Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Expression of a bee venom phospholipase A_2 from *Apis cerana cerana* in the baculovirus-insect cell*

Li-rong SHEN†[1], Mei-hui DING[1], Li-wen ZHANG[1], Wei-guang ZHANG[1], Liang LIU[2], Duo LI[1]

([1]*Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310029, China*)

([2]*Department of Agriculture and Natural Resources, Delaware State University, Dover, DE 19901, USA*)

†E-mail: shenlirong@zju.edu.cn

Received Aug. 8, 2009; Revision accepted Jan. 28, 2010; Crosschecked Mar. 17, 2010

Abstract: Bee venom phospholipase A_2 ($BvPLA_2$) is a lipolytic enzyme that catalyzes the hydrolysis of the sn-2 acyl bond of glycerophospholipids to liberate free fatty acids and lysophospholipids. In this work, a new $BvPLA_2$ ($AccPLA_2$) gene from the Chinese honeybee (*Apis cerana cerana*) venom glands was inserted into bacmid to construct a recombinant transfer vector. Tn-5B-4 (Tn) cells were transfected with the recombinant bacmid DNA for expression. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed a double band with molecular weights of 16 and 18 kDa. Products of hexahistidine $AccPLA_2$ fusion protein accumulated up to 5.32% of the total cellular proteins. The $AccPLA_2$ fusion protein was cross reactive with the anti-$AmPLA_2$ ($BvPLA_2$ of the European honeybee, *Apis mellifera*) polyclonal serum. The reaction resulted in a double glycosylation band, which agrees with the band generated by the native $AmPLA_2$ in Western blot analysis. The PLA_2 activity of the total extracted cellular protein in the hydrolyzing egg yolk is about 3.16 μmol/(min·mg). In summary, the recombinant $AccPLA_2$ protein, a native $BvPLA_2$-like structure with corresponding biological activities, can be glycosylated in Tn cells. These findings provided fundamental knowledge for potential genetic engineering to produce $AccPLA_2$ in the pharmaceutical industry.

Key words: *Apis cerana cerana*, Bee venom phospholipase A_2 ($BvPLA_2$), Insect cell, Expression
doi:10.1631/jzus.B0900254 **Document code:** A **CLC number:** Q781

1 Introduction

Phospholipases A_2 (PLA_2s; EC 3.1.1.4) are a large super-family of lipolytic enzymes that catalyze the hydrolysis of the sn-2 acyl bond of glycerophospholipids to liberate free fatty acids and lysophospholipids (Mingarro *et al*., 1995). PLA_2s have important functions in the cellular processes that modulate the release of arachidonic acid and the precursor of eicosanoids of potent inflammatory mediators (Rodriguez de Turco *et al*., 2002). Additionally, PLA_2s play a critical role in host defense, atherosclerosis, signal transduction processes, membrane remodeling (Murakami and Kudo, 2002; Dennis, 1994; 1997), and delaying oxidant-induced cell death (Zhao *et al*., 2001). PLA_2s are also associated with numerous human disorders such as rheumatoid arthritis, autoimmune uveitis, respiratory distress syndrome, myocardial infarction, and septic and endotoxic shock (Mukherjee *et al*., 1994).

PLA_2s enzymes arise from a variety of sources including the mammalian pancreas, reptile venom and insect venom, and synovial fluids (Scott *et al*., 1990b). These enzymes have been systematically classified into 15 closely structure-related groups on the basis of the level of homology among their nucleotide and amino acid sequences. Based on their properties, PLA_2s can be historically classified into

* Project (No. 2007AA10Z324) supported by the National High-Tech Research and Development Program (863) of China

secretory ($sPLA_2$), cytosolic Ca^{2+}-dependent ($cPLA_2$), and cytosolic Ca^{2+}-independent ($iPLA_2$). Group I gobra/krait venom and mammalian pancreas, Group II crotalid and viper venom, and Group III bee/lizard/scorpion venom are all secretory, low-molecular mass (13–18 kDa) and Ca^{2+}-dependent PLA_2s (Balsinde *et al*., 2002). Bee venom PLA_2 ($BvPLA_2$) is a typical Group III $sPLA_2$ member and makes up approximately 12% of the dry weight of venom in the European honeybee, *Apis mellifera* (Habermann, 1972; Dennis, 1997). $BvPLA_2$ is the most lethal honeybee venom peptide. It acts as an allergen cooperating with other components to defend the colony against predators and intruders (Schmidt, 1995; King and Spangfort, 2000). The activity of $BvPLA_2$ can be enhanced by melittin, the most abundant constituent of honey bee venom (Mingarro *et al*., 1995). $BvPLA_2$ has a wide variety of pharmacological properties including anti-human immunodeficiency virus (HIV) activity, neurotoxicity, myo-toxicity, and neurite outgrowth induction (Fenard *et al*., 1999; 2001; Nakashima *et al*., 2004).

