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Expression of Apalbumin1 of *Apis cerana cerana* in the Larvae of Silkworm, *Bombyx mori*

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Royal jelly (RJ) is a thick, milky material produced by both the hypopharyngeal and the mandibular glands of nurse honeybees. The main proteins of RJ, named apalbumins or major royal jelly proteins (MRJPs), have multiple biological functions. Apalbumin1 is the most abundant glycoprotein of RJ. In this study, Bacmid-*apalbumin1* was constructed for *Apis cerana cerana* using the newly established Bac-to-Bac/BmNPV baculovirus expression system (BES). This procedure allowed us to obtain the recombinant *A. cerana cerana* (*Acc*) apalbumin1 (r*Acc*apalbumin1) from the hemolymph of silkworm larvae through the BmNPV bacmid system, 96 h postinfection. The r*Acc*apalbumin1 was then purified by Ni-NTA spin columns and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis and Western blotting. A 55 kDa protein with good solubility was then obtained. The peptide Ile-Phe was identified from trypsin production of r*Acc*apalbumin1. Such a peptide has been reported to have an antihypertensive ability. Our results have therefore potential applications in biomedical research and open new perspectives for the study of apalbumins.

KEYWORDS: *Apis cerana cerana*; royal jelly; apalbumin1; antihypertensive; Bac-to-Bac/BmNPV baculovirus expression system

INTRODUCTION

The honeybee *Apis mellifera* is an ideal insect model for studies on social behavior, which has long attracted scientists willing to understand this topic at the behavioral, cellular, and molecular levels (*1*). *Apis cerana cerana* is another honeybee species that is common in China, which exhibits great viability, high olfactory sensitivity, and high resistance to environmental stress and varroa mite. *A. cerana cerana* bees are efficient foragers in places where nectar and pollen sources are sporadic and are well-adapted to the Chinese climate. Because of these factors, *A. cerana cerana* has an important economic value in China (*2, 3*).

Royal jelly (RJ) is a thick, milky material with multiple bioactivities, which is secreted by both hypopharyngeal and mandibular glands of nurse honeybees (*3*). In a colony of bees, the queen is fed RJ throughout its whole life, while all of the other larvae are fed RJ only for the first 3 days after hatching. The crude proteins of RJ consist of water-soluble and water-insoluble proteins. The former make up 46−89% of the total proteins of RJ and are named major royal jelly proteins (MRJPs) or apalbumins because their physical and chemical properties are similar to common albumins. Due to their beneficial effects on human health, MJRPs are of potential interest for medical research (*3*). In recent years, with the development of gene engineering, molecular biology, and proteomics, among others, research on apalbumins has acquired a new dimension. The isolation and purification of single RJ proteins (sRJPs) or their hydrolysis or expression in vitro have allowed scientists to ascribe several functions to RJ proteins, such as the immunomodulatory activity of MRJP3 (*4*), the antibacterial activity of Jelleine-I-IV (*5*) and Royalisin (*6*), the cell growth stimulation activity (*7*−*9*), and the antihypertensive ability of peptides hydrolyzed from RJ (*10, 11*). MRJP1 has a molecular mass of 56−57 kDa and is the most abundant glycoprotein of RJ (*12*−*14*). It stimulates the growth of human lymphocytes in a serum-free medium (*15*), enhances cell proliferation of rat hepatocytes (*16*), exerts an antitumor (*17, 18*) and antifatigue (*19*) effect in mice, and modulates the social behavior of honeybees (*20*). These elements, together with the development of protein isolation, gene cloning and expression, and functional researches, make RJ proteins interesting targets for studies in the fields of biological medicine, cell culture, and tissue engineering.

