bee (Apis cerana indica Fabricius). So we performed biochemical studies on the venom and clarified some interactions between the Asian bee venom and human proteins. A low molecular weight protein alpha 1-microglobulin was found in the urine of patients with tubular proteinuria (5). The protein have a molecular weight of about 30 Kd and a carbohydrate content of 23% (5). In our present studies, we have isolated alpha 1-m from urine of patients with chronic cadmium poisoning. We found that the purified alpha 1-m bound to some components in venom of Asian bee (Apis cerana indica). We collected the binding proteins with purified alpha 1-m in venom of Apis cerana indica Fabricius using affinity chromatography and determined the molecular weight by SDS-PAGE.

MATERIALS AND METHODS

VENOM.

Asian bee, Apis cerana indica, was collected at Manipi village in South Sulawesi, Indonesia and venom was collected by the puncture of the venom sacs after removing the sting. The collected venom was brought to Japan in dry ice and immediately stored at-80 C until used. The collected venom was ultrafitrated by using Millipore Mol-cut UFP1 LGC 24 (cut off at 10,000D Nihon Millipore Ltd.), dissolved with some value of PBS (pH 7.0). The venom samples were prefiltred using Millipore Millex AP (Millipore S.A 67120 Molsheim France) before used. 1 ml of purified venom (about 600 insects) was necessary used for our research.

URINE.

Twenty-four hours urine specimens were obtained from two patients with tubular proteinuria Itai-itai disease, patients TA and MU. The urine samples were ultrafitrated and concentrated to ten folds using Diaflo Ultrafiltration membranes, (MA 01915 Amicon USA, cut off point 10,000 MW).

PREPARATION OF GAMMA-GLOBULIN AND DETECTION OF ANTIBODIES :

Gamma globulin (anti PLA2 Ig) was prepared from the serum in male rabbit immunized with PLA2-Apis mellifera (Sigma) by precipitation with Ammonium sulfate. The Ouchterlony, agarose gel-double-diffusion technique was used to detect precipating antibodies.

ISOLATION OF ALPHA -1 MICROGLOBULIN GEL CHROMATOGRAPHY ;

The urine samples were concentrated using amonium sulfate between 20% and 80%. The concentrated urine (5 ml) were applied to the column chromatography on Sephadex G-100. These procedure were performed by the method described by Ekstrom (5).

PURIFICATION OF ALPHA-1 MICROGLOBULIN ;

Alpha - 1 microglobulin were purified by affinity chromatography using CNBr - activated Sepharose 4B according to instructions provided by manufacturer (Pharmacia Biotech, Sweden). Briefly, 1.0 g freeze-dried Sepharose powder was suspended with 1mM HCl and applied to the 10 ml tuberculin syringe used as a column. After washed with 1 mM HCl, rabbit IgG to alpha 1-m (Dako, Denmark) was added to the column as a ligand by using coupling buffer, 0.1M NaHCO3 pH 8.3 containing 0.5 M NaC1. Washed away excess ligand with coupling buffer and then blocked the coupling. Transferred the gel to 0.1M Tris-HCl buffer pH 8.0 and washed with 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl buffer pH 8 containing 0.5 M NaCl. and PBS pH 7.0 Sample urine (2.5 ml) was filtered then applied to the column. After the sample has been loaded, the elution of bound substances was done with

3 cycles buffer, PBS pH 7.0, Acetate buffer pH 4.0 and glycine buffer pH 2.2. The concentration of protein in the effluent was measured by reading the absorbance at 280 nm. Alpha 1-m concentration was measured by using flourescence ELISA. By affinity chromatography using the alpha 1-m as a ligand, the highest concentrations was presumed as alpha 1-m.

POLYACRYLAMIDE GEL ELECTROPHORESIS ;

SDS-Polyacrylamide gel electrophoresis was performed in 10-20% linear gradient gels by carried out following the method of Weber and Osborn, (1969) for estimation of molecular weight.

IMMUNO BLOTTING ANALYSIS :

Alpha 1-m bindings protein obtained from affinity chromatography were shown by SDS-PAGE and then transferred to PVDF membranes (Atto Corp Japan). The membranes were incubated with Rabbit-anti alpha 1-m antibody followed alkaline phosphatase conjugate anti-rabbit IgG antibody staining with BCIP / NBT.

ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY) TO DETECT

The binding between Alpha 1-m and various bee venom proteins were also preformed by ELISA. The wells were coated with peak fraction alpha 1-m in carbonate buffer pH 4.0 and incubated at 4 C onto polystyrene microtiter plates (Maxisorb, Nunc, Denmark) for 3h or overnight, washed and blocking with 1% BSA. Samples (Alpha 1-m binding protein from various bee-venoms) were used with some different pH. buffer from pH 2.2 to pH 11.0. The wells were then coated with Rabbit IgG to PLA2 antibodies labelled with biotin and then incubated for 30 min with Avidin-Biotin complex (ABComplex HRP Dako, Denmark). Phenylenediamine dihydrochloride (o-PDA) was used as a substrate and evaluated on the kinetic microtiter plate reader at the absorbance 492 nm and 620 nm by using Multiskan Bichromatic version 1.03 (Lab. Multiskan Bichromatic USA).

