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Molecular Cloning and Characterization of a Venom Phospholipase A2 from *Apis mellifera spp.*

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ABSTRACT

Honeybee venom plays a central role in the biodiversity and self-defense of honey bee, which protect them by allowing the stinger to immobilize, puncture, and initiate allergy on their predators. Phospholipase A2 (PLA2) is one of the main allergic enzymes of honeybee venom, and it has been extensively studied in biochemical properties due to the toxicity to insects and other invaders causing hemolysis on erythrocytes. The present study described the results of cDNA cloning and chemical characterizations analysis of venom PLA2 from honeybee, *Apis mellifera spp.*, an economically important insect for pollination on crops. The gene encoding Phospholipase A2 from honeybee (*A. mellifera spp.*) venom was isolated, cloned and its nucleotide sequence was determined. The nucleotide of PLA2 is 507 bp in length, and shared 99% and 96% homology with those of *Apis mellifera ligustica* and *Apis cerana cerana*, respectively. The amino acid sequence deduced of PLA2 resembled those of other bee subspecies, such as western honey bee (*A. mellifera lingustica*). Predicted amino acid sequences of PLA2 is 99% identical with that of *A. mellifera lingustica*, and its p*I* is 7.01 and lower than those of *A. mellifera lingustica and A. cerana cerana*. In order to understand better the phylogeny of PLA2, we analyzed its nucleic acid sequence and compared with those of other sources from NCBI database, and modeled its 3D structure based on template d1poca. Amino acid sequences of PLA2 from related species were obtained to construct a phylogenetic tree and revealed that PLA2 of *A.mellifera spp.* was belong to group of the western honey bee, and was most closely related to that of *A. mellifera ligustica* than other bee subspecies.

Key words: Phospholipase A2, molecular clone, properties, venom, *Apis mellifera spp.*

INTRODUCTION

Animal toxins are one of the most largely demonstrated functional substances among the small peptides or proteins. Toxins are peptides or proteins that exist in animal or insect venom glands, which are targeted on invaders that could pose threat on their survival. Recently, numbers of peptides and proteins from toxins were demonstrated from different tissues in non-venomous and venomous animals (Tan *et al*., 2003). These toxin peptides and proteins include protease, protease inhibitors, as

well as secreted protein, which similar cell antigens and growth factors (Whittington *et al*., 2008). Animal toxins are varied in protein types. Some toxin peptides or proteins in length are >400 amino acids, such as metalloproteinases; others are about 200 amino acid or less, including PLA2 from snake and honeybee. Due to the length less than 500 amino acids and high uncertainty in sequence, they have many characteristics that benefit to the health and medical treatment.

Bee venom consists of a few pharmacologically and enzymatically active components, which are as a therapeutic modality in use including antibacterial, anti-inflammatory, hypotension and enhancing immunity, since hundreds of million years ago (Habermann,1972; Gauldie *et al*., 1976; Zhang *et al*., 2003). In fact, honeybee themselves use it as a defence against the invaders (such as wasp) that may threaten their survival or colony population. Honeybee venom are comprehensive mixtures of bioactive substances, most of them are peptides and protein, which play a great role in defensive, attacked and allergy. There are many peptides and proteins in honeybee venoms, which have enzymatic activities, including PLA2. Other than their enzymatic activity, those enzymes could lead to various physical and pharmacological effects, such as haemolytic, hemorrhagic, neurotoxic, procoagulant, anticoagulant and hypersensitization (Heiki *et al*., 2009). Many of these proteins are secreted proteins that act in the extracellular milieu through binding to membrane receptors and ion channels. However, there have still many other venom peptides or components from different bee species that have not been identified.

Among these peptides and proteins in bee venom, PLA2 is major venom component other than melittin. PLA2 hydrolyse the fatty acyl ester bond at *sn*-2 position, leading to the release of 2-acyl lysophospholipid. PLA2 is one of phospholipases belonged to group III that be able to hydrolyse one or more ester linkages in glycerophospholipids and have been classified into four categories, phospholipases A, B, C and D in the light of ester bond targeted in the phospholipid (Wainszelbaum *et al.*, 2001). PLA2 have been reported in many species, including snake and honey bee. Given that the importance of bee source and bee biodiversity in ecosystem of pollination, many PLA2 from honey bee venom have been demonstrated in recent (Tsai *et al*., 2011).