A nucleotide sequence of $BvPLA_2$ ($AmPLA_2$) from the European honeybee has been determined. The deduced amino acid sequence of $AmPLA_2$ consists of a signal peptide of 18 amino acid residues (preregion of PLA_2), a proregion of 15 residues, and a mature peptide of 134 amino acid residues. The mature peptide contains 10 cystine residues which can form 5 disulfide bonds (Kuchler *et al*., 1989; Shipolini *et al*., 1974a; 1974b). The crystal structure and catalyzing activity of PLA_2 were also well documented (Annand *et al*., 1996; Scott *et al*., 1990a; 1990b). A synthetic gene encoding the mature peptide of $AmPLA_2$ was expressed in *Escherichia coli*, but the biological activities of the expressed protein were low because it can form an inclusion body with incorrect folding in the prokaryotic cell (Dudler *et al*., 1992). The similar phenomenon was found for another recombinant bee venom allergen, hyaluronidase (BvHya) from the European honeybee. The biological activity of the BvHya expressed in *E. coli* was only 20%–30% of that of the BvHya expressed in the baculovirus-infected insect cells (Soldatova *et al*., 1998).

The Asiatic honeybee, *Apis cerana*, is another commonly-domesticated species distributed in southern and southeastern Asia. It has been appreciated that the European honeybee derived from its closed relative Asiatic honeybee in western or central Asia and subsequently spread into Europe and Africa (Oldroyd and Wongsiri, 2006; Whitfield *et al*., 2006). The Chinese honeybee, *Apis cerana cerana*, one subspecies of Asiatic honeybee, has more than 3000 years of history. The total number of the Chinese honeybee colonies maintained by Chinese farmers is 1.2 million, while European honeybee has as much as 7.8 million colonies in recent years. This species was the only breeding honeybee in China before the European honeybee was introduced at the beginning of the last century. The Chinese honeybee has unique biological characters and behaviors that are significantly different from the European honeybee, and has played important roles in the formation of the unique botanical layer in China by pollination (Zhang *et al*., 2008; Xu *et al*., 2009). We previously amplified the $BvPLA_2$ ($AccPLA_2$) gene that encodes a mature peptide of the Chinese honeybee (Shen *et al*., 2002). The expression of $AccPLA_2$ appears from 5 to 42 d of the worker bee of the Chinese honeybee. PLA_2 modification between the Chinese honeybee and the European honeybee was compared (Li *et al*., 2005). The aim of the present study was to investigate the expression, glycosylation, immunologic properties, and the enzymatic activity of the recombinant $AccPLA_2$ in insect cells.

2 Materials and methods

2.1 Materials

The plasmid, pGEM®-$AcPLA_2$, was constructed as previously described (Shen *et al*., 2002). *Trichoplusia ni* cell line (Tn-5B-4 (Tn) cell) was maintained in our laboratory. New Zealand white rabbits were purchased from the animal center of Chinese Traditional Medical Institute of Zhejiang. The restriction enzymes *Eco*RI and *Hin*dIII, DL2000 and DL15000 markers were purchased from TaKaRa Company (Dalian, China). The commercially-purified native $AmPLA_2$ was purchased from Sigma Company (St. Louis, MO, USA). The T4 DNA ligase, X-gal, isopropyl beta-D-thiogalactopyranoside (IPTG), Lipofectin, TNM-FH insect cell medium and bovine calf serum, the transfer vector pFastBacHTa, and *E. coli* strains DH10B were purchased from Invitrogen Corporation (Carlsbad, USA). The *Taq* DNA polymerase,

protein molecular marker, nitrocellulose filter (NC filter), and the goat anti-rabbit IgG (Fc) conjugate were purchased from Sino-Promega Company (Shanghai, China). Plasmid DNA extraction kit was purchased from Omega Bio-Tek Corporation (Norcross, USA). The calcium chloride, sodium deoxycholate, and bovine serum albumin (BSA) were purchased from Sangon Company (Shanghai, China). All the biochemical reagents were of highest commercially available purity.

2.2 Construction of baculovirus transfer vector and recombinant bacmid

The pGEM®-AccPLA_2 was amplified in *E. coli* TG1 cells and extracted with a plasmid DNA extraction kit. The *Eco*RI/*Hin*dIII-digested cDNA fragment of AccPLA_2 from pGEM®-AccPLA_2 was directionally inserted into the transfer vector pFastBacHTa. The recombinant transfer vectors were identified by using digestion and polymerase chain reaction (PCR) with the primers AccF1 5′-AGAATTCATGATAA TATATCCAGGAACG-3′ (*Eco*RI site was added and underlined) and AccR1 5′-GAAGCTTAATACTT GCGAAGATCG-3′ (*Hin*dIII site was added and underlined) of AccPLA_2, following the described procedures (Sambrook and Russell, 2002). Then it was sequenced with a DNA sequencer (performed by Sangon Company of Shanghai). After transformed into competent *E. coli* DH10B cells, the AccPLA_2 fragment in recombinant transfer vector was transposed to the recombinant baculovirus shuttle vector, bacmid, by Tn7 transposition. We extracted DNA from the positive clones that had been verified by PCR using primers AccF1 and AccR1 of AccPLA_2, M13F1 (5′-GTAAAACGACGGCCAGT) and M13R1 (5′-AACAGCTATGACCATG) of PUC/M13, following the described procedures (Luckow *et al*., 1993; Shang *et al*., 2007).

