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Identification of a chitinase from *Apocheima cinerarius* nucleopolyhedrovirus

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Baculoviruses are important biological control agents against insect pests. In this work, a chitinase from *Apocheima cinerarius* nucleopolyhedrovirus (ApciNPV) as a fusion protein was highly over-expressed in *Escherichia coli* M 15 strain and affinity purified on Ni Sepharose 6 Fast Flow column. The modeling structure of ApciNPV chitinase indicated the protein contains a polycystic kidney 1(PKD1) domain on N-terminal and a characteristic catalytic domain formed α/β triosephosphate isomerase (TIM) barrel fold like other family 18 glycohydrolases. The ApciNPV chitinase displayed both endo- and exo-chitinase activities using fluorescent oligosaccharides. Moreover, assessed by LC_{50} (50% lethal concentration) values, the ApciNPV chitinase showed insecticidal activity against *Apocheima cinerarius*, *Spodoptera exigua*, *Hyphantria cunea*, *Helicoverpa armigera* and *Lymantria dispar*. The results suggest that ApciNPV chitinase may offer a potential application as a new tool to control pest.

Key words: *Apocheima cinerarius* nucleopolyhedrovirus, chitinase, recombinant protein, insecticidal activity.

INTRODUCTION

Chitinases are enzymes that degrade chitin, which have wide-range roles in biocontrol of pathogens and harmful insects (Cohen-Kupiec and Chet, 1998). Baculovirus chitinases are considered responsible for the liquefaction of insect host (Hawtin et al., 1995; Hawtin et al., 1997) and are used as bioinsecticides for insect control. As reported by Rao et al. 2004 (Rao et al., 2004) in *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV), the chitinase induced 100% mortality for *Bombyx mori* larvae and a significant increase in the number and size of perforations of the peritrophic membrane (PM). The chitinase from *B. mori* nucleopolyhedrovirus (BmNPV) plays an important role in delaying the cell lysis and decreasing the haemolymph turbidity and the degradation of the body in silkworm larvae (Wang et al., 2005). Furthermore, chitinase and V-cathepsin could together advance the liquefaction of the host after death (Hawtin et al., 1997; Ohkawa et al., 1994; Hom et al., 2000; Slack et al., 1995).

Apocheima cinerarius nucleopolyhedrovirus (ApciNPV) was first isolated and identified by the Institute of Forest Ecology and Environment Conservation, Chinese Academy of Forestry in 1979 (Yu and Wang; 1987; Yu and Wang, 1986). It is a member of the Baculoviridae family, Genus

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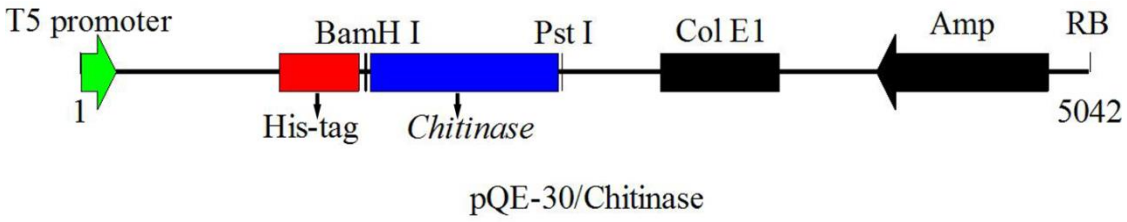

Figure 1. Outline of the pQE 30 expression vector with chitinase from ApciNPV under control of T5 promoter.

of alphabaculovirus. The virus exhibited high virulence against the larvae of *A. cinerarius* (Qi, 2006). In this paper, a gene which encodes a chitinase of ApciNPV is expressed in *Escherichia coli* and its potential use as a biological insecticide is proposed.

MATERIALS AND METHODS

Propagation of viruses

A. cinerarius larvae, ApciNPV strain and artificial diet were provided by the Research Centre of Forest Insect Virus, Chinese Academy of Forestry. ApciNPV was replicated and purified according to the method described by Qu et al. (2011). Briefly, ApciNPV in the concentrations of 3.0×10^7 was used to infect fourth-instar *A. cinerarius* larvae. After 5 days, 30 dead insects were milled and fully homogenized with two volumes phosphate buffer solution (PBS, pH 7.2). To remove insect tissue, the extract was filtered through four layers of gauze and then the filtrate was stirred at 800 rpm for 5 min at 25°C. The crude virus was obtained by centrifugation (8000 rpm, 30 min), and purified in a sucrose gradient of 40-60% (w/w) at 4000 rpm for 30 min at 25°C. The bands containing the virus were collected and washed with sterile water three times by centrifugation at 10000 rpm for 30 min at 4°C.

