

Expression and Regulation of Phospholipase A₂ in Venom Gland of the Chinese Honeybee, *Apis cerana cerana*

Jiang-Hong Li^{1,2} Chuan-Xi Zhang,^{1*} Li-rong Shen,¹ Zhen-Hua Tang,³ and Jia-An Cheng¹

Phospholipase A₂ (PLA₂) is one of the components of bee venom with a wide range of pharmacological functions. It operates as a major allergen working with other venom components to defend the colony from intruder. In the present study, the cDNA sequence of the *Ac-pla₂* gene from cDNA library of the venom gland of *Apis cerana* was compared with the amplified corresponding region of genomic DNA. The result showed that the *Ac-pla₂* gene consisted of four exons and three introns. Southern blot showed that the *Ac-pla₂* gene was a single copy per haploid genome. The most active transcription period was during the first 8 days of adults, which correspondingly was the period of sharp increase of PLA₂ protein. ELISA analysis revealed that the PLA₂ was undetectable in pupal stage and the newly enclosed adult, but increased sharply to a maximum of 10–12 µg per honeybee by 8–10 days of adult life, followed by a gradual decrease to 8 µg for the rest of adult life. Transcriptional or post transcriptional regulation is the key step for *Ac-pla₂* expression. The early secreted Ac-PLA₂ showed a low degree of post-translational modification; with increasing age, glycosylation was detected by Western blot and glycoprotein staining analysis. Different post-translational modifications were found among different individuals in *A. cerana* when compared to *A. mellifera*. Arch. Insect Biochem. Physiol. 60:1–12, 2005. © 2005 Wiley-Liss, Inc.

KEYWORDS: phospholipase A₂; expression profile; venom gland; *Apis cerana*

INTRODUCTION

Phospholipase A₂ (PLA₂s) catalyzes the hydrolysis of the 2-acyl bond of 3-n-phosphoglycerides to liberate free fatty acids and lysophospholipids (Dennis, 1994; Uthe and Magee, 1971). The reaction also produces arachidonic acid, a precursor of eicosanoids including prostaglandins and leukotrienes (Ohsawa et al., 2002). PLA₂s participate in many other cellular processes such as modulating the release of arachidonic acid from neuronal membranes and rat Leydig cells (Rodriguez et al., 2002; Ronco et al., 2002), delaying oxidant-induced cell death (Zhao et al., 2001), host defense,

atherosclerosis, and signal transduction (Murakami and Kudo, 2002; Dennis, 1994). PLA₂ also is related to many human disorders such as rheumatoid arthritis, autoimmune uveitis, respiratory distress syndrome, myocardial infarction, and septic and endotoxic shock (Mukherjee et al., 1994).

PLA₂ from bee venom is a typical group III secreted PLA₂, which represents about 12% of the dry weight of venom in *A. mellifera* (Habermann, 1972). It mainly acts as an allergen working with other components for defense of the colony against predators and intruders (King et al., 2000; Sobotka et al., 1976; Light et al., 1976). Recently, a wide variety of pharmacological properties such as neu-

¹Institute of Applied Entomology, Zhejiang University, Hangzhou, Zhejiang, China

²College of Apiculture, Fujian Agriculture and Forestry University, Fuzhou, Fujian, China

³Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China

Abbreviations used: PLA₂: Phospholipase A₂; Ac-PLA₂: Phospholipase A₂ of *Apis cerana*; Am-PLA₂: Phospholipase A₂ of *Apis mellifera*.

Contract grant sponsor: National Natural Science Foundation of China; Contract grant number: 30271008; Contract grant sponsor: National Basic Research Program of China; Contract grant number: 2003CB114400.

*Correspondence to: Chuan-Xi Zhang, Institute of Applied Entomology, Zhejiang University, Hangzhou, Zhejiang, 310029 China. E-mail: chxzhang@zju.edu.cn

Received 30 November 2004; Accepted 16 March 2005

rite outgrowth induction were also reported (Nakashima et al., 2004).

pla₂ cDNA of *A. mellifera* (*Am-pla₂*) has been screened and sequenced from the venom gland cDNA library (Kuchler et al., 1989). The deduced amino acid sequence consisted of a signal peptide of 18 amino acids (pre region of PLA₂), a pro region of 15 residues, and a mature peptide of 134 residues (Shipolini et al., 1974a). A total of 10 cysteine residues are included in the mature peptide, which could form 5 disulfide bonds (Kuchler et al., 1989; Shipolini et al., 1974b). The crystal structure and catalyzing mechanism of PLA₂ are also well documented (Robert et al., 1996; Scott et al., 1990a,b). cDNA encoding PLA₂ from *Apis cerana* (Shen et al., 2002) and *Apis dorsata* (GenBank B59055) have also been reported. Though PLA₂ is one of the most important components in the venom, many of its basic characteristics such as the gene copy number, transcription, translation, and regulation are still unclear.

The Chinese honeybee, *Apis cerana cerana*, is largely bred in China. In this study, we demonstrated that there is a single gene coding for *A. cerana cerana* PLA₂. The high expression of *Ac-pla₂* mainly occurred during the first 8 days of adult life, and was regulated at a transcriptional and/or post-translational level. The PLA₂ modifications among individuals and between *A. cerana* and *A. mellifera* were also compared.

MATERIALS AND METHODS

Honey Bees

Larvae, pupae, and adults of *A. cerana* were collected from the colonies bred at the Experimental Apiary of Zhejiang University, Hangzhou, China. The developmental stages of workers were identified according to the criteria proposed by Rachinsky et al. (1990) and Michelette and Soares (1993). Bees of known ages were obtained by marking the newly emerged bees from a frame of capped broods kept in an incubator at 34°C and 80% relative humidity (Piulachs et al., 2003), and the bees were returned to the hive and collected as and when required.

Preparation of Polyclone Antiserum

The polyclonal antibody was prepared by immunizing the rabbits with pure PLA₂ (Sigma, HPLC; St. Louis, MO). Prior to immunization, the rabbits were bled to collect serum that served as a control in the studies. Immunized rabbits were bled through the ear vein one week after the last dose and the blood was kept at room temperature for 6 h for clotting. Serum was collected by centrifugation, de-complemented at 56°C for 30 min. Sodium azide to a final concentration of 0.2% was added and then stored at -70°C.