2.3 Culture of insect cells and Lipofectin-mediated transfection

Tn cells were used for the routine transfection and propagation of recombinant bacmid. TNM-FH medium supplemented with 10% (v/v) fetal bovine calf serum (complete medium) was used for propagation of the insect cells. The recombinant bacmid DNA was introduced into Tn cells mediated using a Lipofectin kit according to the supplier's instruction. Then the cells were incubated with serum-free medium. After the cells were exposed to DNA-lipid-medium for 24 h, the medium was replaced by the complete medium. The 2-d-old conditioned medium was collected. After transfection, Tn cells were incubated for 24 h and the medium was replaced with serum-free medium. The 2-d-old conditioned medium was collected. After centrifugation at 1500×*g* at 4 °C for 3 min, the pellet of infected cells was stored at −80 °C, and the supernatant containing the virus particles was used to propagate in Tn cells. After propagation for 4 generations, the genomic viral DNA isolated from the infected cells was confirmed by PCR with primers AccF1 and AccR1.

2.4 Preparation of anti-AmPLA_2 polyclonal serum

New Zealand white rabbits were immunized with the mixture of 300 mg commercially-purified native AmPLA_2 and 1 ml of phosphate buffered saline (PBS)/complete Freund's adjuvant emulsion. The rabbits were bled three weeks after primary immunization with 200 mg of the enzyme emulsified in incomplete Freund's adjuvant (1 ml), followed by injection (200 mg of enzyme in 1 ml of saline) for three weeks. The effect value of the serum extracted from the blood sample after one week of the second enhanced immunization was detected with native AmPLA_2 by the gel diffused protocol as described (Sambrook and Russell, 2002). The serum was stored at −80 °C.

2.5 Recombinant protein analysis

The intracellular proteins of the infected cells, a negative control (normal insect cells), and positive control (native AmPLA_2) were analyzed using 10% (v/v) sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described (Sambrook and Russell, 2002). Gels were stained with Coomassie brilliant blue R250. The concentration of expressed fusion protein was measured by thin layer scanning of the SDS-PAGE gel. For Western blots, the separated unstained proteins were transferred onto unmodified NC membrane in a Bio-Rad transblot apparatus. The fusion protein was analyzed using rabbit antiserum against native AmPLA_2 prepared as above. The proteins on the blot were detected using goat anti-rabbit IgG (Fc) conjugate as described (Sambrook and Russell, 2002).

2.6 Assay of PLA_2 activity

Titrimetric assay of PLA_2 was based on a modified method used by Owen *et al*. (1990). The fresh egg yolk substrate blended in 200 ml of distilled and deionized water was prepared on daily basis. Five milliliters of egg yolk substrate was mixed with 1 ml of 3×10^{-2} mol/L calcium chloride, 1 ml of 1.3×10^{-2} mol/L sodium deoxycholate, 1 ml of 5% (v/v) BSA, and 2 ml of water. This mixture was heated to 37 °C and NaOH was added to bring the pH value of mixture to 8.0 in a thermostatted microreaction vessel. A pH meter system was used to perform pH-stat titration with the pH value held at 8.0 by addition of 0.005 mol/L of NaOH. The concentration of the solubilized proteins of the extracts of the infected cells was measured and then the extracts were added to the substrate mixture. The rate of release of fatty acids was determined by the amount of added NaOH to maintain the pH at 8.0. One unit of PLA_2 activity is defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per minute at pH 8.0 and 37 °C. The purified native $AmPLA_2$ and native bee venom powder diluted in distilled and deionized water, respectively, were used as positive control. The extracts of the normal Tn cells were used as negative control.

3 Results

3.1 Construction of baculovirus transfer vector

The *Eco*RI/*Hin*dIII-digested cDNA fragment of $AccPLA_2$ from pGEM®-$AccPLA_2$ was inserted into the transfer vector pFastBacHTa (Fig. 1). The products of restricted digestion and PCR of the recombinant plasmid had an approximate 400-bp band, which is identical to the $AccPLA_2$ fragment (GenBank accession No. AF321087). Nucleotide sequencing confirmed that the inserted fragment is a correct open-reading frame (ORF) for protein expression (Fig. 2). In accordance with the schematic representation of the recombinant transfer vector pFastBac-$AccPLA_2$, the inserted gene was under the control of the Autographa californica nuclear polyhedrosis virus (AcNPV) polyhedrin promoter. The expressed gene was designed to generate a fusion protein composed of hexahistidine resides (6×His) at amino-terminus, which allows a single-step purification using immobilized Ni^{2+} ion affinity chromatography.

