Cloning and Characterization of Phospholipases A2 and Hyaluronidase Genes from the Venom of the Honeybee *Apis mellifera carnica* (Hymenoptera: Apidae)

by

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ABSTRACT

Bee venom contains the allergic enzymes phospholipases A2 (PLA2) and hyaluronidase. These enzymes have been extensively studied as therapeutic modalities because of their proven effects in pharmaceutical and clinical applications. The cDNA cloning of PLA2 and hyaluronidase was amplified by RT-PCR from the total RNA of the venom gland of a honeybee (*Apis mellifera carnica*). The lengths of the PLA2 and hyaluronidase of *Apis mellifera ligustica* were 504 and 1146bp, respectively. The genes of PLA2 and hyaluronidase shared 90.94% and 96.65% homologies with *A. mellifera ligustica* and *Apis cerana cerana*, respectively. Some similar PLA2 and hyaluronidase were also found in the venom of other bee species, We analyzed their sequences and compared them with those of other sources. A notable finding was that the two genes differed from those of *A. mellifera ligustica* and *A. cerana cerana*. The positions of the disulfide bonds of PLA2 and hyaluronidase were also completely different from those previously reported. We used the available sequences to construct a phylogenetic tree and discovered that these two genes of *A. mellifera carnica* belonged to the western honeybee, and was more closely related to that of *A. mellifera ligustica* than to any other insect.

Keywords: Phospholipase A_2; Hyaluronidase; Honeybee venom; cDNA clone; sequence analysis; *Apis mellifera carnica*

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INTRODUCTION

Bee venom is a natural toxin that contains numerous enzymes, peptides and other active substances. These substances are widely used in medical practice due to their antibacterial, anti-inflammatory, hypotensive, and immunity-promoting activities, among others (Habermann 1972; Gauldie *et al*.1976; Zhang *et al*.2003). A number of phospholipase A2 (PLA2) and hyaluronidase enzymes has already been cloned from different sources (Luo *et al*.2010; Sylvia *et al*.2010). Some of these enzymes have improved cognitive ability in Alzheimer's disease patients (Evelin *et al.*2009),demonstrated anti-inflammatory (Bonfim *et al*.2009) as well as antibacterial activities (Emmanuel & Gérard 2000a), and inhibited the migration effects of cancerous cells (Raoudha *et al*.2009). Thus, several studies have recently focused on isolating the active components of bee venom to determine the underlying action mechanism (Murat *et al*.2009; Timothy *et al*.2009). The enzymes in bee venom are mainly PLA2 and hyaluronidase, which account for 11% to 15% of the bee venom dry weight. PLA2 (Habermann 1972; Kuchler *et al*.1989; Shen *et al*.2002a), and hyaluronidase (Soldatova and Mueller 1998; Gmachl and Kreil 1993; Shen *et al*. 2002b) in the venom glands of *A. mellifera ligustica* and *A. cerana cerana* worker bees have been cloned and their nucleotide sequences have been reported. The structure and catalytic mechanism of bee venom PLA2 and hyaluronidase have also been studied (Robert *et al*.1996; Housley *et al*.2000). The molecular characterization of the PLA2 gene from the bumblebee *Bombus ignites* has been performed as well, and its activity has been verified (Yu *et al*.2009). Hyaluronidase from *Rhynchium brunneum* has been cloned (Xu and Han 2008), and shown to be highly similar with that from *Vepula vulgaris*. Similarly studies on other species such as scorpions and snake have been conducted (Valdez-Cruz *et al*. 2007; Frey Francisco *et al*.2010).

A. mellifera carnica is one of the four most superior bee species. It has a strong resistance against mites as well as excellent foraging and feed-saving abilities (Wang 2005). PLA2 and hyaluronidase are believed to be effective bee venom spreading factors. However, the absence of protein sequences data on these enzymes hinders the verification of their functions. There is no available report on the PLA2 and hyaluronidase genes of *A. mellifera*

carnica, although those of other bee species have been reported. Whether *A. mellifera carnica* also contains these two genes in its venom is not yet clear. The molecular structures of the two genes are also not known.