Extraction of RNA From Venom Gland and Construction of cDNA Library

The honeybees were anesthetized on ice, the venom glands were dissected, and total RNA from the venom glands was isolated using Trizol reagent. mRNA was extracted using Poly (A) Quik® mRNA Isolation Kit (Stratagene, La Jolla, CA). About 5 µg of venom gland mRNA was used to construct a cDNA library, using the ZAP-cDNA® Gigapack III Gold Cloning Kit* (Stratagene).

Amplifying and Assembling of *Ac-pla₂* cDNA

The *Ac-pla₂* cDNA sequence was amplified and assembled from the cDNA library plasmid DNA by two-step PCR, universal primers were used on the cDNA library vectors, and the specific primer based on the cDNA sequence we reported previously (GenBank accession No. AF321087) were used as follows: forward 1: M13+, Reverse 1: 5'-ATAATATATCCAGGAACGTTGTGG-3'; Forward 2: M13-, Reverse 2 5'-TCAATACTTGCGAAGATCGA-ACCA-3'. The amplification conditions were 3 min at 94°C, followed by 30 cycles of 50 s at 94°C, 45 s at 52°C and 55 s at 72°C, and final extension for 10 min at 72°C. The amplified fragments were purified using QIAquick PCR product purification kit (Qiagen, Chatsworth, CA), and cloned into the pGEM-easy T vectors (Promega, Madison, WI) and sequenced at Shanghai Sangon Bio-technology Corporation on ABI377 automated sequencer.

Extraction of DNA

Genomic DNA was isolated from female worker bees according to Beye and Raeder (1993). Briefly, 10 unpigmented pupae were frozen in liquid nitrogen and ground to fine powder and transferred to 2 ml polypropylene tubes containing 300 µl of extraction buffer (0.1 M NaCl, 1 mM EDTA, 1.5% SDS, 50 mM Tris-HCl, pH 7.5) with protease K (0.5 mg/ml) and incubated at 55°C for 2 h, then the homogenate was centrifuged at 12,000g for 10 min and DNA was extracted from the supernatant by repeated phenol-chloroform liquid-phase separation, followed by isopropanol precipitation, the extracted DNA was dissolved in TE buffer, and stored at -20°C.

Amplification of Genomic DNA Corresponding to *Ac-pla₂* Coding Region

Genomic DNA fragment coding *Ac-pla₂* was amplified by PCR, using the primers based on the *Ac-pla₂* cDNA as follows: Forward3: 5'-CTCCACGATGCAAGTCGCGC-3', Reverse3: 5'-AATTATTCTGTTATTTTATTACATC-3'. The amplification conditions were 3 min at 94°C, followed by 30 cycles of 50 s at 94°C, 45 s at 52°C, and 2 min at 72°C, and final extension for 10 min at 72°C. The amplified fragments were purified using QIAquick PCR purification (Qiagen), cloned into the pGEM-easy T vectors (Promega), and sequenced at Sangon Biotechnology Corporation, Shanghai. The genomic DNA sequence was submitted to the EMBL database with the accession number AJ786346.

Southern Blotting

Ac-pla₂ coding region amplified from the cloned cDNA using the Reverse primers 1 and 2 was Dig-labeled (Roche). Ten micrograms of honeybee genomic DNA was digested overnight with restriction enzymes, *EcoR* I, *EcoR* V *Xho* I, *Pst* I, *Sal* I, *Xba* I, and *Hind* III (Takara, Japan), respectively. Five microgram of digested genomic DNA was separated in 0.8% agarose gel in TAE buffer. DNA was denatured and transferred onto Hybond N+ nylon

membrane (Amersham, Arlington Heights, IL) by capillary transfer in 10 × SSC buffer overnight (Sambrook and Russeau, 2001). Afterwards the membrane was washed for 5 min in 6 × SSC and crosslinked with the transferred DNA in a UV-crosslinker (TL-2000, UVP). The membrane was then hybridized and detected using the DIG High prime DNA Labeling and Detection Starter Kit II (Roche).

Northern Blotting

About 4 µg of the isolated RNA samples (two bees equivalent) were separated on formaldehyde agarose gels and then blotted onto the Hybond-N+ membranes (Amersham) for 8 h with 0.05 M NaOH (Sambrook and Russeau, 2001). The transferred RNA was crosslinked with the membranes in a UV-crosslinker (TL-2000, UVP). Prehybridization, hybridization, and detection were performed as per the manufacturer's recommendations (Roche).

Venom Protein Preparation

Bees of known age were collected from the labeled workers. Their venom glands together with the venom sac were dissected and homogenated in 200 µl PBS (pH 7.4), then centrifuged for 5 min at 12,000 rpm. The supernatant was stored at -20°C and used as venom extract.

Enzyme-Linked Immunosorbent Assay (ELISA)

The 96-well microtiter plates with 5-µl venom extracts and 95 µl coat buffer (0.05M Na₂CO₃-NaHCO₃ buffer, pH 9.6) were incubated overnight at 4°C, and then blocked with 200 µl of BSA solution (0.1% in PBS) for 1 h at room temperature. Next, the rabbit anti-PLA₂ polyclonal antiserum (diluted 1:3,000 in PBST/BSA) was added and incubated for 1 h at room temperature. Then the Horse Radish Peroxidase -conjugated goat anti-rabbit IgG (SouthernBiotech; diluted 1:10,000 in PBS, containing 10 mg/ml BSA) was added and incubated for 1 h at room temperature. Lastly, the enzyme substrate (4 mg O-phenylenediamine in 10

ml solution of 0.025 M citric acid, 0.05 M Na₂PO₄, 15 µl H₂O₂) was added and incubated for 30 min at room temperature. Then the reaction was terminated by adding 50 µl of 2 M H₂SO₄. The absorbance was immediately read at 492 nm in Microplate Reader (µQuant, Bio-TEK). Pure PLA₂ (Sigma, HPLC purity) was used to obtain standard curve. The PLA₂ concentrations in other wells were calculated according to the standard curve. Eight repeats for each age were performed. Their average value and standard deviation were calculated.