Purification of viral DNA

The method of isolation of ApciNPV DNA was performed basically according to Wang et al. (Wang et al., 2013). Purified ApciNPV was suspended in 2-3 volumes extraction buffer (0.1 M Na_2CO_3, 0.15 M NaCl and 0.05 M EDTA, pH 10.8) to dissolve the polyhedron matrix. After incubating at 3°C for 1 h, the suspension was adjusted to pH 7.0 with 0.1 M HCl, and SDS (0.5%) and proteinase K (50 µg/mL) were then added successively, and digestion was performed at a temperature of 55°C for 3 h. The solution was extracted with the same volume phenol-chloroform-isoamyl alcohol (25:24:1) and chloroform, respectively. The ApciNPV DNA was precipitated with two volumes of ethanol at -20°C for 2 h, and obtained by centrifugation at 12000 rpm for 10 min at 4°C. The precipitate was dissolved in TE buffer (pH 8.0) and stored at -20°C.

Construction of bacterial expression plasmids

A truncated sequence of *orf38* chitinase gene lacking its C-terminal ER retention motif (HTEL) was amplified from ApciNPV genome (GenBank accession number: FJ914221) using primers P1 (5' -AAAAGGATCCATGCATTGGTGCGTCAACCGCGA-3' and P2 5'-CCCCCTGCAGTTTTATATTAGATTTATAATGT -3') to generate *Bam*H I and *Pst* I restriction sites (underlined) at the 5'end and 3'end, respectively. The 50 µL PCR solution including 40 ng DNA, 0.1 µmol forward and reverse primers and 1× polymerase buffer (containing 15 mM $MgCl_2$, 0.4 mM dNTP, 5U of Expand High Fidelity Taq polymerase). The reaction of PCR operated at 94°C for 5 min and repeated 30 times following 94°C for 30 s, 60°C for 45 s, 72°C for 1 min, and final extension went at 72°C for 10 min. The amplified PCR product was digested using the enzymes of *Bam*H I and *Pst* I, and constructed into the pQE30 vector (Novagen) with 6×his-tag gene and T5 promoter (Figure 1).

Protein production and purification

The ApciNPV chitinase as a recombinant fusion protein with a 6×his-tag was over-expressed in *E. coli* strain M15 (Novagen). Small-scale (3 mL) cells were cultured with a rotary shaker at 37°C until the optical density at 600 nm (OD 600) of 0.6 and the recombinant protein was induced by 0.2 mM IPTG treatment at 16°C for 15 h. The cells were obtained by centrifugation, kept at -20°C for 30 min, and then suspended in FastBreak™ Cell lysis Reagent (Promega). The amount of soluble and insoluble recombinant protein was determined by MagneHis™ Protein Purification System according to the manufacturer's instructions (Promega). Large-scale (1 L) recombinant protein was obtained by adding 0.2 mM IPTG to *E. coli* culture (OD 600=0.6). After induction process for 15 h at 16°C, the cells were harvested and lysed in 80 mL buffer A (20 mM Tris-HCl, 150 mM NaCl, 10 mM Imidazole, pH 7.5), and then sonicated on ice with Sonifier (300W, 3s/2s). After centrifugation, the soluble fraction was adsorbed on Ni Sepharose 6 Fast Flow (GE healthcare) column. The column was equilibrated with buffer A and initially eluted with buffer B (20 mM Tris-HCl, 150 mM NaCl, 20 mM Imidazole, pH 7.5). Adsorbed protein was eluted with buffer C (20 mM Tris-HCl, 150 mM NaCl, 200 mM Imidazole, pH 7.5) and buffer D (20 mM Tris-HCl, 150 mM NaCl, 500 mM Imidazole, pH 7.5), sequentially. The separation and purification of the samples were confirmed by SDS-PAGE (12% gel) and the expected band was visualized staining the gel with Coomassie brilliant blue.