In the current paper, we report the nucleotide sequences of the PLA2 and hyaluronidase genes of *A. mellifera carnica* (the genbank accession numbers are JQ900376 and JQ900377, respectively). We also compare them with those of *A. mellifera ligustica* and *A. cerana cerana*, and perform a phylogenetic analysis.

MATERIALS AND METHODS

Experimental material

Honeybee (*A. mellifera carnica*) workers were obtained from the apiary of the Institute of Jiangxi Apiculture in China. The stingers were collected from the venom gland of the bees and immediately placed in liquid nitrogen for storage until use. The bacterial strain *Escherichia coli* DH5α was from the Laboratory of Biomass Energy of the College of Food Science of the South China Agricultural University. Restriction endonucleases (*Bamh* I and *Xho* I), Taq polymerase, X-gal, isopropyl-β-D-thio-galactoside(IPTG), and DL-DNA 4500 marker were purchased from the Takara Company. A First-strand cDNA Synthesis Kit was obtained from the Shanghai Gereray Company, and a pGEM-T Vector Kit was from the Promega Company. All other chemical reagents used were available from our laboratory.

General experimental procedures

A pair of PCR primers was designed based on the sequences of PLA2 and hyaluronidase from *A. mellifera ligustica* (Nico *et al.*2005; Scott *et al.*1990). The forward and reverse primers of the sequences are listed in Table 1. Total RNA was extracted from the venom glands, frozen in liquid nitrogen, and

Table 1. Primer sets used for the RT-PCR analysis of bee venom gene transcription.

Target gene	Primer	Sequence (5'-3')	Predicted product size (bp)
PLA_2	Forward	TGTAACCTCCGCTTCCCTT	504
	Reverse	TCCGCCCCGTGAATTTATC	
hyaluronidase	Forward	GGTGCGATCGTCGATTCAT	1149
	Reverse	GTCACACTTGGTCCACGCT	

ground into fine powder using a mortar and pestle. Total RNA was isolated using an RNA Kit (Omega) according to the manufacturer's instructions. The two cDNA genes were synthesized from total RNA by a Reverse Transcriptase Kit following the manufacturer's the protocol.

PCR amplification was performed in a 50 uL reaction flask containing 5 μL of 10× Taq buffer (Mg^{2+} Plus), 0.2 mM deoxyribonucleotide triphosphate, 10 μM each primer, 2.5 units of Taq DNA polymerase, and 100 ng of the template genomic RNA of *A. mellifera carnica*. The PCR of PLA2 was carried out as follows: 1.5 min at 94 °C; 33 successive cycles of 40 s at 94 °C, 40 s at 53 °C, and 40 s at 72 °C; and a final extension of 6 min at 72 °C. The PCR of the hyaluronidase was carried out as follows: 1.5 min at 94 °C, 33 cycles of 40 s at 94 °C, 40 s at 55 °C, and 1.5 min at 72 °C; and a final extension of 8 min at 72 °C. The RT-PCR products were examined by electrophoresis in 1.5% (w/v) agarose gels with ethidium bromide staining. The PCR products were purified by PCR Purification Kits and ligated into the pGEM T-easy vector. Component TG1 cells were transformed with the ligation products, and then grown on Luria-Bertani(LB-agar) plates containing 100 μg/mL ampicillin, 80 μg/mL X-gal, and 80 μg/mL IPTG. White colonies were cultured in a 3 mL medium. Plasmid DNA was extracted and identified by *BamH* I and *Xho* I digestion as well as PCR amplification.

Sequence analysis

The positive recombinant plasmid DNA was sequenced by the BGI Company (Shenzhen). The amino acid sequence was deduced from the cDNA data using the NCBI database. Sequence analysis was performed using the DNAman (Version 5.0) program. Two genes homologues of some vertebrates and invertebrates were obtained from NCBI. The full-length amino acid sequences were aligned using the Clustal X1.83 program, and the phylogenetic tree was generated using the MEGA 4.0 software based on the neighbor-joining method. The protein sequences of the representative species, *A. mellifera ligustica* and *A. cerana cerana,* were obtained from published reports. The sequences were analyzed for identity using DNAman version 5.0. Their biological characters were analyzed using online tools (http://www.cbs.dtu.dk/services/NetPhos/, and http://web.expasy.org/cgi-bin/protscale/protscale.pl?1).