As compared with *A. mellifera*, researches on apalbumins of *A. cerana cerana* are scarce. Pothichot et al. found that queen rearing in *A. mellifera* is unsuccessful if the queen is provided with RJ from *A. cerana* and vice versa. This result suggests that the RJ of these two species has different compositions (*21*). Here, we focused on the *apalbumin1* of *A. cerana cerana* (*Accapalbumin1*). To characterize it, we cloned it and constructed the recombinant bacmid DNA Bacmid-*apalbumin1*. The Bac-to-Bac Baculovirus expression system (BES) for nuclear

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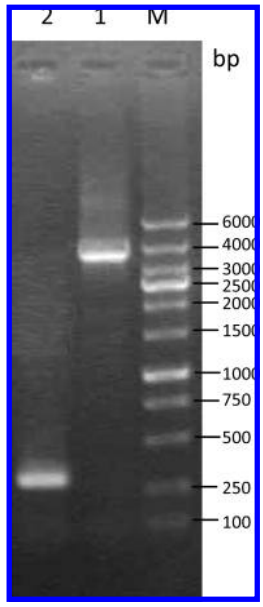

Figure 1. Identification of recombinant Bacmid DNA (lane 1) and Bacmid (lane 2) by PCR. M, DNA molecular weight marker.

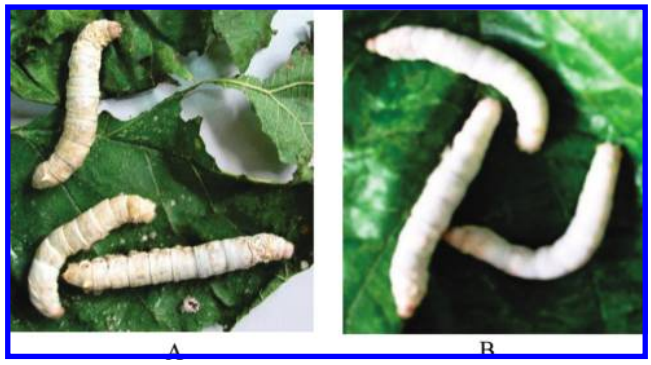

Figure 2. Phenotype of silkworm larvae after infection of recombinant virus. (**A**) The silkworm larvae at the first day of the fifth instar were inoculated with the recombinant bacmid baculoviruses by subcutaneous injection, 5 μL each at about 2 $\times$ 10^5 particles. The worms developed obviously ivory-white bodies and had decreased appetites 96 h post-transfection. (**B**) Normal worms injected with ddH_2O as control.

polyhedrosis virus (AcNPV) was developed by Luckow et al. (*22*). This procedure provides a well-established expression system with high expression efficiency, natural activity for the expressed products, large scale production of heterologous proteins, and safety for both human and livestock. We used a *Bombyx mori* nuclear polyhedrosis virus (BmNPV) bacmid (a baculovirus shuttle vector) including a BmNPV bacmid and its *Escherichia coli* DH10Bac/BmNPV (*23, 24*). This system has a great potential for solving the problems of weak expression of apalbumin1 in *E. coli* (*25*). and transgenic tobacco (*26*). This procedure allowed us to successfully obtain the *A. cerana cerana* apalbumin1 (r*Acc*apalbumin1) from the hemolymph of silkworm larvae through the BmNPV bacmid system 96 h postinfection. Moreover, we determined the peptides from trypsin production of r*Acc*apalbumin1 and found the peptide Ile-Phe, which has been reported to have antihypertensive ability (*10*).

MATERIALS AND METHODS

Materials. The *Accapalbumin1* cDNA template from heads of 8 day old honeybee workers was preserved in our laboratory at 4 °C until needed (*27*). pFastBacHTb was obtained from Invitrogen (San Diego, CA) and FUGENE 6 transfection reagent from Roche Applied