In addition to enzymatic action, PLA2 display a variety of physiological and chemical effects due to the ability to breakup or bind to specific target proteins (Valentin and Lambeau, 2000). Among them, There have been over 12 different groups of secreted PLA2 from mammals, which were involved in large numbers of cellular responses, including digestion, host defence and signal transduction (Dennis,1994). Moreover, there have been some reports about PLA2 from insects, including honey bee (Valdez-Cruz *et al*., 2007; Frey *et al*., 2010; Moreau and Guillot, 2005).

Given that the value of PLA2 in medical and pharmacy, a great number of PLA2 have already been sequenced and characterized from the different sources including vertebrate and invertebrate (Luo *et al*., 2010; Sylvia *et al*., 2010), Some of which had displayed strengthen the consciousness of Alzheimer disease (Evelin *et al*., 2009), anti-inflammatory activity on some disease (Bonfim *et al*., 2009), antibacterial

(Emmanuel and Gérard, 2000), and inhibit cancerous cell migration effects (Raoudha *et al*., 2009). PLA2 is one of mainly enzymes in bee venom, and that accounts for 6% of the bee venom dry weight. Moreover, several studies have recently focused on isolating the active components from bee venom to understand the underlying action mechanism (Murat *et al*., 2009; Timothy *et al*., 2009). The sequences of PLA2 from the venom glands of worker bees of *A. mellifera ligustica* and *A. cerana cerana* have been cloned (Habermann,1972; Kuchler *et al*., 1989; Shen *et al*., 2002) and their nucleotide sequence characterizations were reported, as well as the structure and catalyzing mechanism of PLA2 of bee venom had also been demonstrated (Robert *et al*.,1996). In addition, the molecular characterization of PLA2 from bumblebee *Bombus ignites* had been analyzed, and also verified that its activity correlated with cell death (Yu *et al*., 2009). Similar studies on PLA2 had been obtained from other species containing the venom such as scorpions and snake (Valdez-Cruz *et al*., 2007; Frey *et al*., 2010). However, little known is about the PLA2 gene of *A. mellifera spp.* to date.

In this study, we focused on molecular clone and sequence characterizations of the PLA2 from the venom of the *A. mellifera spp*. We applied RT-PCR to amplify the target gene and analyzed the full-length sequence characterization and compared with those of relative species derived from NCBI database, as well as deduced amino acid sequences were then compared, and constructed the phylogenetic relationship tree of based amino acid between the *A. mellifera spp.* and other related species. To insight into the traits of structure spatially, we modeled the 3D structure of PLA2 based on d1poca.

MATERIAL AND METHODS

Bee samples

Honeybee (*A. mellifera spp.*) samples were obtained from the apiary of Institute of Heilongjiang Apiculture in China, and it was raised under the natural conditions in the Department of Food Science, South China Agricultural University. The stinger were collected from the worker bees' venom gland of *A. mellifera spp.* using sterile forceps, and immediately grounded in liquid nitrogen and lapped for used. Briefly, live bee samples was brought to laboratory and stayed for 40 minutes, and extracted total RNA of bee venom as described Yu *et al.,* (2009).

Chemicals

Bacterial strain, *E. coli* DH5α, was kept in the laboratory of biomass energy of College of Food Science of South China Agricultural University. Restriction endonucleases (*Bam* HI, *Xho* I), *Taq* polymerase, X-gal, IPTG and DL-DNA 4500 marker were obtained from the Takara Company. pGEM T-easy Vector Kit was obtained from Promega Company. Other chemical reagents were available in biomass energy of College of Food Science of South China Agricultural University. All the procedures were performed following the manufacturer's instructions.

Extraction RNA and PCR

About 15 to 20 bees' venom apparatus were pulled from abdomen of worker bees using sterile forceps and immediately put into liquid nitrogen and ground using a pestle and mortar into fine powder until used, and the protocol of total RNA isolated as described of RNA extract Kit (Promega). The total RNA of bee venom was extracted from stingers of worker bees of *A. mellifera spp.*, and the quality of RNA in each sample was measured using a spectrophotometer.