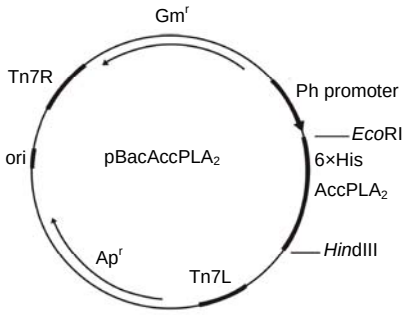

Fig. 1 Schematic representation of the donor vector

Forward primer (AccF1)

```
AGAATTC   ATA ATA TAT CCA GGA ACG TTG TGG TGC GGG CAT GGT AAC GTG TCG
            I   Y   P   G   T   L   W   C   G   H   G   N   V   S   S
TCC GGC CCG AAC GAG CTA GGT CGG TTC AAG CAC ACG GAT GCA TGC TGT CGA ACC
 G   P   N   E   L   G   R   F   K   H   T   D   A   C   C   R   T   H
CAC GAC ATG TGC CCG GAC GTG ATG TCA GCT GGT GAA TCG AAG CAC GGT CTG ACC
 D   M   C   P   D   V   M   S   A   G   E   S   K   H   G   L   T   N
AAT ACG GCC TCC CAC ACC AGG TTG TCG TGC GAC TGC GAC GAC ACG TTC TAC GAT
 T   A   S   H   T   R   L   S   C   D   C   D   D   T   F   Y   D   C
TGT CTT AAA AAT TCG GGG GAC AAG ATT AGC TCG TAT TTC GTA GGA AAG ATG TAC
 L   K   N   S   G   D   K   I   S   S   Y   F   V   G   K   M   Y   F
TTC AAT CTG ATA GAC ACC AAA GTG TTC AAA CTG GAG CAT CTG TGA CCG GGT GCG
 N   L   I   D   T   K   C   Y   K   L   E   H   P   V   T   G   C   G
GTG AGA GGA CCG AGG GTC GTT GTC TTC GCT ACA CGG TGG ACA AAA GCA AAG CCG
 E   R   T   E   G   R   C   L   R   Y   T   V   D   K   S   K   P   K
                                  Reversed primer (AccR1)
AAA GCG TAC  CAA TGG GTC GAT CTT CGC AAG GAT TAA  GCTTC
 A   Y   Q   W   F   D   L   R   K   Y   I   /
```

Fig. 2 cDNA sequence of $AccPLA_2$

The deduced amino acid sequence (matured peptide) is given below the nucleotide sequence. Forward primer and reversed primer for PCR are indicated with arrows, respectively

3.2 Construction of recombinant of bacmid and transfection of insect cells

After the recombinant transfer vector pFastBac-$AccPLA_2$ was transformed into *E. coli* DH10B competent cells that contain baculovirus shuttle vector bacmid, the recombinant colonies were further cultured. Then the PCR identification was conducted with the bacmid DNA isolated from the positive colonies as a template using primers $AccPLA_2$ and PUC/M13, respectively. Two fragments, about 400 bp (Fig. 3a) and 2700 bp (Fig. 3b), were generated. These fragments are identical to $AccPLA_2$ fragment within the multiple cloning sites (MCSs) and the bacmid fragment of 2300 bp within the left and right ends of bacterial transposon Tn7 plus $AccPLA_2$ fragment, which confirms that the recombinant bacmid, rBacmid-$AccPLA_2$, had been correctly constructed.

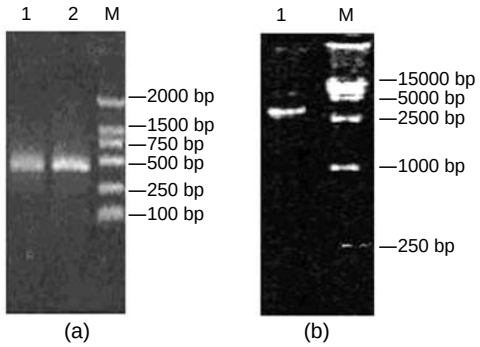

Fig. 3 PCR assays with AccPLA$_2$ and PUC/M13 primers
(a) AccPLA$_2$ primer. Lane 1: rBacmid-AccPLA$_2$ PCR; Lane 2: pGEM-AccPLA$_2$ PCR product; Lane M: DNA molecular weight marker (DL2000); (b) PUC/M13 primer: Lane 1: rBacmid-AccPLA$_2$ PCR product; Lane M: DNA molecular weight marker (DL15000)

With mediation of the Lipofectin, the rBacmid-AccPLA$_2$ DNA was introduced into Tn cells in medium with free bovine calf serum. AccPLA$_2$ appeared in 3 d after infection of rBacmid-AccPLA$_2$ DNA. After centrifugation for harvesting cells, the amplified rBacmid in Tn cells, rTnV-Bac-AccPLA$_2$, in supernatants was used to propagate.

3.3 PCR identification of rTnV-Bac-AccPLA$_2$

After propagation for four generations, PCR identification was performed using the rTnV-Bac-AccPLA$_2$ genomic DNA isolated from the infected cells as a template. The agarose electrophoretic analysis profile showed that the PCR products were of approximate 400 bp, identical with AccPLA$_2$ gene, confirming that rTnV-Bac-AccPLA$_2$ had been propagated in Tn cells.