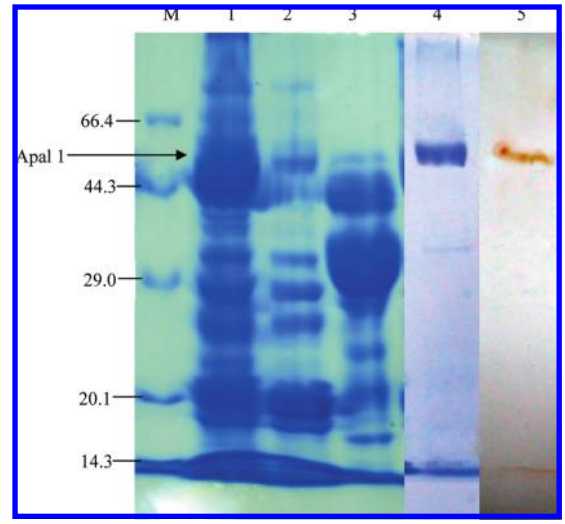

Figure 3. SDS-PAGE result of the expressed product of r*Acc*apalbumin1 and Western blotting analysis using antihis tag antibody. M, protein molecular weight marker (low): BSA (66.4 kDa), ovabumin (44.3 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa). Lane 1, the supernate of the hemolymph collected 96 h post-transfection; lane 2, control; lane 3, the water soluble substance of RJ of honeybee, *A. cerana cerana*; lane 4, purified r*Acc*apalbumin1 from the supernatant of hemolymph; and lane 5, Western blotting of r*Acc*apalbumin1. Gel was stained with Coomassie Brilliant Blue R-250. The arrow shows the position of r*Acc*apalbumin1.

Science (United States). A Ni-NTA Spin Column and YM-10 Ultracel Amicon Ultrafiltration Disc were obtained from Qiagen (Japan) and Millipore (United States), respectively. The DH10Bac/BmNPV *E. coli* was supplied by Prof. Y. Miao (*24*).

Cell Line and Silkworm. *B. mori* cell line, BmN, originating from the ovary of silkworms, was provided by Prof. Y. Miao (*28*). It was cultured at 27 °C in a HyQ SFX-INSECT insect cell culture medium (HyClone, United States) with 10% fetal bovine serum (FBS). The silkworm strain P50 (Dazhao) supplied by Prof. Y. Miao (*28*) was reared on mulberry leaves at 26 $\pm$ 2 °C.

Cloning of *Accapalbumin1* Gene. A pair of primers termed FP and RP were designed as follows according to the sequence of *Accapalbumin1* cDNA. FP: 5′-CCATGGACATGACAAGGTGGTTGTT-3′; RP: 5′-GCGGCCGCTTACAGATGTATTGAAAT-3′. *Nco* I and *Not* I recognition sites were introduced into the 5′-terminal of FP (underlined) and RP (underlined), respectively, to facilitate cloning. The polymerase chain reaction (PCR) was done under the following conditions: preheating at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54.6 °C for 1 min, and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 10 min. The PCR products were analyzed by agarose gel electrophoresis and recovered using a TaKaRa agarose gel DNA purification kit (Japan). The fragments were then ligated into the TaKaRa pMD18-T TA cloning vector and sequenced using the dideoxy-chain termination method with ABI 3730 DNA Analyzer. We named the plasmid obtained pMD18-T-*Accapalbumin1*.

Construction and Proliferation of Recombinant Bacmid Baculoviruses. pMD18-T-*Accapalbumin1* was digested by *Nco* I and *Not* I. The resulting *Accapalbumin1* fragments were cloned into compatible sites of pFastBacHTb and transformed into *E. coli* DH10Bac/BmNPV. Recombinant bacmid-containing target gene *Accapalbumin1* was generated by transposing a mini-Tn7 element from the donor plasmid to the mini-*att*Tn7 attachment site of the bacmid. The transformed *E. coli* DH10Bac/BmNPV was plated on the LB agar plate containing 50 μg/mL kanamycin, 10 μg/mL tetracycline, 7 μg/mL gentamicin, 100 μg/mL X-gal, and 40 μg/mL IPTG to select positive clones containing recombinant bacmid (http://tools.invitrogen.com/content/sfs/manuals/baculogatewayman.pdf). The white colonies (Lac$^-$) were selected by PCR analysis using the M13 forward and M13 reverse primers.