Western Blotting

The venom extract mixed with same volume of 2 × loading buffer (63 mM Tris.Cl, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue, pH 6.8) was denatured by boiling for 5 min and electrophoresed on 12% SDS-PAGE. The separated peptides were electrophoretically transferred to the PVDF membrane (0.22 µm, Millipore, Bedford, MA) using a Bio-Rad Transblot (Bio-Rad). The membrane was firstly immersed in 1% BSA in PBST (PBS+0.3% Tween 20) blocking for 1–2 h, and then the anti-PLA₂ polyclone antiserum was added and incubated 1–2 h at room temperature with constant agitation. Lastly, the Horse Radish Peroxidase (HRP) conjugated rabbit anti-serum (1:2,000) was added and incubated for 30–60 min at room temperature with agitation. The resulting complexes were visualized in color development solution (2 mg diaminobenzidine in 10 ml, 0.1 M PBS, pH 6.4, 60 µl 10% H₂O₂). The individual venom extract from the same colony or different colonies were analyzed.

Determination of PLA₂ N'-Terminal Sequences

Venom extract was subjected to SDS-PAGE and transferred to the PVDF membrane in the same way as Western blot. After staining with 0.1% Coomassie Brilliant Blue, 40% methanol, and 1% acetic acid for 30 s and destaining with 50% methanol, the specific peptide band on PVDF membrane was excised and sequenced on a protein sequencer (Applied Biosystems, model 492cLC) at Shanghai Genecore Bio Technologies Co.

Glycoprotein Staining

Glycoprotein in venom was stained using the Glycoprotein staining Kit (Pierce, Rockford, IL). Glycoprotein staining was performed according to the protocol recommended by the supplier. Briefly, venom extract was firstly subjected to SDS-PAGE, then the gel was immersed in 50% methanol for 30 min, washed for 2 × 10 min in 3% acetic acid, oxidized for 15 min, stained and then the background was reduced, respectively, using reagents provided by the supplier. Soybean trypsin inhibitor and horseradish peroxidase provided by the supplier were used as the negative control and positive control, respectively.

RESULTS

cDNA Sequence

Ac-pla₂ cDNA sequence was 642 nt in length, consisted of a 7-nt 5'-UTR, a 510-nt coding region, a 99-nt 3'-UTR, and a 26-nt poly A tail. Both the coding nucleotide sequence and the corresponding amino acid sequence showed 93.5% identities with those of *A. mellifera* (Fig. 1). The 3'-UTR of *Ac-pla₂* cDNA was longer than that of *Ac-pla₂* cDNA, which was 51 nt in length (XM_391951).

The deduced amino acid sequence consisted of a 20 amino acid signal peptide (preregion of Ac-PLA₂), a 15 amino acid proregion, and a 134 amino acid mature peptide. Alignment analysis with Am-PLA₂ (GenBank AAL30844) revealed two residue insertions (Leu and Phe) sited before the 13th residue, and the residues of 4th, 8th, and 12th in signal peptide were different with the corresponding residues, which were Ala, Trp, and Ala in *A. cerana* and Val, Leu, and leu in *A. mellifera*. A total of 6 residues sited at 14, 15, 66, 75, 77, and 115 in the mature peptide were different between the two honeybees. They were Val, Ala, Thr, Gly, Lys, and Arg in *A. cerana* and Lys, Ser, Lys, Ala, Thr, and His in *A. mellifera*, respectively. Though both belong to *A. cerana*, the 15th residue of the mature peptide in *A. cerana* differed from that in *A. cerana indica*; the residue was Ser in Indian honeybee (GenBank A59055) and Ala in *A. cerana cerana* (Fig. 2).

```

1  CTCCACGATGCAAGTCGCGCTCGGATCGTGGTTCTCTCTCGCTCTCTTCCTCTCCACCTC  60
    M  Q  V  A  L  G  S  W  F  L  L  A  L  F  L  S  T  S
61  TCACGGATGGCAAATCAGGGATAGGATCGGGGATAACGAGTTGGAGGAGCGAATAATATA  120
    H  G  W  Q  I  R  D  R  I  G  D  N  E  L  E  E  R  I  I  Y
    Intron 64 bp
121  TCCAGTGAACGTTGTGGTGCGGGCATGGTAACGTGGCGTCCGGCCCGAACGAGCTAGGTCG  180
    P  G  T  L  W  C  G  H  G  N  V  A  S  G  P  N  E  L  G  R
181  GTTCAAGCACACGGATGCATGCTGTGCAACCCACGACATGTGCCCGGACGTGATGTCAGC  240
    F  K  H  T  D  A  C  C  R  T  H  D  M  C  P  D  V  M  S  A
    Intron 523 bp
241  TGGTGAATCGAAGCACGGTCTGACCAATACGGCCTCCACACCAGTGTGTCGTGCGACTG  300
    G  E  S  K  H  G  L  T  N  T  A  S  H  T  R  L  S  C  D  C
301  CGACGACACGTTCTACGATTGTCTTAAAAATTCGGGGGACAAGATTAGCTCGTATTTTCGT  360
    D  D  T  F  Y  D  C  L  K  N  S  G  D  K  I  S  S  Y  F  V
361  AGGAAAGATGTACTTCAATCTGATAGACACCAAGTGTACAACTGGAGCATCCTGTGAC  420
    G  K  M  Y  F  N  L  I  D  T  K  C  Y  K  L  E  H  P  V  T
    Intron 112 bp
421  CGGGTGCGGTGAGTAGGACCGAGGGTCGTTGTCTTCGCTACACGGTGGACAAAAGCAAGCC  480
    G  C  G  E  R  T  E  G  R  C  L  R  Y  T  V  D  K  S  K  P
481  GAAAGTGTAACCAATGGTTCGATCTTCGCAAGTATTGATAAAATTCACGGGGCTGATCTTG  540
    K  V  Y  Q  W  F  D  L  R  K  Y  *
541  AACATCCACTTGAAGATCGATTGTTTATCGAATTCACATCGATGTTAATTGATGTAATA  600
601  AAATAACAGAATAATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  642

```

Fig. 1. cDNA sequence and deduced amino acid sequence of PLA₂ gene from *Apis cerana*. The coding region of prepropla2 is in bold. The underlined and italic amino

acids represent the preregion and proregion of PLA₂, respectively. Positions of introns are indicated.