Enzyme activity analysis

The enzyme activity of the recombinant ApciNPV chitinase was analyzed as previously described (Rao et al., 2004; McCreath et al., 1992) with 4-methylumbelliferyl β-D-N,N′ diacetilchitobioside (4MU-$(GluNAC)_2$) and 4- methylumbelliferyl β-D-N, N′,N″ triacetilchito-trioside (4MU-$(GluNAC)_3$) as substrates for the quantified of exo-chitinase and endo-chitinase activities, respectively. For each standard sample, 20 µL McIlvaine's buffer (0.1 M citric acid, 0.2 M dibasic sodium phosphate, pH 5.0) and 5 µL appropriate substrate was mixed and then seven different concentrations (0, 5, 10, 15, 20, 25, 30 µg/µL) of protein were added into each tube. After incubating

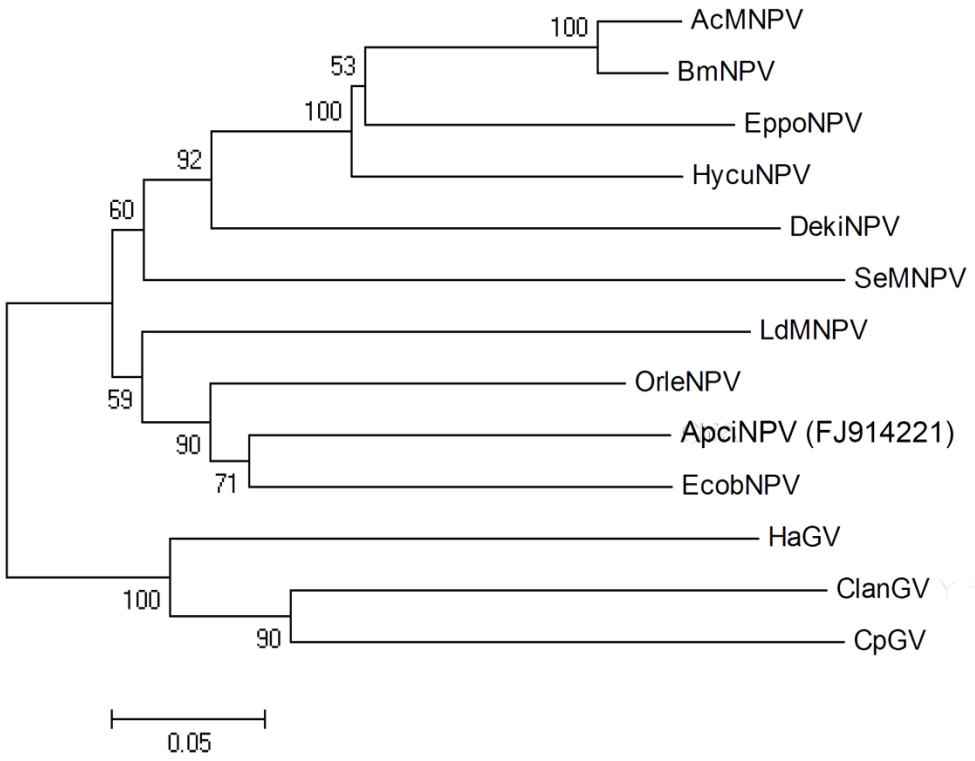

Figure 2. Constructed phylogenetic tree of chitinases from baculovirus using the neighbor-joining method. The chitinases come from ApciNPV (FJ914221), AcMNPV (NP_054156), BmNPV (NP_047523), EppoNPV (NP_203279), HycuNPV (YP_473218), DekiNPV (AFP66961), SeMNPV (NP_046280), LdMNPV (NP_047707), OrleNPV (YP_001650934), EcobNPV (YP_874243), HaGV (YP_001649087), ClanGV (YP_004376217) and CpGV (YP_148794).

at 30°C for 30 min, the reaction was terminated by 120 µL of 1 M glycine/NaOH buffer, pH10.6 for 5 min. Fluorescence was carried out using a Fluoroskan fluorimeter (Thermo Scientific Fluoroskan Asecent FL, Waltham, United States) with an excitation light at 360 nm and an emission light at 460 nm. All experiments were repeated three times.