3.4 SDS-PAGE and Western blot analysis

The SDS-PAGE analysis showed that the expressed product has a double band of 16 to 18 kDa (Fig. 4a). The 18-kDa band agrees with the expected molecular weight for the expressed fusion protein of AccPLA$_2$ (134 amino acids, 10.45 kDa) and 25 amino acids of *N*-terminal 6×His tag protein (2.8 kDa), a spacer region and rTEV protease cleavage site (3 kDa).

The gel diffusion detection showed that there was precipitation between the anti-AmPLA$_2$ polyclonal serum (1:8, 1:16, 1:32, 1:64, and 1:128) and antigen (1:1000). It has been confirmed that the prepared rabbit antibody could cross-react with native AmPLA$_2$. Following resolution on SDS-PAGE, the proteins of the infected cells were transferred electrophoretically into unmodified NC membrane probed with the anti-AmPLA$_2$ polyclonal serum (1:3000). The expressed fusion protein was recognized by the AmPLA$_2$-antibody, which confirms the expression of AccPLA$_2$ proteins, and shows that the Tn cells infected with the recombinant virus exhibited a double-immunoreaction band with molecular weights of 16 to 18 kDa (Fig. 4b). Meanwhile, the blots of native AmPLA$_2$ and native bee venom (positive control) yielded a similar double-band. By contrast, no immunoreactivity was detected in negative control. The results indicate that there was cross-reaction between the expressed fusion protein in Tn cells and anti-AmPLA$_2$ polyclonal serum. One mixed venom sample of Chinese honeybee was reported to have three bands detected for the natural AccPLA$_2$ with molecular weight 15 kDa using the anti-AmPLA$_2$ antibody, which was caused by the characters of BvPLA$_2$ glycosylation (Li *et al*., 2005). These results also indicate that the expressed AccPLA$_2$ protein can be glycosylated in Tn cells.

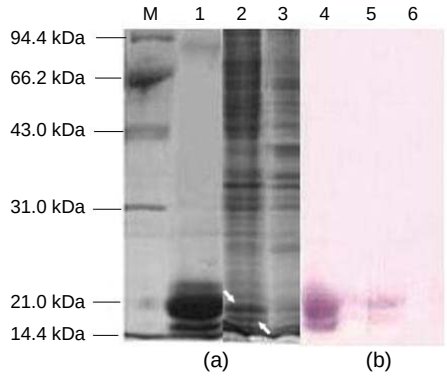

Fig. 4 SDS-PAGE analysis and Western blot of AccPLA$_2$ expression products
(a) SDS-PAGE analysis: Lane M: protein molecular weight marker proteins; Lane 1: native AmPLA$_2$; Lane 2: extracts from Tn cells infected with rBacmid-AccPLA$_2$ (the expressed bands were indicated with arrows); Lane 3: extracts from normal Tn cells; (b) Western blot: Lane 4: native AmPLA$_2$; Lane 5: extracts from Tn cells infected by rBacmid-AccPLA$_2$; Lane 6: extracts from normal Tn cells

3.5 Enzymatic activity assay of the expressed fusion protein

The hydrolyzation analysis of the total infected cellular protein on egg yolk substrate revealed that the PLA$_2$ enzymatic activity is about 3.16 μmol/(min·mg)

(Table 1). The result demonstrates that the crude fusion protein extracts of $AccPLA_2$ expressed in Tn cells could directly yield enzymatic activity of hydrolyzation on egg yolk substrate. The crude enzymatic activity of the pure fusion protein of $AccPLA_2$ was estimated at 63.3 μmol/(min·mg), which is about 20 times that of the total protein of the cells. However, the enzymatic activity of the purified $AccPLA_2$ from Tn cells cannot be detected because of the low expression of $AccPLA_2$.

Table 1 Enzymatic activity of the expressed $AccPLA_2$

Samples	Enzymatic activity (μmol/(min·mg))
Native $AmPLA_2$	193.75±8.84
Native bee venom	15.84±1.18
Extracts from Tn cells infected with rBacmid-$AccPLA_2$	3.16±1.13
Extracts from normal Tn cells	Not detected

4 Discussion

Previous studies have concluded that the amino acid sequences deduced from the cDNAs of $AccPLA_2$ and $AmPLA_2$ are composed of 134 amino acids with 5 disulfide bridges, 2 catalytic domains, and 3 calcium binding sites. The sequences of $AccPLA_2$ and $AmPLA_2$ share 95% homologies (Shen *et al*., 2002). This study confirmed that the activity and immunological properties of $AccPLA_2$ fusion protein expressed in Tn cells are in agreement with those of the native $AmPLA_2$ isolated from the worker bee venom glands of the European honeybee. Based on the molecular and biochemical homologies of $AccPLA_2$ and $AmPLA_2$, it could be speculated that the recombinant protein of $AccPLA_2$ might have a native-like conformation in Tn cells.