To amplify and clone the PLA2, PCR amplification was conducted using a pair of mixed-base oligonucleotide primers that were designed according to the highly conserved cDNA regions of venom PLA2 from *A. mellifera ligustica* (Nico *et al*., 2005). The forward and reverse primer of the sequence of PLA2 were TGTAACCTCCGCTTC-CCTT (5'-3') and TCCGCCCCGTGAATTTATC (5'-3'), respectively. The cDNA of PLA2 was synthesized by Reverse Transcriptase Kit as the procedure recommended of the manufacturer. PCR amplification for obtaining target gene was performed in a 50 µL reaction volume containing 5 µL of 10×Taq buffer (Mg^+ plus), 0.2 mM dNTPs, 10 µM of each primer, 2.5 units of Taq DNA polymerase, and 100 ng of the template total RNA from bee venom of *A. mellifera spp*. PCR amplification of the PLA2 was carried out as 1.5 min at 94°C, then for successive 33 cycles consisting 40 s at 94°C, 40 s at 53°C and 40 s at 72°C, with a final extension of 6 min at 72°C. PCR product was examined through electrophoresis in 1.5% (w/v) agarose gels stained with ethidium bromide and visualized by UV transillumination and photographed using a Nikon Digital Camera and analysis system. PCR product fragment consisted with target size and was purified using PCR purification Kits. Then, it was confirmed by sequenced and the cDNA of PLA2 purified was inserted and ligated into the pGEM T-easy vector (Promega) and transformed into *E.coli* strain DH5α, and then grown on LB-agar plates containing 100 µg/mL ampicillin, 80 µg/mL X-gal and 80 µg/mL IPTG. Then, plasmid DNA from white transformants picked was extracted as described Kit instructions and identified with *Bam* HI and *Xho* I restricted digestion.

Sequence analysis

cDNA sequence was determined by sequenced and assembled. The cDNA sequence for PLA2 from other species was searched using BLAST at NCBI database (www.ncbi.nlm.nih.gov). The amino acid sequence of PLA2 from *Apis mellifera spp.* was deduced and analyzed using a translator program available with database from NCBI. Sequence identities were verified using the BLASTp program from NCBI database. CLUSTALW 1.8 (available on GenomeNet CLUSTALW Server (Kyoto Center)-http://clustalw.-genome.ad.jp) was used to align the PLA2 from *A.mellifera spp.* with known amino acid sequences deposited in GenBank.

The biological characters of PLA2 were analyzed using on-line tools (http://www.cbs.dtu.dk/services/NetPhos/,http://web.expasy.org/cgi-bin/protscale/protscale.pl?1), such as molecular mass and isoelectric point, and the prediction for three dimension structure of PLA2 based on amino acid sequence availed was computed using model tools (Schwede *et al*., 2003; Guex and Peitsch, 1997).

The multiple sequence alignment of PLA2 was imported to the MEGA program version 5.0 to construct the phylogenetic tree based on deduced amino acid sequences. Phylogenetic analysis was performed using neighbor-joining. Bootstrap confidence limit probabilities were estimated from 1000 replications. These protein sequences of the representative species, such as *A. mellifera ligustica* and *A. cerana cerana,* were obtained from published reports and NCBI database.

RESULTS

Detection and sequence analysis of the cDNA encoding PLA2

To obtain the full-length sequence of cDNA of PLA2, we cloned and sequenced the cDNA encoding the PLA2 from venom gland of honey bee, *Apis mellifera spp*. PCR amplification using primers as described yielded a diagnostic fragment of the expected size from RNA extracts of the bee venom. The result of the agarose electrophoresis of PCR products amplified from venom cDNA of *A. mellifera spp*. was shown in Fig. 1A, and PCR amplification products were sequenced, and indicated that cDNA full-length of PLA2 was obtained successfully. To verify the validity of the sequence, the PCR products were purified following the manufacture's instructions and cloned into the pGEM T-easy vector and the recombinant plasmids were extracted as described by Extract Kits. Then, recombinant plasmid was identified with restriction site, *Bam* HI and *Xho* I (Fig. 1 B). Sequencing results showed that the amplified fragments were 507 bp in length encoding 167 amino acids. The expected fragment size (~500 bp) of the PLA2 from PCR amplification and enzyme digestion shown that the PLA2 differed slightly with that of the corresponding fragment reported by Kuchler (1989) from *A. mellifera ligustica*, 504 bp in length. The sequence was deposited in GenBank as the accession JQ900380.

Alignment and comparative analysis nucleic acid for the PLA2 gene cloned indicated that PLA2 sequence was highly identity with that of *A. mellifera ligustica*, and shared 99% homology with that of *A. mellifera ligustica,* only different at position 39, and has 6 sites different compared to those of *A. cerana cerana*.