PLA2 PURIFIED FROM VARIOUS VENOMS :

Small amount (100ul) of each venoms (Apis cerana indica, A. c. negrocinta, A. dorsata, Polistes) were applied to anti-PLA2 IgG-affinity chromatography column. Elution proteins with acetate buffer pH 4.0 and Glycine HCl buffer pH 2.2 were fractionated and measured the protein concentration at A590 nm by used Tonein-TP II kit (Otsuka Pharm, Japan). The peak fractions of each venoms were compared by SDS-PAGE and then gel were transferred to PVDF membranes. The membranes were incubated with rabbit anti-Alpha 1-m antibodies and followed by alkaline phosphatase labelled anti rabbit IgG antibody and staining with BCIP / NBT.

RESULT :

SDS - POLYCRYLAMIDE GEL ELECTROPHORESIS :

The electrophoretic pattern of ACI was shown to be similar with ACN, ACI had similar bands with AD at 25 kDA and 50 kDa, which were not observed in AM.

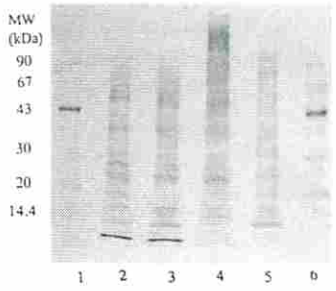
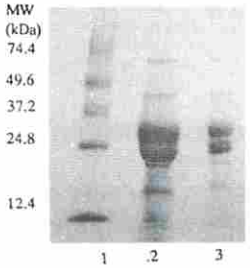


Fig. 1 : SDS-Polyacrylamide gel electrophoresis of bee venom.
The proteins were separated by gradient page(T10%-20%polyacrylamide) and stained with silver stain. Venom proteins of AC.indica were shown in lane 2.AC.negrocinta in lane 3. A.dorsata in lane 4. Apis mellifera in lane 5 and MW-marker proteins in lane 1,6.

PURIFICATION OF ALPHA-1 MICROGLOBULIN ;

Alpha-1 microglobulin was isolated separately from several concentrated urine of the patients with chronic cadmium poisoning, which gave us similar results. Gel chromatography on Sephadex G-100 : 5 ml of concentrated urine (100 folded concentrated urine) were separated on column chromatography of Sephadex G-100 (column 33 x 2 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.8 containing 1.0 M NaCl. and peak protein were observed. The alpha 1-m was present in a highest protein peak. Fractions containing alpha-1 microglobulin were pooled and concentrated by ultrafiltration. Affinity Chromatography on CNBr-activated Sepharose 4B. The pooled alpha-1 fractions from the gel-column chromatography was dialyzed and applied to a CNBr-activated Sepharose 4B column. The purified alpha 1-microglobulin had a brown color which could be observed during the entire isolation procedure. Purified alpha 1-m was shown on SDS-Polyacrylamide gel electrophoresis at 30 kDa. (Fig. 2)

Fig. 2 : SDS-Polyacrylamide gel electrophoresis of Alpha 1-m.
MW-marker protein in lane 1,concentrated urine by using ammonium sulfate buffer from a patient with chronic cadmium poisoning in lane 2,and purified Alpha 1-m obtained by aff. chrom.using anti Alpha 1-m IgG-gel column in lane 3.



ALPHA 1-MICROGLOBULIN BINDING PROTEIN

Using this Alpha 1-m as a ligand on affinity chromatography, we showed that the purified alpha 1-m was binding to three or more proteins in venom. Their molecular weight of the alpha 1-m binding protein (ABP) were from 10 kDa to 70 kDa on SDS-PAGE (Fig. 3). Immunoblotting was performed using ABP transferred membrane. The membrane was incubated with alpha 1-m followed by anti-PLA2 IgG, Alkaline-phosphatase conjugated anti-IgG antibody and staining. ABP from AM showed clear band at 19 kDa (Fig. 4a). However ABP from ACI showed clear bands at 19 kDa and 50 kDa. Furthermore, supporting the finding that Alpha 1-m were able to bind to protein in various venoms are involved in the measurement of ELISA.