Insect bioassays

Apocheima cinerarius, *Spodoptera*. *exigua*, *Hyphantria cunea*, *Helicoverp armigera* and *Lymantria dispar* were provided by the Research Centre of Forest Insect Virus, Chinese Academy of Forestry. Larvae were fed on artificial diet and reared at 26±°C, under a photoperiod of 14 h light/10 h dark, with 60-70% relative humidity. The insecticidal activity assay for determination of the median lethal concentrations (LC_{50}) was performed as previously described (Rajamohan et al., 1996) and partly modified. Briefly, 100 µL volume of purified ApciNPV chitinase protein solution in the concentrations of 50, 100, 200, 400 and 800 ng dissolved in elution buffer D were added to the surface of artificial diet in each 2 cm^2 well (diet surface area). Control diet was mixed with elution buffer D. The experiment was repeated three times with 20 third instar larvae (3 per well) per concentration. Starting from the fifth day after feeding with diet containing ApciNPV chitinase, the number of larvae was recorded until death or pupation. LC_{50} values were estimated by Probit analysis on the SPSS 19.0 statistical software (Finney, 1971).

Structural modeling of chitinase from ApciNPV

The homology modeling of ApciNPV chitinase was constructed by the SWISS-MODEL server (http://swissmodel.expasy.org/), and structure template was the chitinase of *S. marcescens* (PDB code: 1CTN) (Perrakis et al., 1994).

RESULTS

Analysis of chitinase gene of ApciNPV

The 1581bp *orf38* gene from ApciNPV encoded a putative chitinase at amino acid level with 65% identity to AcMNPV, 62% identity to *S. marcescens* and 58% identity to *Cydia pomonella* granulovirus (CpGV). Phylogenetic analyses of baculovirus chitinases consisted of two main branches (Figure 2) which might have much alike in properties. ApciNPV chitinase showed to belong to Alphabaculovirus genus with different percentages of identity with *Ectropis oblique* nucleopolyhedrovirus (EcobNPV,YP_874243), *Orgyia leucostigma* nucleopoly-hedrovirus (OrleNPV, YP_001650934), *L. dispar* multiple nucleopolyhedrovirus (LdMNPV, NP_047707), AcMNPV (NP_054156), BmNPV

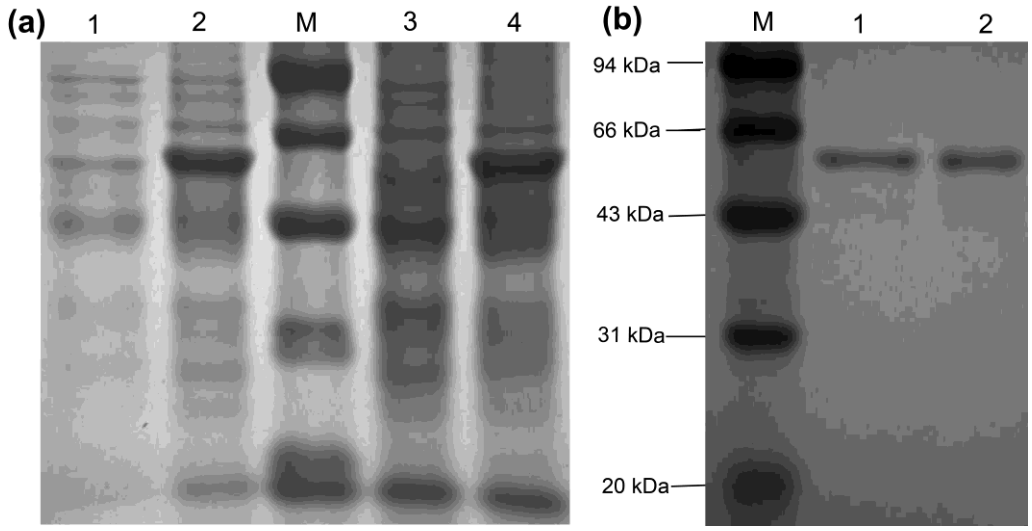

Figure 3. SDS-PAGE analysis of recombinant and purified native ApciNPV chitinase. **(a)** Expression of the recombinant ApciNPV chitinase from small-scale by IPTG induced bacterial cultures. Lane 1, the insoluble fraction of induced *E. coli* cells; lane 2, the soluble fraction; lane M, molecular mass markers (from top to down 94.0, 66.2, 43.0, 31.0, and 20.0 kDa); Lane 4, bacterial culture pellet; Lane 5, induced culture pellet using 0.2 mM IPTG at 16°C). **(b)** Large-scale IPTG-induced ApciNPV chitinase purified by Ni^{2+}-affinity chromatography. 10 µL samples containing 3 µg of protein were loaded to each lane. Lane 1 indicates the fractions washed with 20 mM Tris-HCl, 150 mM NaCl, 200 mM Imidazole, pH 7.5. Lane 2 displays the fractions eluted by 500 mM Imidazole.