Ac-pla₂ Gene Structure

The genomic DNA corresponding to *Ac-pla₂* region was amplified by PCR using the primers designed based on the *Ac-pla₂* cDNA sequence. The

amplified fragment was 1,314 bp in length. Comparison of the DNA sequence with the cDNA sequence revealed that the genomic sequence of *Ac-pla₂* consisted of four exons of 126, 160, 148, and 182 bp (Fig. 1), separated by three introns of 64, 523,

```

Ac  MQVALGSWFLALFLSTSHGQWQIRDRIGDNELEERIIYPGTLWCCHGNVASGPNELGRFK  60
Am  ...V...L...L--.....KS.....  58

Ac  HTDACCRTHTDMCPDVMSAGESSKHGLTNTASHTRLSCDCDDTFYDCLKNSGDKISSYFVGK  120
Am  .....R.....A.T.....  118

Ac  MYFNLIDTKCYKLEHPVTGCGERTEGRCRLRYTVDKSKPKVYQWFDLRKY  169
Am  .....H.....  167

```

Fig. 2. Comparison of the protein sequences of PLA₂ from *A. cerana* and *A. mellifera*. Alignment of amino acid residues was done employing the program CLUSTAL W.

Points indicate residues in Am-Pla2 are identical to those in Ac-Pla2. The sequences of the signal peptides are given in italic letters, and the proregions are in bold letters.

and 112 bp, respectively. All introns contained the consensus GT-AG borders (Breathnach and Chambon, 1981).

Gene Copy Number

To determine the copy numbers of *Ac-pla₂* in genome, Southern blot analysis was performed using the genomic DNA of worker bees digested with 6 nt recognizing restriction endonucleases. The blot was probed with the Dig-labeled *Ac-pla₂* cDNA fragment. There was only one positive band detected in each of the seven restriction enzyme digested DNA samples. The sizes of positive bands ranged from 3.0 to 20 kb (Fig. 3). This result indicated that the *Ac-pla₂* gene was likely present as a single copy gene per haploid honeybee genome.

Age-Based Transcription

Northern blot results (Fig. 4) showed that *Ac-pla₂* mRNA was undetectable in the pupal and the adult stage from the 10th day onwards, but was abundantly detected during the first 6 days of adult stage. Two positive bands were detected, the larger one was about 1,700 nt, and the smaller one was about 700 nt, and they showed a similar transcriptional profile.

Age-Based Expression

The content of PLA₂ in a venom reservoir of different ages was determined by ELISA, and the result is shown in Figure 5. PLA₂ was nearly undetectable in the pupal stage, then gradually increased at the first 2–3 days of adult ages, followed by a rapid increase to its maximum (about 12 µg per bee) from the 4th to the 7th day, and slowly decreased to 8.0 µg per honeybee for the rest of the adult stage.

Western blot analysis was also performed to measure the content of PLA₂ in a venom reservoir of different ages. To exclude the effect of individual differences, the mixed samples from 8 bees were selected, and the results showed that PLA₂ was undetectable in the pupal stage and the

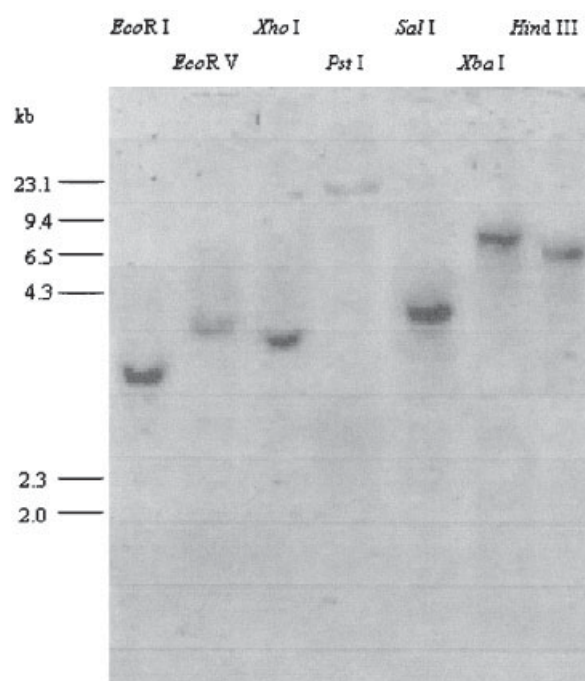


Fig. 3. Southern blot analysis using a Dig-labeled *Ac-pla₂* cDNA as the probe. DNA samples digested with different enzymes (from left to right: *EcoRI*, *EcoRV*, *XhoI*, *PstI*, *SalI*, *XbaI*, *HindIII*). DNA molecular weight standards (in kilobases) are shown on the left.

first 2 days of adult life (Fig. 6); then it was continuously detectable in the samples of age from the 8th day to the rest of the adult's life. Two bands with a molecular weight around 15 kDa were detected in 8-day-old bees. With increasing age, three bands of Ac-PLA₂ were detected in the sample from day 12 onwards (Fig. 6). The peptide sequencing showed that all three bands of protein had the same N'-terminal sequence, IIYP-GTLW, which was the N'-terminal sequence of the mature PLA₂. The lowest molecular weight of Ac-PLA₂ at an early adult stage might not have undergone post-translational modification. With increasing age, various extents of post-translational modification such as glycosylation were observed in Ac-PLA₂. Glycoprotein staining showed that the biggest molecular weight of the Ac-PLA₂ band and the two biggest Am-PLA₂ bands were glycosylated; two other smaller Ac-PLA₂ or Am-PLA₂ bands with no or low glycosylation could not be detected (Fig. 7).

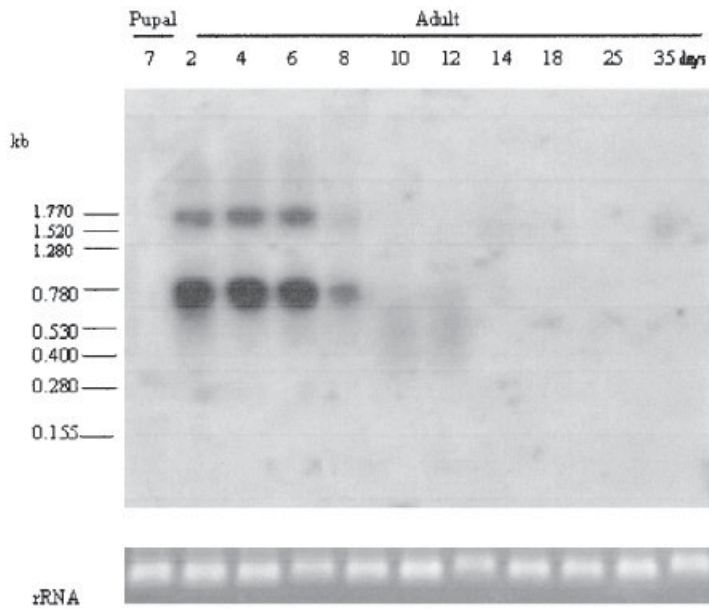


Fig. 4. Northern blot analysis using a Dig-labeled *Ac-pla₂* cDNA as a probe. Time points (development stages) are indicated above the corresponding lanes. Sizes of RNA molecular weight standards (in kilobases) are shown on the left. RNA samples (4 μ g each) were pooled from 30 bees at different ages. The portion of the gel containing rRNA stained with ethidium bromide are shown (lower panel).