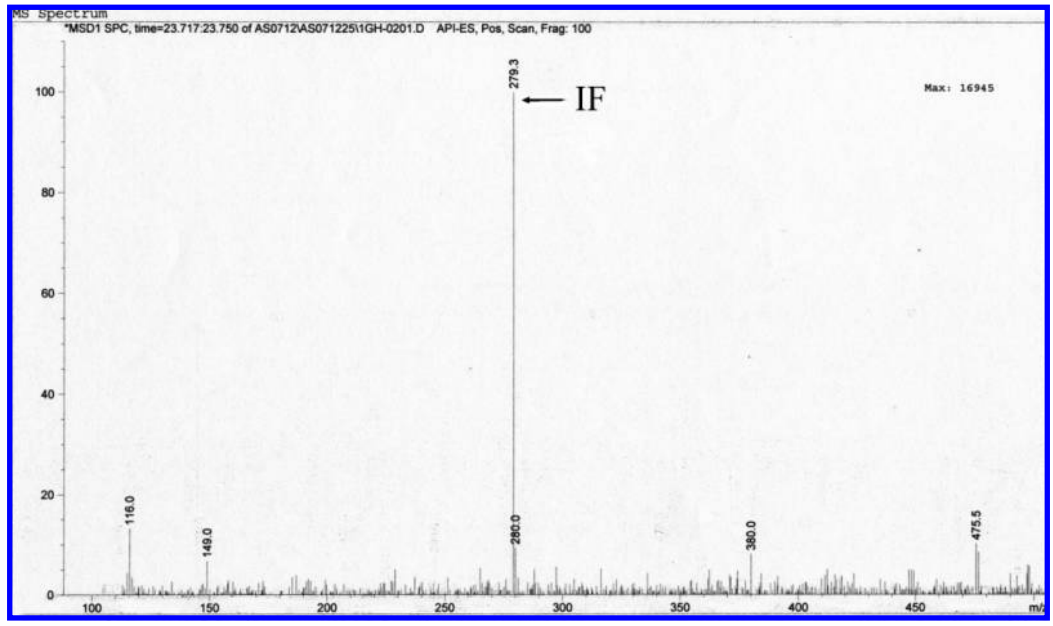

Figure 4. LC-MS analysis of peptides from the trypsin productions of r*Acc*apalbumin1. The arrow shows the ACE inhibitory peptide IF (MW = 278 kDa). Use of the purified r*Acc*apalbumin1 as a control.

The recombinant bacmid baculoviruses were then purified to transfect BmN cells using the transfection reagent FUGENE 6 under the ratio 3:2 (6 μL of FUGENE 6 and 4 μg of bacmid DNA) based on Roche handbook (https://www.roche-applied-science.com/pack-insert/1815091a.pdf).

The P1 viral stock was gathered from the cell medium containing virus 120 h post-transfection. We amplified the viral stock through further infection of BmN cells using the P1 stock until the P3 stock was obtained. The P3 stock was stored at 4 °C and preserved from light.

Expression of *Accapalbumin1* Gene in Silkworm Larvae and Its Purification. P3 stock (ddH_2O as control) was introduced into the silkworm larvae of the fifth instar by means of subcutaneous injections, delivering 5 μL of P3 stock at about 2 × 10^5 particles per larva (*28*). The hemolymph was collected 96 h postinfection and centrifuged for 5 min at 8000 rpm and 4 °C. The supernatant was preserved at −70 °C for further use.