The full protein sequence of PLA2 was deduced from the cDNA nucleic acid sequences. The alignment to deduce amino acid sequence of PLA2 from different bee species and related species suggested that PLA2 from *A. mellifera spp.* was more similar western honeybee species. Although *A. mellifera carnica* and *A. mellifera spp*. all belong to the western honey bee family, there exists some difference between them in amino acid, and also different from that of *A. mellifera lingustica*. The result in Fig. 2 indicated PLA2 from *A. mellifera spp.* was totally different from that of bumble bee and *Drosphila* (Fig. 2), suggesting that there were huge differences between species during the course of evolution.

For the biochemistry traits of deduced amino acid of PLA2 analyzed by using online analysis tool (ExPASy), there were 5 phosphorylation sites, 4 Ser (position at 48,75,79,93) and 1 Thr sites (position at 110) as shown in Fig. 3, and found that there was a kinase-specific phosphorylation site at position 7, Ser site. The isoelectric points (pIs) and molecular weight of PLA2 were 7.05 and 19.03 kDa through online

predicted tools (ExPASy), respectively. PLA2 belongs to hydropathic amino acid as predicted of the Bioedit program.

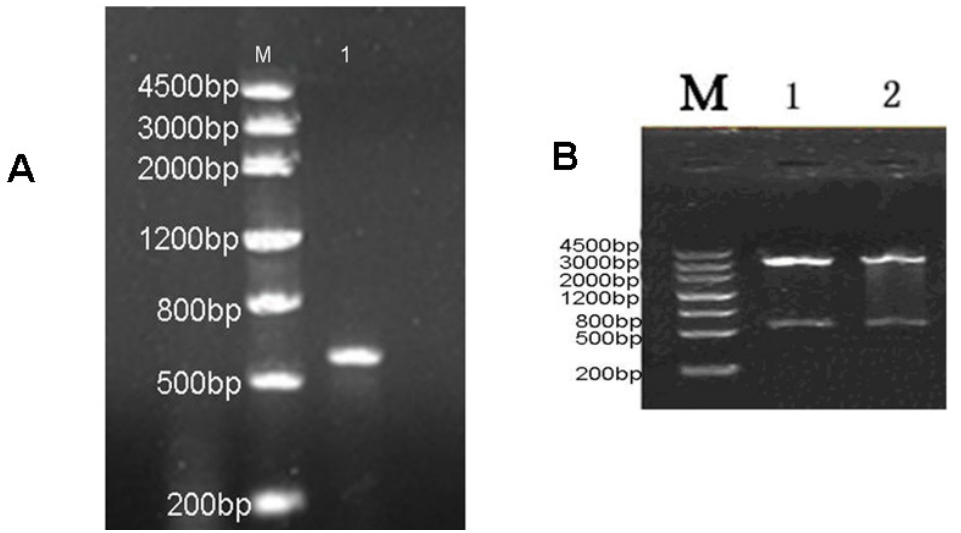

Fig. 1. The RT-PCR amplification of PLA2 from total RNA of *A.mellifera ssp*. A. identified the target fragment. (Lane M, DNA ladder; Lane 1, detedtion of PLA2). B. identified recombinant plasmid DNA by enzyme digestion. (Lane M, DNA ladder;Lane 1 and 2,recombinant plasmid containing PLA2.)Analysis of amino acid sequence