Baculovirus-mediated expression in insect cells is well established for the production of recombinant glycoproteins. With regard to protein folding and post-translational processing, insect cells are second only to mammalian cell lines. Evidence indicated that many processing events known in mammalian systems also occurred in insects (Luckow *et al*., 1993; Altmann *et al*., 1999). Numerous experiments were conducted with insect cells. For instance, human β-interferon and *O*-glycan core 2β-1,6-*N*-acetylglucosaminyltransferase (C2GnT) with high levels of activity have been successfully expressed in Sf-9 cells (Smith *et al*., 1983; Toki *et al*., 1997), and Tn cells showed high glycosylation potential for the recombinant insect glycoprotein, BvHya from the European honeybee (Soldatova *et al*., 1998). However, its capability to produce human-like complex type *N*-glycans has been a matter of controversy for many years. The lack of complex glycosylation has limited the use of the insect cell baculovirus expression vector system. Tn cells have been found to produce a small amount of galactose-terminated *N*-glycans, which prevent glycoproteins from being used as therapeutics proteins (Altmann *et al*., 1999). Therefore, two novel cell lines, A7S from *Pseudaletia unipuncta* and DpN1 from *Danaus plexippus* that possess the capability of complex glycosylation, can overcome such a problem (Palomares *et al*., 2003). However, the similar problems should not be taken account of for the naturally occurring glycoproteins such as $BvPLA_2$ and BvHya from insects to be expressed in Tn cell system. The Bac-to-Bac system and Tn cells may serve as a general probe for elucidation of some of the regulatory factors influencing glycosylation of insect protein.

$BvPLA_2$ is the most important bee venom glycoprotein with a single oligosaccharide attached to asparagin-13, which is responsible for its enzymatic activity and allergenicity (Scott *et al*., 1990a; 1990b; Kubelka *et al*., 1993). Prior to this study, $BvPLA_2$ has not been expressed in insect cells. Here, the $AccPLA_2$ gene was expressed in Tn cells. The expressed fusion protein was active, indicating that Tn cells could express properly modified $AccPLA_2$. The appearance of recombinant protein in SDS-PAGE and Western blot is a double band and the glycosylation capacity of Bac-to-Bac system and Tn cells for $AccPLA_2$ was verified, which provides further insights into the advantages of baculovirus and its active foreign eukaryotic insect protein. Our previous work on the difference of $BvPLA_2$ glycosylation among individuals of Chinese honeybee showed that $AccPLA_2$ was glycosylated during 8–12 d old at adult stage, and the $AccPLA_2$ in venom was a mixture of three different glycosides. The sequencing for all three bands of proteins excised from the NC membrane of Western blot showed that they had the same *N*-terminal sequence, IIYPGTLW, which is the *N*-terminal sequence of the mature $AccPLA_2$. The difference in the

molecular weights of the three bands was caused by their different glycosylations (Li *et al*., 2005). Altmann *et al*. (1991) found that $AmPLA_2$ from the European honeybee consisted of three isoforms with approximate molecular masses of 16, 18, and 20 kDa, respectively. The sequencing data on *N*-terminal amino acid sequence showed that the PLA_2-18 and PLA_2-20 carried oligosaccharide residues, and PLA_2-16 escaped glycosylation during biosynthesis. The electrophoretic separation of the three isoforms was based on structural features of glycosylation of $AmPLA_2$. Soldatova *et al*. (1998) also found that recombinant BvHya expressed in Tn cells appeared as a double band of 43 to 44 kDa with different glycosylations. Our results are consistent with these previous reports (Li *et al*., 2005; Altmann *et al*., 1991; Soldatova *et al*., 1998). Since the glycosylation of recombinant protein is an important factor that affects protein function, the present study combined with our previous findings may lay a new scientific basis for the molecular biological utilization of $AccPLA_2$ in the future.

The present study showed a low level of expression of $AccPLA_2$ protein in Tn cells, which might be caused by the allergen and toxic characters of $BvPLA_2$ and may have harmed insect cells. It also showed that the splicing time of the insect cells infected by the bacmid containing $AccPLA_2$ protein is much less than that of the cells infected by the bacmids containing other foreign genes.

In conclusion, we constructed Tn cells by transfecting the recombinant bacmid DNA that was inserted in a new $BvPLA_2$ ($AccPLA_2$) gene from the Chinese honeybee (*Apis cerana cerana*) venom glands to express the recombinant $AccPLA_2$ protein, a native $BvPLA_2$-like structure with corresponding biological activities that can be glycosylated in Tn cells. Our work may provide fundamental knowledge for potential genetic engineering to produce $AccPLA_2$ in the pharmaceutical industry.

5 Acknowledgement

We are grateful to Prof. Chuan-xi ZHANG and Prof. Jia-an CHENG in the Institute of Insect Science, Zhejiang University for their kind instruction in experiment. We are also grateful to Dr. Jian-xiang WU in Zhejiang University for his assistance in preparing the antibody against $AmPLA_2$. We would like to acknowledge Dr. Jia-cai WU in Zhejiang University for his technical assistance, and Dr. Zhen-hua LIU in Tufts University and Dr. Song-bai LIU in Harvard University for editing the manuscript.