(NP_047523), *Epiphyas postvittana* nucleopolyhedrovirus (EppoNPV, NP_203279), *H. cunea* nucleopolyhedrovirus (HycuNPV, YP_473218), *Dendrolimus kikuchii* nucleopolyhedrovirus (DekiNPV, AFP66961) and *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV, NP_046280).

Production and purification of native ApciNPV chitinase

The ApciNPV chitinase contained 523 amino acid residues with a calculated molecular mass of 58.2 kDa. The gene sequence was amplified according to the primers from the ApciNPV genome (GenBank accession number: FJ914221) by PCR. The PCR product was constructed into the pQE30 vector with the N-terminal 6×His-tag and then over-expressed in *E. coli* under T5 promoter transcription-translation *in vitro*. The cellular soluble fraction of recombinant protein was induced by 0.2 mM isopropyl-β-d-thiogalactoside (IPTG) at 16°C (Figure 3a, Lane 2). The recombinant His-tagged chitinase was purified using Ni^{2+}- affinity chromatography and analyzed by SDS-PAGE (Figure 3b, Lane 1 and 2).

Enzyme activity analysis of ApciNPV chitinase

The exo- and endo-chitinase activities of ApciNPV chitinase were measured by 4-MU-$(GlcNAC)_2$ and 4-MU-$(GlcNAC)_3$ fluorescent substrates, respectively. From Figure 4, both exo- and endo-chitinase activities increased linearly with the ApciNPV chitinase concentration up to 4.3-fold and 5.2-fold, respectively, over their lowest levels. It suggested that the ApciNPV chitinase produced in *E. coli* was active and exhibited its native exo- and endo-chitinolytic activities.

In vivo assays on larvae

A. cinerarius, *S. exigua*, *H. cunea*, *H. armigera* and *L. dispar* larvae were exposed to artificial diet treated with different concentrations of ApciNPV chitinase. The LC_{50} values of the larvae are shown in Table 1. The toxicities of ApciNPV chitinase response of different larvae from high to low in order were *A. cinerarius*, *S. exigua*, *H. cunea*, *H. armigera* and *L. dispar* larvae. *A. cinerarisus* larvae were the most susceptible of all tested larvae treated with the ApciNPV chitinase and LC_{50} values was 175.6 ng/cm^2 while the LC_{50} value to *L. dispar* larvae was the highest of 382.7 ng/cm^2. These results implicate the ApciNPV chitinase have an obvious insecticidal activity.

Modeling three-dimensional structure of ApciNPVchitinase

To clarify the mechanism of ApciNPV chitinase insecticidal activity, the three-dimensional structure was obtained by computer modeling (Figure 4). The model

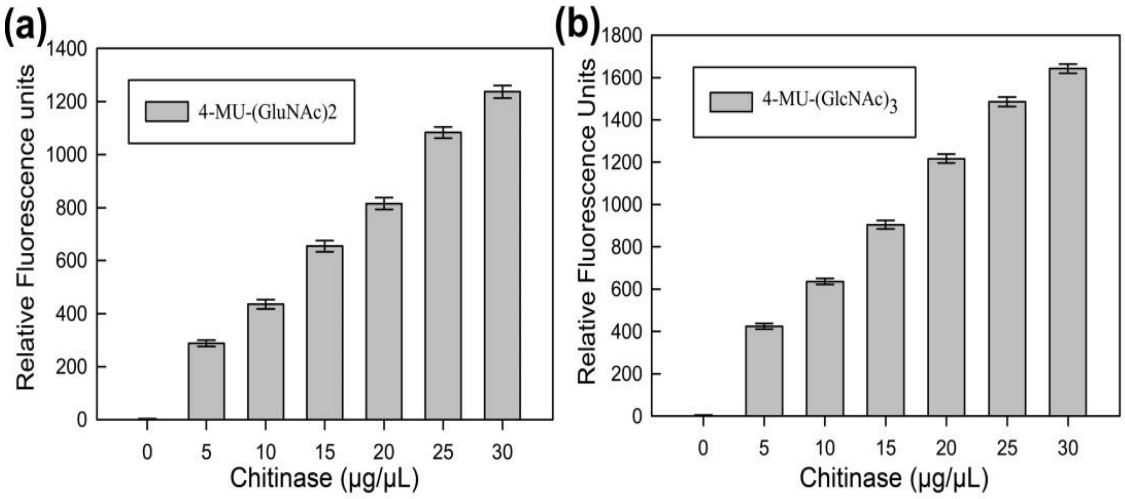

Figure 4. Enzyme activity of the recombinant ApciNPV chitinases. **(a)** The exo-chitinase activity of ApciNPV chitinase was detected using 4-MU-$(GlcNAc)_2$ substrate. **(b)** The endo-chitinase activity of ApciNPV chitinase was analyzed against 4-MU-$(GlcNAc)_3$ substrate. Results are indicated means±SE (n=3).