The Ni-NTA Spin Column was used to purify the 6 × His-tagged r*Acc*apalbumin under native conditions as described in Qiagen manual (http://www1.qiagen.com/HB/NiNTASpinColumns_EN). The supernatant of hemolymph was first thawed and mixed with lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole; pH 8.0) and centrifuged for 2 min at 12000 rpm and 4 °C. We loaded the clear lysate into the Ni-NTA Spin Column and centrifuged it for 2 min at 8000 rpm and 4 °C. The r*Acc*apalbumin1 with a cluster of six histide residues bound to the column was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole; pH 8.0) and finally collected with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole; pH 8.0).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis. Both the hemolymph and the purified r*Acc*apalbumin1 were subjected to 10% SDS-PAGE after boiling with the same volume of loading buffer (100 mM Tris·Cl, pH 6.8; 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) for 5 min. The gel was stained with Coomassie Brilliant Blue R-250. The proteins were electrophoretically transferred to a PVDF membrane after SDS-PAGE. The membrane was first blocked in PBST (137 mM NaCl, 2.68 mM KCl, 8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4, and 0.1% Tween 20) plus 2% nonfat milk for 2 h at 25 °C and washed three times with PBST. The film was then incubated with PBST containing 2% nonfat milk and diluted mouse anti-His antibody (1:300, Invitrogen) for 2 h at 25 °C and subsequently washed three times with PBST, for 10 min each time. After incubation for 1 h at 25 °C in the same PBST with peroxidase-labeled antimouse IgG (1:500, Invitrogen) and washing with PBS (137 mM NaCl, 2.68 mM KCl, 8.06 mM Na_2HPO_4, and 1.47 mM KH_2PO_4), the immunoactive protein was detected by adding DAB chromogenic substrate (2 mg DAB, 20 μL of 30% H_2O_2, and 10 mL of PBS).

Trypsin Digestion of r*Acc*apalbumin1 and LC-MS Analysis. The purified r*Acc*apalbumin1 (10 mg) in elution buffer was treated with trypsin (100 μg, Amresco, United States) in a water bath at 37 °C for 6 h, followed by 100 °C for 5 min to inactivate the trypsin. After centrifugation at 13000 rpm for 30 min through YM-10 Ultrafiltration Disc, the peptide fragments (MW < 10 kDa) were separated in elution buffer in which the phosphate was removed by adding $CaCl_2$. After centrifugation at 12000 rpm for 1 min, the supernatants were collected and subjected to liquid chromatographic mass spectrum (LC-MS) analysis. Liquid chromatographic separations were carried using a DIKMA c-18 column (4.6 mm × 250 mm, 5 μm) at a flow rate of 0.3 mL/min at 35 °C. The injection volume was 8 μL. The mobile phase A consisted of formic acid−water (0.1%, v/v), while the mobile phase B consisted of acetonitrile only. The gradient varied from 15 to 40% B (0−60 min) and from 40 to 80% B (60−70 min). The elution was detected at 220 nm with a UV-diode array detection detector. Ions were generated using electrospray ionization (ESI) and detected in the positive-ion mode. We repeated the experiments using the purified r*Acc*apalbumin1 as control.

RESULTS

Cloning of the *Accapalbumin1* Gene. A 1302 bp long *Accapalbumin1* gene fragment was synthesized by PCR and cloned into a pMD18-T TA vector. The results of sequencing (data not shown) were consistent with those that we have deposited in GenBank (accession number: AY279539).

Construction and Proliferation of Recombinant Bacmid Baculoviruses. The pFastBacHTb containing the target gene *Accapalbumin1* was transformed into *E. coli* DH10Bac/BmNPV in which the transposition may occur. The white colonies (Lac^-, about 8−10% of all of the colonies) were selected from the plate containing 50 μg/mL kanamycin, 10 μg/mL tetracycline, 7 μg/mL gentamicin, 100 μg/mL X-gal, and 40 μg/mL IPTG. The large recombinant plasmids Bacmid/BmNPV-*Accapalbumin1* were isolated from the white colonies for analysis. PCR was done using the M13 forward and M13 reverse as primers and recombinant Bacmid DNA as template, which is too large to perform a restriction analysis. We found a ∼3700 bp band

(**Figure 1**), which was consistent with the prediction size of PCR product (2430 bp plus the insertion gene 1302 bp), thus indicating the successful generation of Bacmid/BmNPV-*Accapalbumin1*.

The purified recombinant baculoviruses were then used to transfect the BmN cells of *B. mori* by liposomes. The transfected cells typically increased their cell diameter and nuclei, stopped growing, and exhibited lysis and mass 120 h post-transfection. After three transfection cycles, a high viral titer of P3 viral stock was collected and protected from light at 4 °C.