Data processing

The amino acid sequence was deduced from the cDNA data using DNAman 5.0. Sequence analysis was performed using the Bioedit program, and the phylogenetic tree was generated using the Mega 4.0 software based on the neighbor-jointing method.

RESULTS

The result of the agarose electrophoresis of the RT-PCR products amplified from the venom cDNA of *A. mellifera carnica* is shown in Fig. 1**.** The fragment size of the PLA2 of *A. mellifera carnica* was consistent with that of *A. mellifera ligustica* in the corresponding region reported by (Kuchler *et al.*1989), but differed from that of *A. cerana cerana*. The fragment size of the hyaluronidase of *A. mellifera carnica* was not consistent with those of *A. mellifera ligustica* and *A. cerana cerana* in the corresponding region reported by (Gmachl and Kreil 1993). The PCR products were purified and cloned into the pGEM T-easy vector. The recombinant plasmids were identified by *Bam*HI I and *Xho* I digestion (Fig. 2) and the PCR amplifications were sequenced. The two recombinant plasmids were named pGEM- PLA2 and pGEM- hyaluronidase.

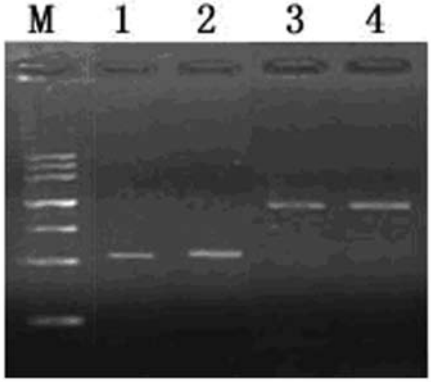

Fig. 1. PCR products of the two genes from *Apis mellifera carnica* by agarose electrophoresis. M:molecular weight marker(DL4500); lanes 1 and 2: phospholipase A_2; lanes 3 and 4: hyaluronidase.

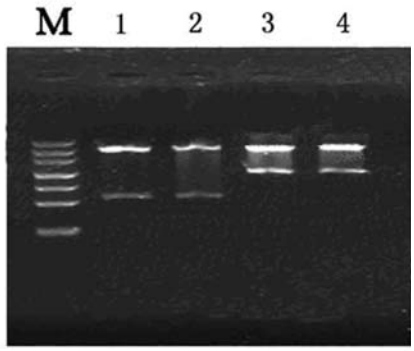

Fig. 2. Enzyme cut identification of the recombinant plasmids containing the target gene from *Apis mellifera carnica.* M: molecular weight marker (DL4500); Lanes 1 and 2: pGEM-phospholipase A_2; lanes 3 and 4: pGEM-hyaluronidase.

Sequencing results showed that the amplified fragments were 504 and 1146bp long, respectively. The multiple alignments showed that the PLA2 and hyaluronidase genes shared more than 90.9% and 96.6% homologies with those of *A. mellifera ligustica* and *A. cerana cerana* in terms of nucleotide sequences.

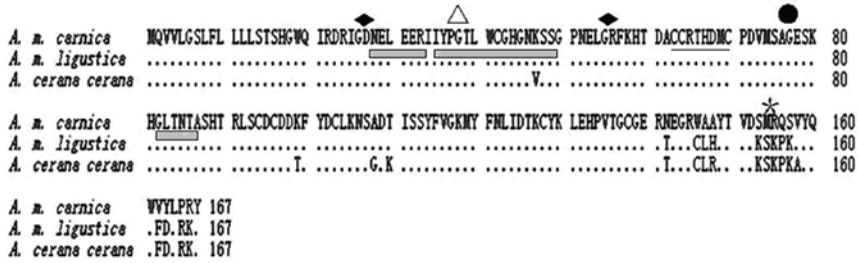

Fig. 3. Alignment of the deduced amino acid sequences of the phospholipase A2. Diamond represents Ca^{2+}-binding site; underline represents active site; square represents N-myristoylation site; triangle represents N-glycosylation site; black dot represents casein kinase II phosphorylation site; asterisk represents protein kinase C phosphorylation site.