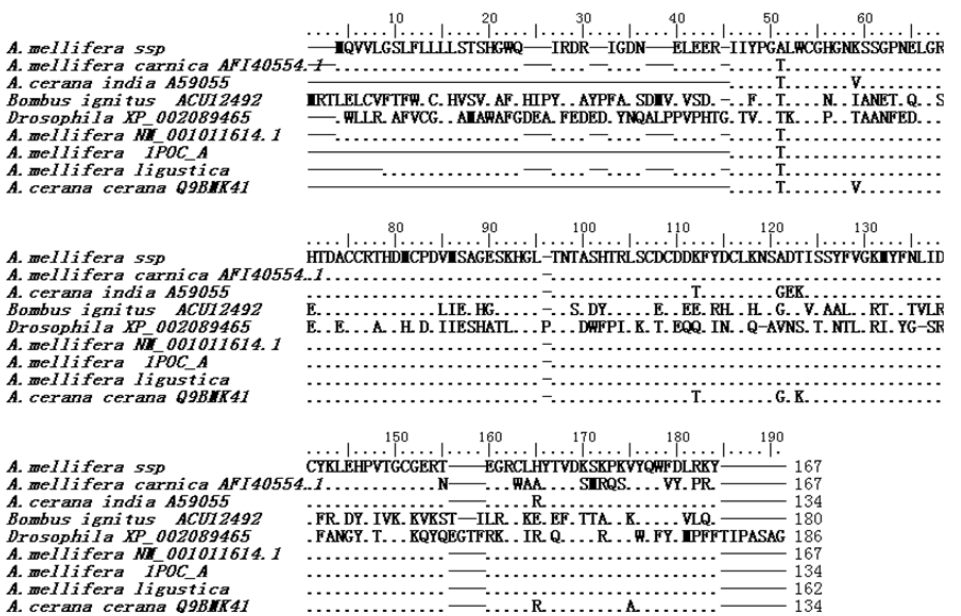

Fig. 2. The alignment of amino acid sequences of PLA2 from different species.

The component of secondary structure of PLA2 was α-helix (25.75%), extended strand (19.16%), beta turn (4.19%), and random coil (50.9%). To understand the mechanism of between proteins and interaction with other substances, we modeled the 3D structure of PLA2 protein based on template d1poca (Kelley and Sternberg,2009; Wass,2010), and shown that there was a protein binding site in position 107, His (Fig. 3). Amino acids His, Asp, Tyr and Asp, which are important for enzymatic activity, were conserved in Tgc-E6. This indicated that this site may pay tribute to the binding with other surface of protein, which can cause the allergy or other actions.

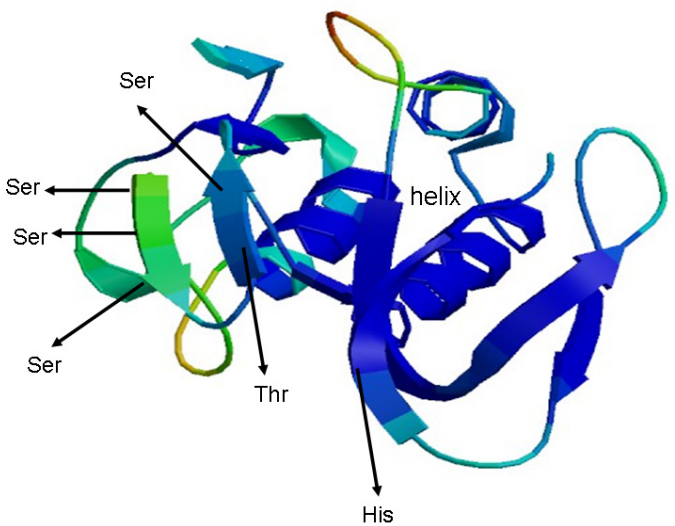

Fig. 3. The 3D structure of PLA2 deduced amino acid based on model of d1poca. Ser, Thr and His represents serine, threonine and histidine site, respectively.

Phylogenetic analysis

Phylogenetic analysis was performed based on the neighbor-joining method using the amino acid sequences of known bees and other species, for which full-length sequences are available from NCBI database. The results revealed that *A. mellifera spp.* and *A. mellifera ligustica* were nearly identical, forming a parent cluster, and shown that the PLA2 from *A. mellifera spp.* was more closely related to that of *A. mellifera ligustica* relative to other bee subspecies, and also illustrated the evolutionary relationship of different selected honeybees (Fig. 4).

DISCUSSION

In this study, PLA2 gene from bee venom of *A. mellifera.spp.* was isolated and its biological characterization was described for the first time. Due to abundance of PLA2 and the variety of components toxic activities in bee venom, PLA2 increasingly becomes one of the most mainly studied components of bee venom, especially applied in medicine.

To understand the structure traits of PLA2, we examined its amino acid characterization of *A. mellifera.spp*. PLA2 protein contained different amino acid at position 39 compared to other species (Fig. 2), even *A. mellifera linguistic*, although they all belong to the same families. The PLA2 of *A. mellifera.spp.* contained the same His and Asp amino acid residue as reported (Scott *et al*., 1990), which is composed of the enzyme activity and proton transfer system.