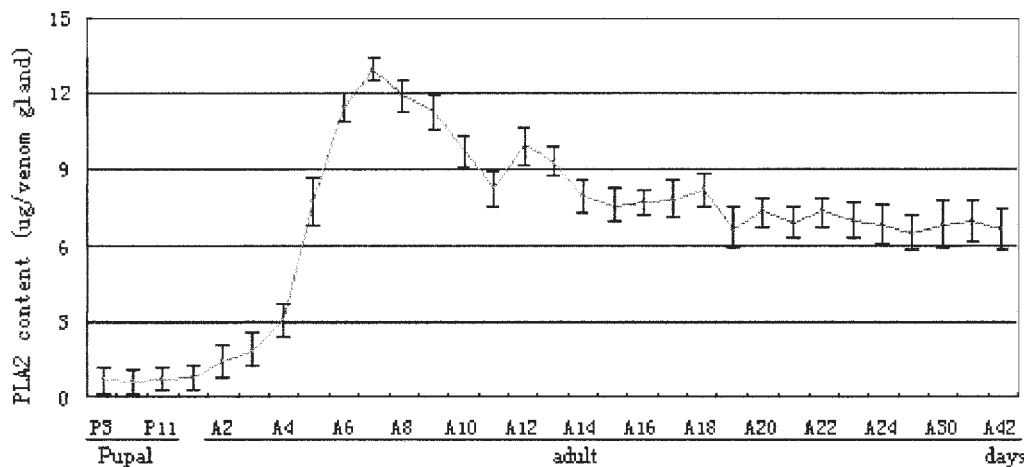


Fig. 5. PLA₂ or its precursor contents in venom reservoir of different development stage from pupal stage (P3, P7, and P11) and adult stage (A1–A42) of *A. cerana*.

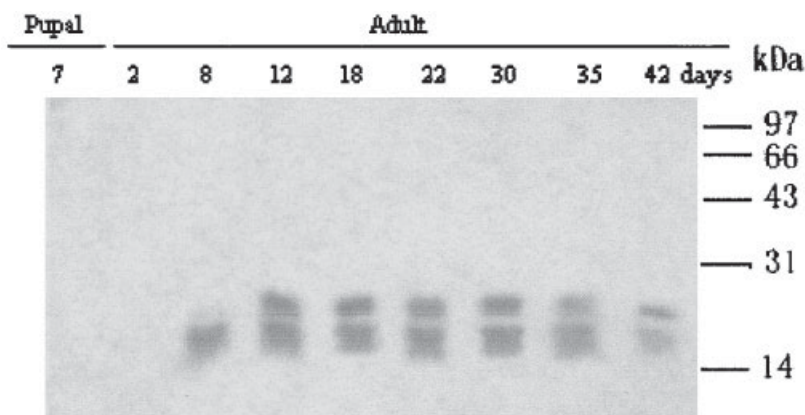


Fig. 6. Western blot analysis of PLA₂ from the venom reservoir at different development stages of *A. cerana*.

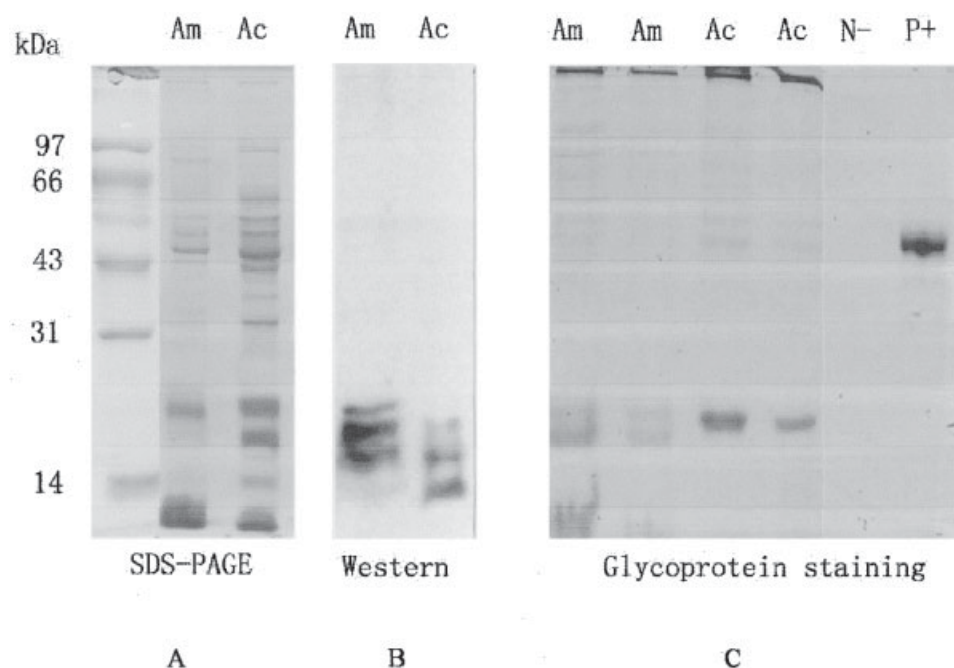


Fig. 7. SDS-PAGE, Western blot, and glycoprotein staining analysis of venom sample from *A. mellifera* and *A. cerana*. A: SDS-PAGE analysis of venom sample from *A. mellifera* and *A. cerana*. B: Western blot analysis of the corresponding samples of *A*. C: Glycoprotein staining of venom samples from *A. mellifera* and *A. cerana*. Am and Ac: The venom samples from *A. mellifera* and *A. cerana*, N- and P+: the negative control (soybean trypsin inhibitor) and positive control (horse-radish peroxidase).