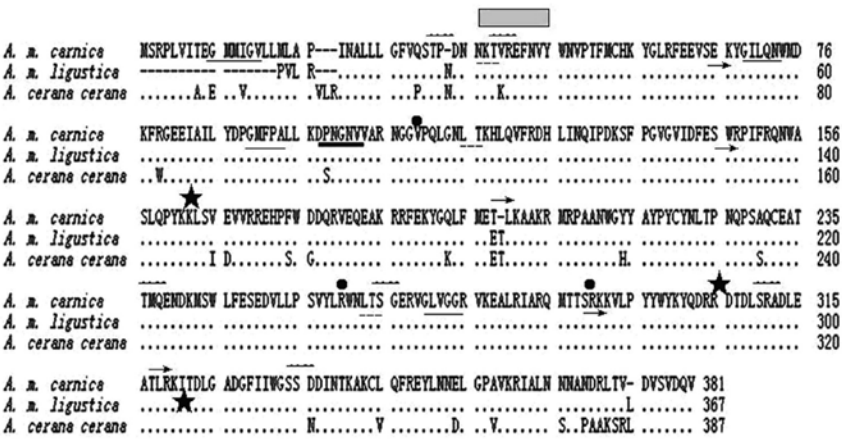

Fig. 4. Alignment of the deduced amino acid sequences of hyaluronidase. Underline represents N-myristoylation site; broken line represents casein kinase II phosphorylation site; dotted line represents N-glycosylation site; square represents tyrosine kinase phosphorylation site; arrowheads represents protein kinase C phosphorylation site; asterisk represents cAMP-and cGMP-dependent protein kinase phosphorylation site; black dot represents the B-cell epitope.

Using the Bioedit program, the deduced amino acid sequences of the two genes were determined to be 168 and 382 amino acid residues in length, and predicted molecular weights of 41.2 and 92.257kDa, respectively. Multiple alignment (Fig. 3) showed that the amino acid sequence of venom PLA2 from *A. mellifera carnica* shared a high degree of homology with *A. mellifera ligustica* (the GenBank accession number: NP_001011607.1) and *A. cerana cerana* (the GenBank accession number: Q8LW54.1). On the other hand,

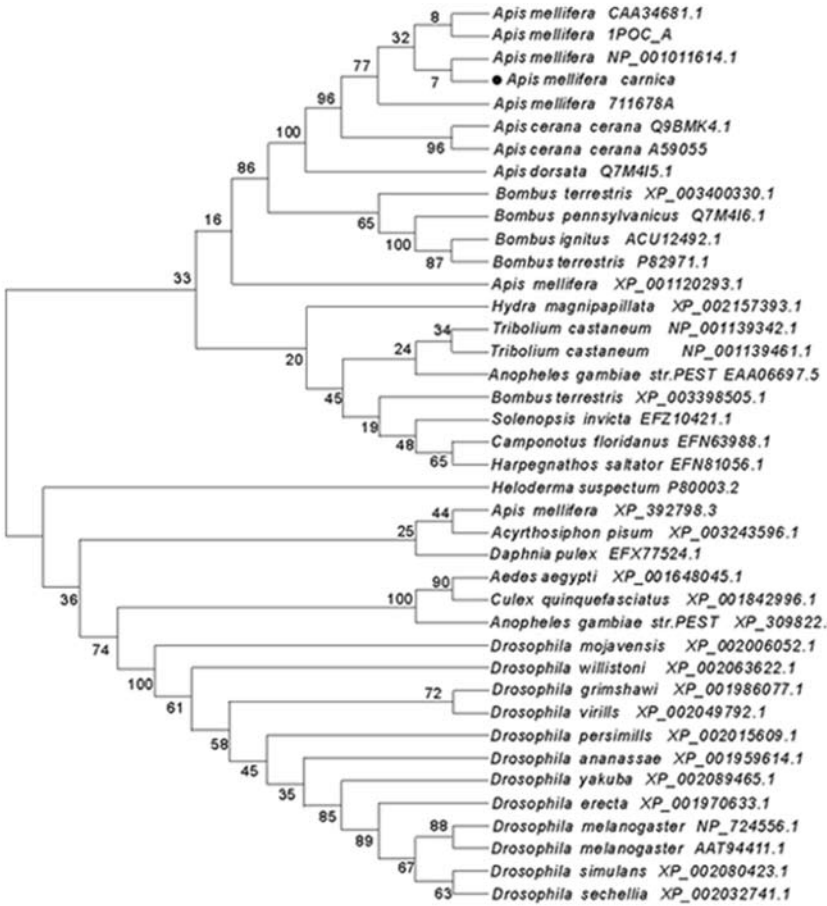

Fig. 5. Phylogenetic tree constructed based on the alignment of the amino acid sequence of phospholipase A2 homologues. Black dot represents the *Apis mellifera carnica*.

the hyaluronidase gene had differences in many positions with those from *A. mellifera ligustica* and *A. cerana cerana* (Fig. 4).