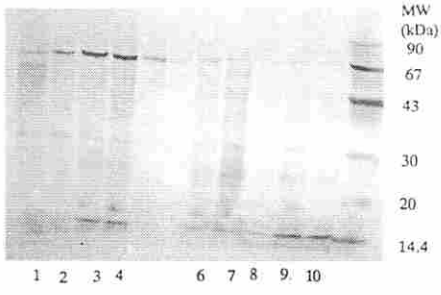


Fig. 3 : SDS-Polyacrylamide gel electrophoresis of Alpha 1-m binding protein in venom. ABP was collected using Alpha 1-m coupled aff.chrom. from various venoms. Fractions eluted with Glycine-HCl buffer from A.mellifera in lane 1,2.and Acetate-buffer in lane 3,4,5. ABP of venom AC.indica eluted with Glycine-HCl buffer in lane 6,7 and Acetate-buffer in lane 8,9,10. MW-marker proteins for SDS-PAGE were shown in lane 11.

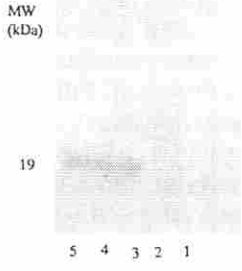


Fig. 4a : Immunoblotting of ABP in venom of A.mellifera after SDS-PAGE and ABP was transferred to PVDF membrane.The membrane was incubated with rabbit IgG-anti PLA2,followed AlP-phosphatase conjugated anti rabbit IgG. In lane 1,2; ABP-Apis mellifera (Sigma) with Glycine -HVCl buffer. Clear band was shown at 19 kDa in lane 3,4,5 eluted with acetate-buffer from A.mellifera.

IMMUNOBLOTTING USING PURIFIED PLA2 :

As various venoms were subjected to aff. chromatog. column by using anti-PLA2 IgG aff-chrom-column and the protein which were eluted from aff. chrom were subjected to SDS-PAGE. Several protein bands were shown in each venom.

A clear band was observed at 50 kDa in ACN, AD and polistes but not in AM., see Fig. 5a. Some difference were observed when gel transferred to PVDF membranes for blotting (Fig. 5b). PLA2-A. mellifera were shown in clear band of mol-mass size 19 kDa in Fig. 5b. PLA2 of AC. indica, AC. negrocinta, A. dorsata and polistes were shown different band in molecular size (50 kDa), but another bands were not appeared, these finding were strongly suggested that PLA2 of AC. indica, AC. negrocinta, A. dorsata have different mol-mass size with PLA2 of Apis mellifera.

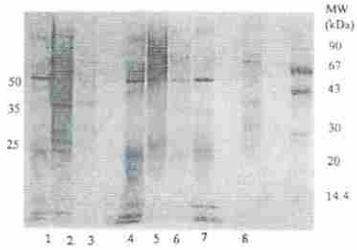


Fig. 5a :SDS-PAGE of PLA2 purified from various venoms using anti PLA2 IgG -aff.chrom.column. Bee venoms were subjected to aff-chrom on an rabbit anti-PLA2 serum aff.chrom.column. The proteins eluted with acetate-buffer pH 4.0 and Glycine-HCl buffer. PLA2 purified from venom of polistes in lane 1,4,two main bands were shown,about 25kDa and 50 kDa,from AC.negrocinta (lane 2,6) were 25 kDa ,35 kDa and 50 kDa mol.sizes were shown. PLA2-A.mellifera was shown in lane 3,8 and A.dorsata in lane 5 bands of 25 kDa and 50 kDa were shown.In the lane 7, AC.indica were shown in three clear bands,19 kDa 30kDa and 50 kDa. This gel electrophoresis then transferred to PVDF membrane.

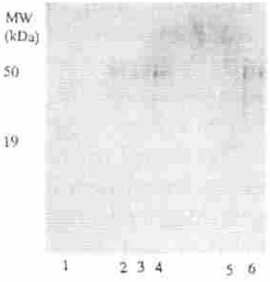


Fig. 5b :Immunoblotting of PLA2 purified various venoms using anti-PLA2-IgG aff-column. After transfer of proteins,PVDF membrane were incubated with alpha 1-m purified followed rabbit-anti Alpha 1- antibodies. PLA2 of A.mellifera was shown in lane 1,5. PLA2 purified AC.negrocinta,A.dorsata and polistes were shown in lane 2,3,4 and 6,respectively.

ALPHA 1-M BINDING CAPACITY BY USING ELISA ;

The capacity binding of Alpha 1-m and protein of various venoms were determined by ELISA. Samples fractions of ABP from aff. chromatography column were used with different pH. buffer (from pH 2.2-11.0). We found that the Alpha 1-m binding capacity of PLA2 in AC. indica, AC. negrocinta and A. mellifera to Alpha 1-m were stronger at pH. buffer 4.0-8.6 more than that of A. dorsata. (Fig. 6). These result indicate that binding capacity of ABP to Alpha 1-m, are responsible for the change of the pH. buffer.