Venom samples from individuals of different development stages of adults were subjected to SDS-PAGE and Western blot analysis to determine the individual differences in post-translational modification. Among 35 worker venom samples of 15-day-old bees, 27 showed three Ac-PLA₂ bands, which represent various extents of post-translational modification such as glycosylation, 8 individuals had only one Ac-PLA₂ band, which was completely glycosylated and had a high molecular weight (Fig. 8). Venom samples of 25-day-old bees had a similar profile (Fig. 8). The difference in post-translational modification of PLA₂ could be detected among the individuals of *A. cerana* from the same colony or different colonies. Thus, the post-translational modification of Ac-PLA₂ among individuals was not identical.

Different post-translational modifications or glycosylation were also found between two bee species, *A. cerana* and *A. mellifera*. Three PLA₂ bands in *A. cerana* and four PLA₂ bands in *A. mellifera* were detected by SDS-PAGE and Western blot analysis, respectively, when the mixed venom samples were used (Fig. 9). Moreover, unlike in *A. cerana*, such a post-translational process among individuals was identical in *A. mellifera*. Post-trans-

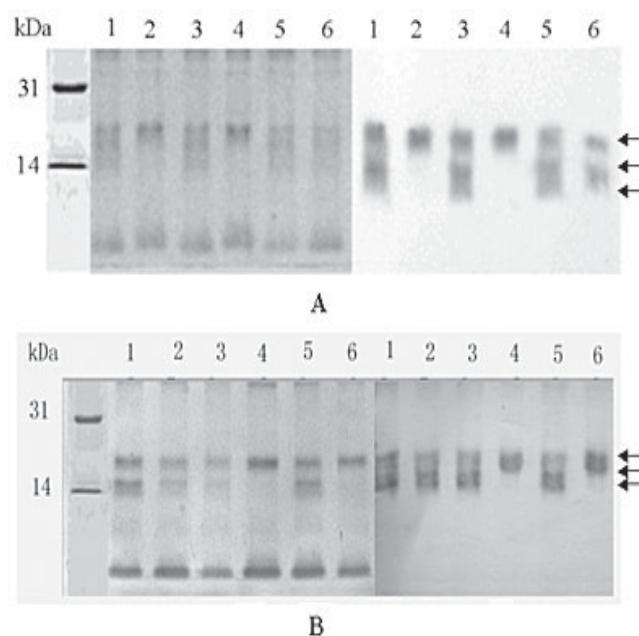


Fig. 8. SDS-PAGE and Western blot analysis of venom extracts with PLA₂ antiserum. A: SDS-PAGE and Western blot analysis of the venom sample prepared from 15-day-old adults. Three Ac-PLA₂ bands were detected in lanes 1, 3, 5, and 6, and only one band was detected in lanes 2 and 4. B: SDS-PAGE and Western blot analysis of 25-day-old bee venom extracts. Three bands of PLA₂ were detected in lanes 1–3 and 5, only one band in lanes 4 and 6. The arrows represent the three Ac PLA₂ types.

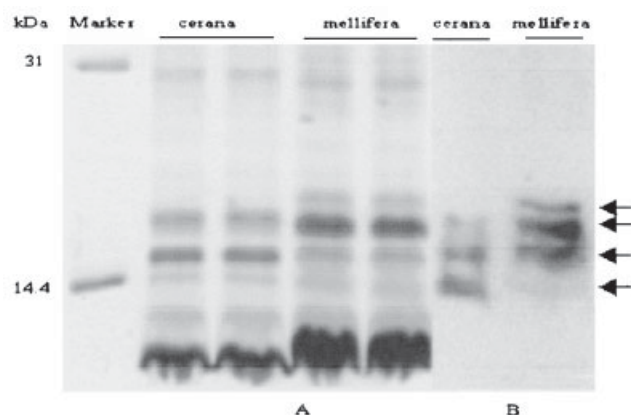


Fig. 9. SDS-PAGE (A) and Western blot (B) analysis of mixed venom from workers >15 days old of *A. cerana* and *A. mellifera*. The arrows indicate the corresponding PLA₂ bands.

lational modification was more complicated in *A. mellifera* than in *A. cerana*.

DISCUSSION

Our results showed that the *Ac-pla₂* was a single copy gene per haploid honeybee genome and its expression was active during the first week of adult age. *Ac-PLA₂* was undetectable in pupae and newly emerged adults, increased sharply during the first week of adult stage, and reached the maximum of 10–12 µg on the 10th day, then slowly decreased to about 8 µg for the rest of adult life. This is the first work that elucidates the *Ac-pla₂* gene expression regulation. The interesting discovery is that the post-translational modification of *Ac-PLA₂* among individuals of the Chinese honeybee and between *A. cerana* and *A. mellifera* was not identical. The results are biologically significant and will lay a basis for related research and future utilization.

The quantity of PLA₂ in the venom sac of *A. cerana* was 10–12 µg per worker, much less than that in *A. mellifera*, which was 30–40 µg per worker (Owen et al., 1990). Similar observations were also made on other venom components such as melittin (Owen and Pfaff, 1995; Li et al, 2005). Moreover, it has been documented that the total venom content in *A. mellifera* and *A. cerana* was 138 and 43 µg, respectively (Schmidt, 1995). More venom con-

tent in *A. mellifera* was thought to have its biological significance. Worker honeybees use their venom for defense of the colony against predators or intruders, often against large mammals and other vertebrates. Comparatively, *A. mellifera* often maintains a big colony and fearlessly defends their nest while the Chinese honeybee usually has a small colony and is more willing to flee when confronting adverse circumstances. More venom is helpful for *A. mellifera* to defeat enemies and guard their nest, but has no equal biological significance for the Chinese honeybee though their body size is similar to that of *A. mellifera*. The main venom component including melittin and phospholipase A₂ reach their maximum by the age of around 10 days when the bees are ready to be guarders (Winston, 1987). Venom secretion seems to be adapted to their behavior development and biological characters.

Ac-pla₂ mRNA was present at a high level during the first 6 days of adult ages, undetectable in the pupal stage and the adult life. The result suggested that the transcriptional activity occurred mainly during this period. Synthesis of PLA₂ or its precursor in the early adult stage measured by ELISA correlated with the *Ac-pla₂* transcriptional profile, which meant that the transcribed mRNA was immediately translated. During the later adult stage, *Ac-pla₂* mRNA was undetectable and, therefore, had little influence on PLA₂ synthesis in the protein level. Transcriptional or post-translational regulation was the key step in *Ac-pla₂* expression.