Phylogenetic analysis was performed based on the neighbor-joining method using the amino acids of these two gene sequences. The two genes from *A. mellifera carnica* were found to be most closely related to those of *A. mellifer ligustica*, and the trees illustrated the evolutionary relationship of the different bee species studied (Figs. 5 and 6).

DISCUSSION

Due to the abundance of PLA2 and hyaluronidase in bee venom and their various toxic activities, they are two of the most commonly studied components of honeybee venom. In this study, the genes encoding these two proteins were

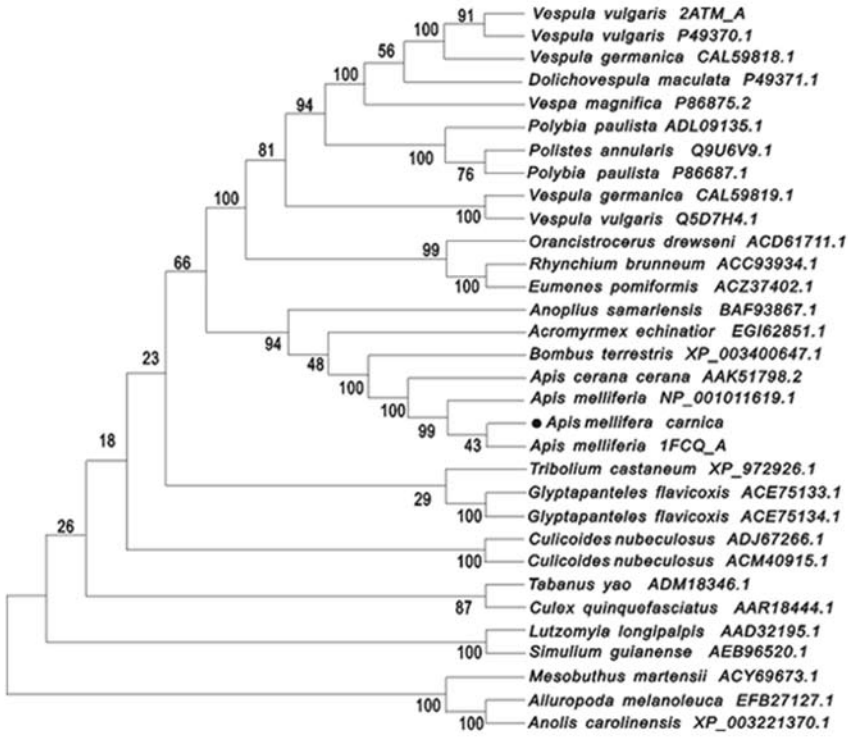

Fig. 6. Phylogenetic tree constructed based on the alignment of the amino acid sequence of hyaluronidase homologs. Black dot represents *Apis mellifera carnica*.

obtained from the venom of the honeybee *A. mellifera carnica*. The genes were cloned as well as identified, and their sequences were analyzed. There are two main interesting findings. First, the fragment size of hyaluronidase (1146bp) had a difference with those from *A. mellifera ligustica* (1149bp) and *A. cerana cerana* (1164bp). On the other hand, the fragment size of PLA2 is the same as that of *A. mellifera ligustica*, although their sequences differ. Second, the amino acid residues of the signal peptides of genes of the *A. mellifera carnica* genes are significantly different from that of *A. cerana cerana* (Figs. 3 and 4). The phylogenetic tree shows that the relationship between the honeybee and other species, including some vertebrates, involves the conservation and variation of these genes during long-term evolution.