References

Altmann, F., Kubelka, V., Staudacher, E., Uhl, K., Marz, L., 1991. Characterization of the isoforms of phospholipase A_2 from honeybee venom. *Insect Biochem.*, **21**(5):467-472. [doi:10.1016/0020-1790(91)90099-Z]

Altmann, F., Staudacher, E., Wilson, I.B.H., Marz, L., 1999. Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconjugate J*., **16**(2):109-123. [doi:10.1023/A:1026488408951]

Annand, R.R., Kontoyianni, M., Penzotti, J.E., Dudler, T., Lybrand, T.P., Gelb, M.H., 1996. Active site of bee venom phospholipase A_2: the role of histidine-34, As-partate-64 and Tyrosine-87. *Biochemistry*, **35**(14):4591-4601. [doi:10.1021/bi9528412]

Balsinde, J., Winstead, M.V., Dennis, E.A., 2002. Phospholipase A_2 regulation of arachidonic acid mobilization. *FEBS Lett*., **531**(1):2-6. [doi:10.1016/S0014-5793(02)03413-0]

Dennis, E.A., 1994. Diversity of group types, regulation and function of phospholipase A_2. *J. Biochem*., **269**(18):13057-13060.

Dennis, E.A., 1997. A growing phospholipase A_2 superfamily of signal transduction enzymes. *Trends Biochem. Sci*., **22**(1):1-2. [doi:10.1016/S0968-0004(96)20031-3]

Dudler, T., Chen, W.Q., Wang, S.S., Schneider, T., Annand, R.R., Dempcy, R.O., Crameri, R., Gmachl, M., Suter, M., Gelb, M.H., 1992. High-level expression in *Escherichia coli* and rapid purification of enzymatically active honey bee venom phospholipase A_2. *Biochim. Biophys. Acta*, **1165**(2):201-210.

Fenard, D., Lambeau, G., Valentin, E., Lefebvre, J.C., Lazdunski, M., Doglio, A., 1999. Secreted phospholipases A_2, a new class of HIV inhibitors that block virus entry into host cells. *J. Clin. Invest*., **104**(5):611-618. [doi:10.1172/JCI6915]

Fenard, D., Lambeau, G., Maurin, T., Lefebvre, J.C., Doglio, A., 2001. A peptide derived from bee venom-secreted phospholipase A_2 inhibits replication of T-cell tropic HIV-1 strains via interaction with the CXCR4 chemokine receptor. *Mol. Pharmacol.*, **60**(2):341-347.

Habermann, E., 1972. Bee and wasp venoms. *Science*, **177**(4046):314-322. [doi:10.1126/science.177.4046.314]

King, T.P., Spangfort, M.D., 2000. Structure and biology of stinging insect venom allergens. *Int. Arch. Allergy Imm.*, **123**(2):99-106. [doi:10.1159/000024440]

Kubelka, V., Almann, F., Staudacher, E., Tretter, V., Marz, L., Hard, K., Kamerling, J.P., Vliegenthart, J.F., 1993. Primary structures of the *N*-linked carbohydrate chains from

honeybee venom phospholipase A_2. *Eur. J. Biochem.*, **213**(3):1193-1204. [doi:10.1111/j.1432-1033.1993.tb17870.x]

Kuchler, K., Gmachl, M., Sippl, M.J., Krell, G., 1989. Analysis of the cDNA for phospholipase A_2 from honeybee venom glands-the deduced amino acid sequence reveals homology to the corresponding vertebrate enzymes. *Eur. J. Biochem.*, **184**(1):249-254. [doi:10.1111/j.1432-1033.1989.tb15014.x]

Li, J.H., Zhang, C.X., Shen, L.R., Tang, Z.H., Cheng, J.A., 2005. Expression and regulation of phospholipase A_2 in venom gland of the Chinese honeybee, *Apis cerana cerana*. *Arch. Insect Biochem. Physiol.*, **60**(1):1-12. [doi:10.1002/arch.20075]

Luckow, V.A., Lee, S.C., Barry, G.F., Olins, P.O., 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *J. Virol.*, **67**(8):4566-4579.

Mingarro, I., Prez-Paya, E., Pinilla, C., Appel, J.R., Houghten, R.A., Blondelle, S.E., 1995. Activation of bee venom phospholipase A_2 through a peptide-enzyme complex. *FEBS Lett.*, **372**(1):131-134. [doi:10.1016/0014-5793(95)00964-B]

Mukherjee, A.B., Miele, L., Pattabiraman, N., 1994. Phospholipases A_2 enzymes: regulation and hysiological role. *Biochem. Pharmacol.*, **48**(1):1-10. [doi:10.1016/0006-2952(94)90216-X]

Murakami, M., Kudo, I., 2002. Phospholipase A_2. *J. Biochem.*, **131**(2):285-292.

Nakashima, S., Kitamoto, K., Arioka, M., 2004. The catalytic activity, but not receptor binding, of PLA_2s plays a critical role for neurite outgrowth induction in PC12 cells. *Brain Res.*, **1015**(1/2):207-211. [doi:10.1016/j.brainres.2004.04.069]

Oldroyd, B.P., Wongsiri, S., 2006. Asian Honeybees. Biology, Conservation, and Human Interactions. Harvard University Press, Cambridge, Massachusetts, USA, p.36-64.