Two bands were detected in the Northern blot; the smaller one was about 700 bp corresponding to the matured *Ac-pla₂* mRNA, and the larger one was about 1,700 bp. Two reasons might be responsible for the larger transcript. Firstly, though the PLA₂ gene is single copied, two different transcriptional initiation sites were possible. Secondly, the larger *Ac-pla₂* mRNA was identical to the genomic DNA PCR amplified fragments of PLA₂ gene. The larger transcript, therefore, might be the primary transcribed PLA₂ mRNA that was not processed in time.

Phospholipases A₂ had been quantified by titration assay according to the enzyme activity in *A. mellifera* (Owen et al., 1990), which gives a similar

expression profile of low levels at the time of eclosion, increasing steadily through the 10 days after eclosion, and maintaining the maximal levels through the rest of the life of a worker bee in summer. Because of the possible synergism with melittin (Cajal and Jain, 1997), phospholipase A₂ content might be over-estimated. ELISA is an ideal method for detecting phospholipase A₂ depending on the immunoreactions and has been successfully used for quantitative analysis of spider venom antigens (Chávez-Olórtegui et al., 2001). Not the catalyzing activity, but the allergenicity and immunogenicity are the main biological functions of PLA₂ in bee venom. Quantification of PLA₂ by ELISA in bee venom is better than by enzyme activity assay in reflecting the immunogenic function of PLA₂.

A total of three types of Ac-PLA₂ were detected in SDS-PAGE and Western blot analysis. In adults less than 8 days old, only a small molecular size of Ac-PLA₂ was detected. With the increase in age, a larger molecular size of Ac-PLA₂ appeared, which resulted from a post-translational modification such as glycosylation (Lai and Her, 2000; Haslam et al., 1994; Kubelka et al., 1993; Staudacher et al., 1992; Weber et al., 1986). Such modification among individuals was different in *A. cerana*. While in Am-PLA₂ it was uniform among individuals, four types of PLA₂ were detected in all individuals, and the fourth PLA₂ had the highest molecular weight. Prediction of N-Glycosylation sites of Ac-PLA₂ and Am-PLA₂ with NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) revealed that they share the same glycosylation sites. But our results showed that the PLA₂ was not glycosylated in the same way between the two bee species. The allergenicity of the carbohydrate content of PLA₂ is well documented (Okano et al., 1999; Nakano et al., 1996; Dudler et al., 1995; Gomi et al., 1994; Mori et al., 1993; Prenner et al., 1992; Ohno et al., 1990). A more complicated glycosylation of PLA₂ in *A. mellifera* was thought to have a more intensive allergenicity, which is essential for honeybees to defend their colony and is, therefore, an adaptation to their venom utilization strategy. In the Chinese honey bee, the selection pressure for allergenicity of venom components was less. Understanding the

mechanism involved in the difference of glycosylation for PLA₂ in the two honeybee species would make the honeybee an excellent material to explore the glycosylation mechanism and its biological functions.

ACKNOWLEDGMENTS

We are thankful to Dr. V. Shyam Kumar at Zhejiang University for proofreading and advice on the manuscript. We are also grateful to Dr. Su Song-kun at Zhejiang University for his help in bee culture. 

LITERATURE CITED

- Beye M, Raeder U. 1993. Rapid DNA preparation from bees and %GC fractionation. *Biotechniques* 14:372–374.
- Breathnach R, Chambon P. 1981. Organization and expression of eukaryotic split genes coding for proteins. *Ann Rev Biochem* 50:349–383.
- Cajal Y, Jain MK. 1997. Synergism between mellitin and phospholipase A₂ from bee venom: apparent activation by intervesicle exchange of phospholipids. *Biochemistry* 36:3882–3893.
- Chávez-Olórtegui C, Bohórquez K, Alvarenga LM, Kalapothakis E, Campolina D, Maria WS, Diniz CR. 2001. Sandwich-ELISA detection of venom antigens in envenoming by *Phoneutria nigriventer* spider. *Toxicon* 39:909–911.
- Dennis EA. 1994. Diversity of group types, regulation and function of phospholipase A₂. *J Biochem* 269:13057–13060.
- Dudler T, Altmann F, Carballido JM, Blaser K. 1995. Carbohydrate-dependent, HLA class II-restricted, human T cell response to the bee venom allergen phospholipase A₂ in allergic patients. *Eur J Immunol* 25:538–542.
- Gomi H, Tagaya Y, Nakano T, Mikayama T, Ishizaka K. 1994. Antigen-binding glycosylation inhibiting factor from a human T-cell hybridoma specific for bee venom phospholipase A₂. *Proc Natl Acad Sci* 91:2824–2828.
- Habermann E. 1972. Bee and wasp venoms. *Science* 177:314–322.
- Haslam SM, Reason AJ, Morris HR, Dell A. 1994. Core fucosylation