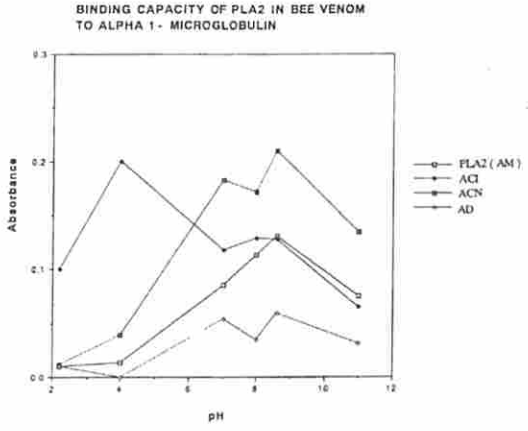


Fig. 6 Binding capacity of PLA2 in bee venom to Alpha 1-m were measured by ELISA. Binding capacity of PLA2 in venoms of ACI, AM and ACN to Alpha 1-m were stronger in pH 4.0-8.6, however, the capacity of venom AD was not significantly different when change the pH buffer

DISCUSSION

The present study demonstrated by SDS-PAGE that the component of the venom from Asianbees showed different patern from AM. Biochemical comparison of Asian bees (ACI, ACN, AD) venom and European honey bee venom (AM) have not yet reported until now. The European honeybee venom and Africanized honeybee venom have compared by thin-layer isoelectric focusing and SDS-PAGE (11). Furthermore, biochemical differences study between venom from individual honeybees, venom sacs from European bees and Africanized bees respectively different colonies and dry weight of venom from each bee have determined (9).

Phospholipase A2 purified from various venoms using anti-PLA2 IgG affinity chromatography have demonstrated that the PLA2 purified from ACI, ACN, AD have higher mol. mass at about 50 kDa, which can not observed in AM venom. One of the major allergens in bees venom is reported to be PLA2 (16). According to our findings that mol. size of one of the PLA2 from Asian bees is larger than that of AM. Further study will be needed to determine the detaiked characteristic of allergen in Asian bee is a consistent finding. This protein band (50 kDa) that is unique to Asian bees that might be considered as potential neo-allergens.

In this work we also have isolated Alpha 1-m from urine patient of chronic cadmium poisoning (5), by SDS-PAGE have a mol. weight, 30 kDa. The true function of Alpha 1-m is still unknown (1). The binding capacity with ABP in venom would suggest some aspect of characteristic of this protein.

The effect of pH on the binding of ABP in various bee venom to Alpha 1-m were further examined by ELISA. The pH-dependent curve shown in this experiment might be some relationship with PLA2 enzyme activity (3).

Histamine release in the preliminary investigation, by PLA2-AM as well as pear pollen extracts were demonstrated using blood leukocytes from allergic patient (Fig. 7). It would be reasonable to think that bee venom binding protein

with alpha 1-m described in the paper might have some important roles in allergic and / or pathological mechanism.

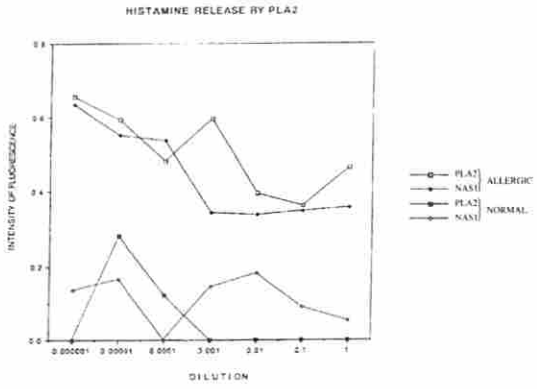


Fig. 7 : Preliminary study of the histamine release in vitro provocation by two allergens, PLA2-A. mellifera and pear pollen extract. 50 ul from each blood samples in six allergic patient were placed in glass microfiber-prepared microtiter plates and incubated for 90 min at 37°C with 50 ul of diluted allergen. After washed with distilled water histamine was released by addition of 75 ul of OPT (o-phthaldehyde, Fluka), incubated for 10 min at 15-30°C. The coupling reaction was terminated after 10 min by addition of 75 ul of HClO4. The histamin release from allergic patient blood were shown to be higher than that of nomal blood

CONCLUSION :

1. We found that there are several proteins which react with human alpha 1-microglobulin (or protein HC) in venom of Asian bee, Apis cerana indica. Three or more proteins are included in such proteins and these molecular sizes are shown to be between 10 kDa and 70 kDa by SDS-PAGE.
2. The bee venom binding proteins with alpha 1-m might have some important roles in sting allergy and / or immunopathology because the important roles of alpha 1-m as an immunomodulator were proposed by Akerstrom and the other researchers.

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