Table 1. Insecticidal activities of ApciNPV chitinase.

Insect species	LC_{50} (ng/cm^2)[1]	95%CL[2]	Slope
Apocheima cinerarius	175.6	73.5-543.2	2.4
Spodoptera exigua	178.5	64.2-574.5	1.8
Hyphantria cunea	279.1	87.5-628.4	3.1
Helicoverp armigera	325.4	84.3-419.7	2.8
Lymantria dispar	382.7	97.5-643.6	1.5

[1]LC_{50}, 50% lethal concentration. [2]CL=confidence limit.

was constructed using the SmChiA from *S. marcescens* which had 62% identity with chitinase from ApciNPV by SWISS-MODEL server. The ApciNPV chitinase showed three structural domains (Figure 5a), domain I was called a polycystic kidney 1(PKD1) domain producing an immunoglobulin-like fold; domain II consisted of an $(\alpha/\beta)_8$ TIM barrel, including residues 94-404 and 474-517 and domain III was composed of five antiparallel β-strands, one of which was interrupted. Furthermore, the conserved aromatic residues of ApciNPV chitinase in the hydrolysis mechanism were deduced on positions similar to SmChiA from *S. marcescens* and other chitinases (18, 19). These aromatic residues contained W28 and W31 in the immunoglobulin-like fold, W192, W205 and Y130 contributing chitin to the catalytic cleft and W127, W498, W235, Y379 and W357 binding native chitin (Figure 5b).

DISCUSSION

In this paper, the chitinase from ApciNPV was characterized. Based on amino acid sequences identity, the ApciNPV chitinase shared 65, 62 and 58% homology with the gene of AcMNPV, *S. marcescen* and CpGV, respectively. It suggests that the ApciNPV chitinase has essentially similar to the chitinase of AcMNPV and closely related to the chitinase of *S. marcescens*. The ApciNPV chitinase contained a C-terminal ER-retention sequence (HTEL) that has probably played a part in maintaining the hydrolysis in the cell at the late infection (Saville et al., 2002; Saville et al., 2004). Furthermore, compared with the known structure of SmchiA by computer model, the ApciNPV chitinase displayed a PKD1 domain and a family 18 glycohydrolase catalytic domain (Figure 5). The immunoglobulin-like fold of PKD1 domain is related to carbohydrate splitting and guiding the substrate into the catalytic groove (Bork and Doolittle, 1992; Perrakis et al., 1997). The catalytic domain possesses an open substrate-binding cleft (Uchiyama et al., 2001; Young et al., 2005). In addition, conserved tryptophan residues along the PKD1 fold and other aromatic residues in the catalytic domain have been found on the surface of SmChiA and other chitinase (Uchiyama et al., 2001; Young et al., 2005). The conserved residues of SmChiA included W69, W33, and W245, which involve in the chitin binding, and F-232 introduces the chitin chain into the catalytic pocket (Uchiyama et al., 2001). Analogously, the conserved residues from ApciNPV chitinase included