- sylation of honeybee venom phospholipase A₂. *Glycobiology* 4:105–106.
- King TP, Spangfort MD. 2000. Structure and biology of stinging insect venom allergens. *Int Arch Allergy Imm* 123:99–106.
- Kubelka V, Altmann F, Staudacher E, Tretter V, Marz L, Hard K, Kamerling JP, Vliegenthart JF. 1993. Primary structures of the N-linked carbohydrate chains from honeybee venom phospholipase A₂. *Eur J Biochem* 213:1193–204.
- Kuchler K, Gmachl M, Sippl MJ, Kreil G. 1989. Analysis of the cDNA for phospholipase A₂ from honeybee venom glands. *Eur J Biochem* 184:249–254.
- Lai CC, Her GR. 2000. Analysis of phospholipase A₂ glycosylation patterns from venom of individual bees by capillary electrophoresis/electrospray ionization mass spectrometry using an ion trap mass spectrometer. *Rapid Communications in Mass Spectrometry* 14:2012–2018.
- Li JH, Zhang CX, Tang ZH. 2005. Expression of melittin gene in the venom gland of the Chinese honeybee, *Apis cerana cerana*. *Apidologie* (in press).
- Light WC, Reisman RE, Ilea VS, Wypych JI, Okazaki T, Arbesman CE. 1976. Studies of the antigenicity and allergenicity of phospholipase A₂ of bee venom. *J Allergy Clin Immun* 58:322–329.
- Michelette ERF, Soares AEE. 1993. Characterization of preimaginal developmental stages in Africanized honey bee workers (*Apis mellifera* L.). *Apidologie* 24:431–440.
- Mori A, Thomas P, Tagaya Y, Iijima H, Grey H, Ishizaka K. 1993. Epitope specificity of bee venom phospholipase A₂-specific suppressor T cells which produce antigen-binding glycosylation inhibiting factor. *Int Immunol* 5:833–842.
- Mukherjee AB, Miele L, Pattabiraman N. 1994. Phospholipases A₂ enzymes: regulation and physiological role. *Biochem Pharmacol* 48:1–10.
- Murakami M, Kudo I. 2002. Phospholipase A₂. *J Biochem* 131:285–292.
- Nakano T, Ishii Y, Ishizaka K. 1996. Biochemical characterization of antigen-specific glycosylation-inhibiting factor from antigen-specific suppressor T cells. I. Identification of a 55-kilodalton glycosylation-inhibiting factor peptide with TCR alpha-chain determinant. *J Immunol* 156:1728–1734.
- Nakashima S, Kitamoto K, Arioka M. 2004. The catalytic activity, but not receptor binding, of sPLA₂ s plays a critical role for neurite outgrowth induction in PC12 cells. *Brain Res* 1015:207–211.
- Ohno H, Iwata M, Katamura K, Ishizaka K. 1990. Effect of phospholipase A₂ inhibitors on mouse T lymphocytes. II. Phospholipase A₂ inhibitors induce T cell hybridomas and a T cell clone for the formation of glycosylation-inhibiting factor. *Int Immunol* 2:257–266.
- Ohsawa K, Mori A, Horie S, Saito T, Okuma Y, Nomura Y, Murayama T. 2002. Arachidonic acid release and prostaglandin F₂α formation induced by phenylarsine oxide in PC12 cells: possible involvement of secretory phospholipase A₂ activity. *Biochem Pharmacol* 64:117–124.
- Okano M, Nishizaki K, Satoskar AR, Yoshino T, Masuda Y, Harn DA. 1999. Involvement of carbohydrate on phospholipase A₂, a bee-venom allergen, in in vivo antigen-specific IgE synthesis in mice. *Allergy* 54:811–818.
- Owen MD, Pfaff LA. 1995. Melittin synthesis in the venom system of the honey bee (*Apis mellifera* L.). *Toxicon* 33:1181–1188.
- Owen MD, Pfaff LA, Reisman RE, Wypych J. 1990. Phospholipase A₂ in venom extracts from honey bees (*Apis mellifera* L.) of different ages. *Toxicon* 28:813–820.
- Piulachs MD, Guidugli KR, Barchuk AR, Cruz J, Simoes ZL, Belles X. 2003. The vitellogenin of the honey bee, *Apis mellifera*: structural analysis of the cDNA and expression studies. *Insect Biochem Mol* 33:459–465.
- Prenner C, Mach L, Glossl J, Marz L. 1992. The antigenicity of the carbohydrate moiety of an insect glycoprotein, honey-bee (*Apis mellifera*) venom phospholipase A₂. The role of alpha 1,3-fucosylation of the asparagine-bound N-acetylglucosamine. *Biochem J* 284:377–380.
- Rachinsky A, Strambi C, Strambi A, Hartfelder K. 1990. Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *Gen Comp Endocr* 79:31–38.
- Robert RA, Maria K, Julie EP, Thomas D, Terry PL, Michael HG. 1996. Active site of bee venom phospholipase A₂: the role of histidine-34, Aspartate-64 and Tyrosine-87. *Biochemistry* 35:4591–4601.
- Rodriguez De Turco EB, Jackson FR, DeCoster MA, Kolko M,

- Bazan NG. 2002 Glutamate signalling and secretory phospholipase A₂ modulate the release of arachidonic acid from neuronal membranes. *J Neurosci Res* 68:558–567.
- Ronco AM, Moraga PF, Llanos MN. 2002 Arachidonic acid release from rat Leydig cells: the involvement of G protein, phospholipase A₂ and regulation of cAMP production. *J Endocrinol* 172:95–104.
- Sambrook J, Russell DW. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press. p 7.42–7.50.
- Schmidt JO. 1995. Toxicology of venom from the honeybee Genus *Apis*. *Toxicon* 33:917–927.
- Scott DL, Otwinowski Z, Gelb MH, Sigler PB. 1990a. Crystal structure of bee-venom phospholipase A₂ in a complex with a transition-state analogue. *Science* 250:1563–1566.
- Scott DL, White SP, Otwinowski Z, Yuan W, Gelb MH, Sigler PB. 1990b. Interfacial catalysis: the mechanism of phospholipase A₂. *Science* 250:1541–1546.
- Shen LR, Zhang CX, Cheng JA. 2002. Cloning and sequencing of gene encoding phospholipase A₂ from the venom of *Apis cerana cerana* and *A. mellifera*. *Acta Agri Biol* 10:29–32 (in Chinese, with English abstract).
- Shipolini RA, Callewaert GL, Cottrell RC, Vernon CA. 1974a. The amino-acid sequence and carbohydrate content of phospholipase A₂ from bee venom. *Eur J Biochem* 48:465–476.
- Shipolini RA, Doonan S, Vernon CA. 1974b. The disulphide bridges of phospholipase A₂ from bee venom. *Eur J Biochem* 48:477–483.
- Sobotka AK, Franklin RM, Adkinson NFJ, Valentine M, Baer H, Lichtenstein LM. 1976. Allergy to insect stings. II. Phospholipase A: the major allergen in honeybee venom. *J Allergy Clin Immun* 57:29–40.
- Staudacher E, Altmann F, Marz L, Hard K, Kamerling JP, Vliegenthart JF. 1992. Alpha 1-6(alpha 1-3)-difucosylation of the asparagine-bound N-acetylglucosamine in honeybee venom phospholipase A₂. *Glycoconjugate J* 9:82–85.
- Uthe J, Magee W. 1971. Phospholipase A₂: action as affected by deoxycholate and divalent cations. *Can J Biochem* 49:776.
- Weber A, Marz L, Altmann F. 1986. Characteristics of the asparagine-linked oligosaccharide from honey-bee venom phospholipase A₂. Evidence for the presence of terminal N-acetylglucosamine and fucose in an insect glycoprotein. *Comp Biochem Phys B* 83:321–324.
- Winston ML. 1987. The biology of the honey bee. Cambridge, MA: Harvard University Press.
- Zhao M, Brunk UT, Eaton JW. 2001 Delayed oxidant-induced cell death involves activation of phospholipase A₂. *FEBS Lett* 509:399–404.