The isoelectric points (pIs) of PLA2 and hyaluronidase are 6.36 and 8.82, respectively, and both are hydropathic. The secondary structure prediction of PLA2 is α-helix (26.35%), extended strand (18.56%), beta turn (5.39%), and random coil (49.7%). The hyaluronidase secondary structure is α-helix (35.96%), extended strand (19.69%), beta turn (4.72%), and random coil (39.63%). The pIs of PLA2 and hyaluronidase from *A. mellifera carnica* differ from those (7.05 and 8.67) of a previous report (Nico *et al.* 2005), although they all belong to the alkaline amino acid family.

Their structures of the two enzymes could explain their properties. PLA2 contains the same His and Asp amino acid residues as previously reported, which are involved in enzyme activity and the proton transfer system. PLA2 also possesses Trp, Gly and Asp amino acid residues with Ca^{2+}-binding site and active sites (Scott *et al.* 1990). PLA2 contains the same Gly amino acid at position 143 as *A. cerana cerana*, although they belong to different families with different biological characterizations. Using online anaylsis tools (http://clavius.bc.edu/~clotelab/DiANNA/), we find that the amino acid sequence of PLA2 has nine Cys amino acid residues, which can be formed into four disulfide bonds. The PLA2 sequence also differs from those of *A. mellifera ligustica* and *A. cerana cerana,* its positions (42-64, 63-128, 94-138 and 96-103) also differed from that reported (9-31, 30-70, 37-63, 61-95 and 105-113)(Sylvia *et al.* 2010). The hyaluronidase has only one disulfide bond in position 232 to 344, which also differs from a previous report wherein only two disulfides were observed (Gmachl & Kreil 1993). This result also differs from that of previous studies indicating that the mature peptide of the bee

enzyme is a single polypeptide chain, given that PLA2 belongs to group III phospholipases (Valdez-Cruz *et al*. 2007), which contain ten Cys residues and are composed of one or two subunits (Emmanuel & Gérard 2000b). This discrepancy may have a relationship with allergens.

Hyaluronidase has the same structure as that reported, with a catalytic active center at D141 and E143 (Housley *et al*. 2000). However, positions 141 and 143 are occupied by Val and Asp, and a conserved region with a hydrophilic ring, DPNGNV, is present. Moreover, only three out of nine B-cell epitopes have been reported (Paclavattan *et al*. 2007). The amino acid sequences of PLA2 and hyaluronidase, share four sites, namely, N-myristoylation, N-glycosylation, casein kinase II phosphorylation and protein kinase C phosphorylation. There is a Tyr kinase phosphorylation site in hyaluronidase, but none in PLA2.

PLA2 and hyaluronidase are the main components of bee venom. They play important roles in anti-inflammatory and hemolytic actions, among others (Habermann 1972; Gauldie *et al*. 1976). We predict that their different functions could be related with their different structures, because they both cause hemolysis. However, PLA2 causes indirect hemolysis, meaning that it dissolves phospholipids after melittin dissolves the lipoprotein layer of the erythrocyte surface. At the same time, both have activities related to allergic reactions (histamine release), which affects nearly 20% of the population (Zhang *et al*. 2003; Sutton and Gould 1993). We do not know which the strongest allergy cause is. The severest allergy is induced by PLA2 in some people and by hyaluronidase in others (Wang *et al*. 1997). An allergic reaction results from the lack of aromatic amino acids, which can increase the rigidity of protein molecules, although this has not been proved by experiments.

Bee venom requires a posttranslational route independent of a signal identification particle and a docking protein, such as PLA2 (Boman *et al*. 1989). Whether the two gene precursors have the same route when they slip into the signal peptide and proregion or the natured peptide is unknown. *A. mellifera carnica* and *A. cerana cerana* have different signal peptides (Figs. 3 and 4), which could be useful in more in-depth studies of the mechanism of related proteins.

This study is the first to describe *A. mellifera carnica* venom PLA2 and hyaluronidase sequences, and provides a significant contribution to the honeybee

database. The main proteins of bee venom have been widely studied and used in many fields. Further studies are required to clarify the structure, biological characteristics, and expression mechanisms of the two genes. Detailed studies on the effects of the two enzymes are important, considering that the complexity of the mechanisms involved hinder therapeutic approaches. Therefore, studies on the molecular biology of bee venom proteins are necessary for further applications. Our further studies shall focus on the expression of the recombinant forms of these two genes and their biological activities.

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