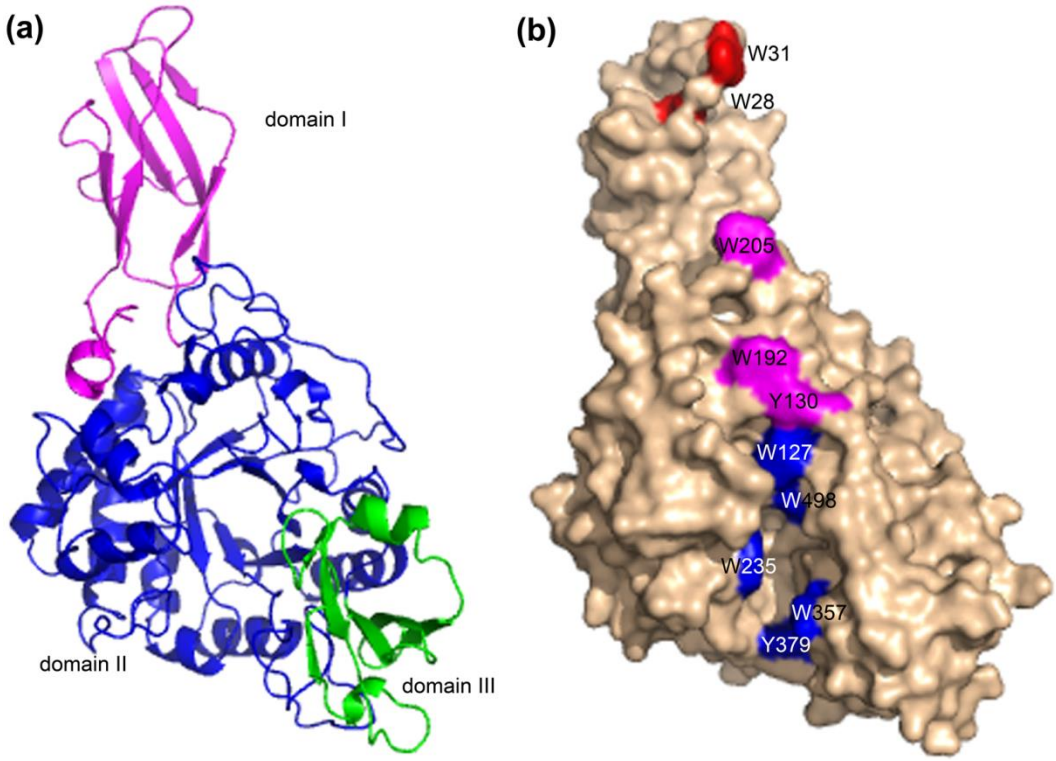

Figure 5. Modeled three-dimensional structure of ApciNPV chitinase by computer. **(a)** Ribbon drawing of ApciNPV chitinase showing the three domains in magenta (I), blue II) and green (III). **(b)** Representation of the molecular surface of ApciNPV chitinase. The conserved aromatic residues of immunoglobulin-like fold are shown red. The catalytic cleft is colored magenta. The catalytic binding-site is displayed in blue.

W28 and W31 in the corresponding position along the immunoglobulin-like fold, and W192, W205 and Y130 may also be essential for leading chitin chain into the catalytic pocket during crystalline chitin hydrolysis, and W127, Y498, W235, Y379 and W357 probably interact with GlcNAc units of chitin to forming the binding sites.

To obtain the biological activity, the truncated ApciNPV chitinase gene lacking the C-terminal ER-retention sequence (HTEL) was over-expressed in *E. coli*. The recombinant ApciNPV chitinase stored as a large amount of soluble cytosolic components (Figure 3a). The protein was efficiently purified in its native form and further identified by SDS-PAGE (Figure 3b). Furthermore, the ApciNPV chitinase was detected exo- and endo-chitinase activities with 4-MU-$(GlcNAc)_2$ and 4-MU-$(GlcNAc)_3$ substrates, respectively (Figure 4). It was in accord with previous reports stating that baculovirus chitinases have both exo- and endo-chitinases activities (Hawtin et al., 1995; Hawtin et al., 1997; Rao et al., 2004). Compared to chitinase from AcMNPV (Rao et al., 2004) and DekiNPV (Qu et al., 2011; Wang et al., 2013), the ApciNPV chitinase were higher exo- and endo-chitinase activities than the DekiNPV chitinase, yet lower exo-chitinase activity than the AcMNPV chitinase. In addition, the chitinase of ApciNPV showed an obvious insecticidal activity agianst *A. cinerarius*, *S. exigua*, *H. cunea*, *H. armigera* and *L. dispar* (Table 1). Previous studies have also indicated the role of baculovirus chitinase in pest control. For example, ChiA of AcMNPV at a sub-lethal doses (0.56 µg/g of larval body weight, LW) decreased larval body weight of *B. mori* and caused 100% mortality at a dose of 1 µg/g LW after 24 h (Rao et al., 2004). ChiA from BmNPV and AcMNPV play an important role in damaging the chitinous PM in the *B. mori* larval midgut (Rao et al., 2004; Wang et al., 2005).

The overall results indicate a chitinase from ApciNPV chitinase may offer valuable opportunities to kill insect pests under lab condition.

Conflict of Interests

The authors did not declare any conflict of interests.

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