Owen, M.D., Pfaff, L.A., Reisman, R.E., Wypych, J., 1990. Phospholipase A_2 in venom extracts from honey bees (*Apis mellifera* L.) of different ages. *Toxicon*, **28**(7):813-820. [doi:10.1016/S0041-0101(09)80004-4]

Palomares, L.A., Joosten, C.E., Hughes, P.R., Granados, R.R., Shuler, M.L., 2003. Novel insect cell line capable of complex *N*-glycosylation and sialylation of recombinant proteins. *Biotechnol. Prog.*, **19**(1):185-192. [doi:10.1021/bp025598o]

Rodriguez de Turco, E.B., Jackson, F.R., DeCoster, M.A., Kolko, M., Bazan, N.G., 2002. Glutamate signalling and secretory phospholipase A_2 modulate the release of arachidonic acid from neuronal membranes. *J. Neurosci. Res.*, **68**(5):558-567. [doi:10.1002/jnr.10239]

Sambrook, J., Russell, D.W., 2002. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York, USA.

Schmidt, J.O., 1995. Toxinology of venoms from the honeybee genus *Apis*. *Toxicon*, **33**(7):917-927. [doi:10.1016/0041-0101(95)00011-A]

Scott, D.L., White, S.P., Otwinowski, Z., Yuan, W., Gelb, M.H., Sigler, P.B., 1990a. Interfacial catalysis: the mechanism of phospholipase. *Science*, **250**(4987):1541-1546. [doi:10.1126/science.2274785]

Scott, D.L., Otwinowski, Z., Gelb, M.H., Sigler, P.B., 1990b. Crystal structure of bee-venom phospholipase A_2 in a complex with a transition-state analogue. *Science*, **250**(4987):1563-1566. [doi:10.1126/science.2274788]

Shang, J.Y., Shao, Y.M., Lang, G.J., Yuan, G., Tang, Z.H., Zhang, C.X., 2007. Expression of two types of acetylcholinesterase gene from the silkworm, *Bombyx mori*, in insect cells. *Insect Sci.*, **14**(6):443-449.

Shen, L.S., Zhang, C.Z., Cheng, J.A., 2002. Cloning and sequencing of genes encoding phospholipase A_2 from the venom of *Apis cerana cerana* and *A. mellifera*. *J. Agric. Biotechnol.*, **10**(1):29-32 (in Chinese).

Shipolini, R.A., Callewaert, G.L., Cottrell, R.C., Vernon, C.A., 1974a. The amino-acid sequence and carbohydrate content of phospholipase A_2 from bee venom. *Eur. J. Biochem.*, **48**(2):465-476. [doi:10.1111/j.1432-1033.1974.tb03787.x]

Shipolini, R.A., Doonan, S., Vernon, C.A., 1974b. The disulphide bridges of phospholipase A_2 from bee venom. *Eur. J. Biochem.*, **48**(2):477-483. [doi:10.1111/j.1432-1033.1974.tb03788.x]

Smith, G.E., Summers, M.D., Fraser, M., 1983. Production of human belta interferon in insect cell with baculovirus expression vectors. *J. Mol. Cell. Biol.*, **3**(12):2156-2165.

Soldatova, L.N., Crameri, R., Gmachl, M., Kemeny, D.M., Schmidt, M., Weber, M., Mueller, U.R., 1998. Superior biologic activity of the recombinant bee venom allergen hyaluronidase expressed in baculovirus infected insect cells as compared with *Escherichia coli*. *J. Allergy Clin. Immunol.*, **101**(5):691-698. [doi:10.1016/S0091-6749(98)70179-4]

Toki, D., Sarkar, M., Yip, B., Reck, F., Joziasse, D., Fukuda, M., Schachter, H., Brockhausen, I., 1997. Expression of stable human *O*-glycan core 2β-1,6-*N*-acetylglucosaminyltransferase in Sf9 insect cells. *Biochem. J.*, **325**(1):63-69.

Whitfield, C.W., Behura, S.K., Berlocher, S.H., Clark, A.G.J., Johnston, S., Sheppard, W.S., Smith, D.R., Suarez, A.V., Weaver, D., Tsutsui, N.D., 2006. Thrice out of Africa: ancient and recent expansions of the honey bee, *Apis mellifera*. *Science*, **314**(5799):642-645. [doi:10.1126/science.1132 772]

Xu, P., Shi, M., Chen, X.X., 2009. Antimicrobial peptide evolution in the Asiatic honey bee *Apis cerana*. *PLoS ONE*, **4**(1):e4239. [doi:10.1371/journal.pone.0004239]

Zhang, C.X., Tang, X.D., Cheng, J.A., 2008. The utilization and industrialization of insect resources in China. *Entomol. Res.*, **38**(S1):S38-S47. [doi:10.1111/j.1748-5967.2008.00173.x]

Zhao, M., Brunk, U.T., Eaton, J.W., 2001. Delayed oxidant-induced cell death involves activation of phospholipase A_2. *FEBS Lett.*, **509**(3):399-404. [doi:10.1016/S0014-5793(01)03184-2]