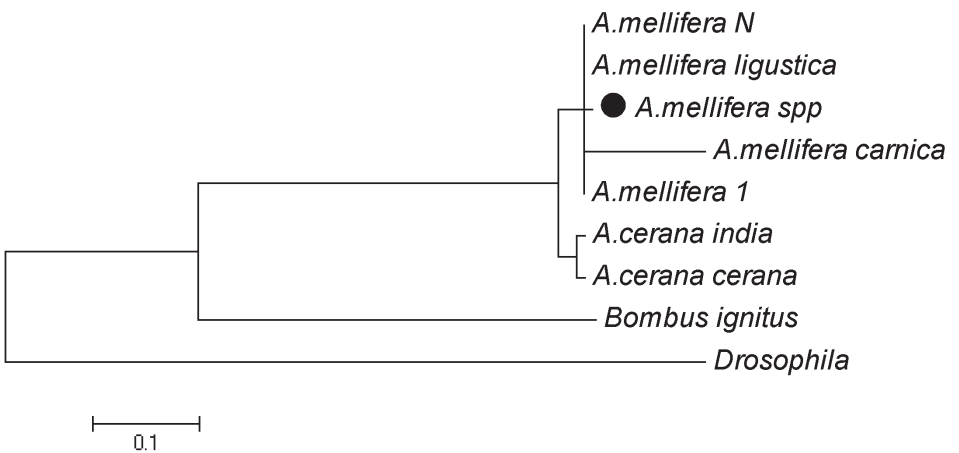

Fig. 4. Phylogenetic tree constructed based on alignment of the amino acid sequence of PLA2 from related species.

In fact, the major functional components of bee venom are melittin and PLA2, which cause toxic to invaders that pose a seriously threat on their survival at both individual and colony level. Eukaryotic organisms rely on intricate signaling networks to connect recognition of microbe with the activation of efficient defense reactions. In addition, molecular diversity of PLA2 occurs in animal venom and mammalian tissue, which show different reactivity at T-cell and humoral level that are relative to cell immunity (Sin, *et al*., 2011). Bees attack predators and intruders by injecting venom using their stingers, an effective weapon for both their own defense and that of the colony. Some report shown that PLA2 involved the important defense signaling pathway in plants (Joanne,*et al*., 2011). PLA2 is a well-established antimicrobiology proteins in the defense system of animals, and experiments verified that it played an important role in the defense system of marine sponges and was a key precursor of secondary metabolite synthesis (Joseph *et al*., 2009). Some toxins acquired the ability to cause paralysis and death on tissue and cell. A publication of Kini and Evans (1989) proposed a unifying concept to explain the pharmacological activities of venom PLA2 (Bruno and José, 2012). This model hypothesized that the action of PLA2 was based on interactions between protein and protein instead of on protein-phospholipid interactions, as previously believed. These two authors proposed that toxic PLA2 had a ‘pharmacological site’ in their surface, which was distinct from the catalytic site (Arnold *et al*., 2006). PLA2 from honeybee venom belongs to group III phospholipases

and displays the apparent feature of group III PLA2 proteins containing 10 Cys, which compose of one or two subunits (Valdez-Cruz *et al*., 2007). By online analysis tools (http://clavius.bc.edu/~clotelab/DiANNA/) that we found that the amino acid sequence of PLA2 has 10 Cys amino acid residue, which can be formed 5 disulfide, and their positions at 42-64, 42-128, 63-128, 96-103 and 103-128, and differentiated from those of the *A. mellifera ligustica*, *A. cerana cerana* and *Apis mellifera carnica* (Sylvia *et al*., 2010). From the amino acid sequence deduced of PLA2, there are both ca^{2+} binding site and other active site (Yu *et al*., 2009). Moreover, the results shown that there are another four active sites: N-myristoylation, N-glycosylation, casein kinase II phosphorylation and protein kinase C phosphorylation in PLA2 from bee venom of *A. mellifera spp*.

Molecular phylogeny has been widely used in classifying invertebrate and vertebrate animal protein families and identifying different functional subtypes (Fry and Wuster, 2004). The results of phylogenetic analysis on the basis of the amino acid sequence homology demonstrated that PLA2 from *A. mellifera spp.* grouped with that of western honeybee and they appeared to share a close evolutionary relationship with the western honeybee.

PLA2 is one of the most mainly components of bee venom, and play an important role in anti-inflammatory, hemolytic action, and so on (Habermann, 1972; Gauldie *et al*.,1976). Among the bee venom, PLA2 belongs to indirect hemolysis, and means that PLA2 will dissolve the phospholipids when the melittin dissolve lipoprotein layer of erythrocyte surface. So PLA2 could has some functions in posttranslational route of bee venom, which is independent of signal identification particle and docking protein (Boman *et al*.,1989). Since the complexity of the mechanisms involved represents an obstacle to therapeutic approaches, detailed studies on the effects of the PLA2 is of important. Therefore, the more work needs to be done in interaction and recognition between PLA2 and other proteins.

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