Expression of *Accapalbumin1* Gene in Silkworm Larvae. The P3 stock (ddH_2O as control) was used to inoculate silkworm larvae through subcutaneous injection. No significant symptoms were observed during the first 3 days after infection. The infected silkworm showed obviously ivory-white bodies and decreased appetites 96 h post-transfection (**Figure 2**); 120 h later, the body began to expand, and a turbid hemolymph leaked. Finally, the silkworm developed acute lethality, and the body turned black. The hemolymph was harvested 96 h post-transfection and centrifuged for 5 min at 8000 rpm and 4 °C. The supernatant and the r*Acc*apalbumin1 were purified from the supernatant using a Ni-NTA Spin Column and were examined using 10% SDS-PAGE and Western blotting. A band around 55 kDa was visualized and positively identified using Western blot analysis (**Figure 3**). This band indicated the successful expression of r*Acc*apalbumin1 in the silkworm.

LC-MS Analysis of r*Acc*apalbumin1 Fragments Digested by Trypsin. The r*Acc*apalbumin1 fragments (r*Acc*apalbumin1 as control) digested by trypsin were subjected to LC-MS analysis. Several di- and tripeptides were identified. Such an analysis revealed that the fraction Ile-Phe (IF, MW = 278 kDa, **Figure 4**) was the same as the dipeptide found in the gastrointestinal enzyme production of intact RJ by Matsui et al. (*10*). Ile-Phe can retard the action of angiotensin I-converting enzyme (ACE) and contributes to antihypertensive ability (*10*). This result suggests that the trypsin production from r*Acc*apalbumin1 is one of the sources of ACE inhibitory peptides.

DISCUSSION

The completion of the genome sequencing of the honey bee, *A. mellifera* (*1*), has intensified molecular studies in this insect. *A. cerana cerana*, the Asian honey bee, has irreplaceable application value and resource superiority in China. However, few molecular researches have been so far undertaken in this insect. We performed a molecular study whose goal was to clone the *apalbumin1* gene of *A. cerana cerana*, express it in vitro, and learn more about its function. We expressed r*Acc*apalbumin1 in the larvae of the silkworm *B. mori* using the BmNPV bacmid system, and we purified it using Ni-NTA spin column. Given that silkworm rearing is easy and economic, our method constitutes a promising tool for industrial large-scale production of r*Acc*apalbumin1 and other heterologous proteins.

Up to now, several functions of RJ have been characterized such as cell growth stimulation (*7*−*9, 15, 16*), potential modulatory activity of honeybee social behavior (*20*), and antibacterial (*5, 6*), antitumor (*17, 18*), antifatigue (*19*), antihypertensive (*10, 11*), and immunomodulatory activities (*4*). Unfortunately, the function of sRJP is poorly understood because of their complex and easily degradable composition. Isolation technologies have been the bottleneck for developing further studies on sRJP, while gene engineering has been used for easy generation of sRJP. We obtained the purified r*Acc*apalbumin1 and confirmed the presence of antihypertensive peptides from the trypsin productions of r*Acc*apalbumin1. According to the predicted amino acid sequence of r*Acc*apalbumin1, the ACE inhibitory peptides Ile-Phe (IF), Lys-Phe (KF), Ile-Val-Tyr (IVY), Val-Tyr (VY), and Leu-Thr-Phe (LTF) (*10, 11*) were found in the structure of r*Acc*apalbumin1 [(77−78, 369−370), (39−40, 237−238), (215−217), (204−205, 216−217), and (156−158), respectively]. Our trypsin digestion generates IF only, which may be due to our experimental conditions. Multiple enzymes may be needed to produce various antihypertensive peptides.

Apalbumin1 contains high amounts (48%) of essential amino acids such as Leu (9.5%), Ile (6.0%), and Arg (3.4%), among others (*12*). The codons for Arg are AGG, AGA, and CGA, which are scarcely used in conventional *E. coli* (*18*). Inefficient expression levels and insoluble forms of rApa1 (MRJP1) were detected in both *E. coli* (*25*) and transgenic tobacco (*26*), while higher efficiency expression level of soluble rApa1 and its recombinant fragments were observed with pET28b(+) system in modified *E. coli* BL21-CodonPlus(DE3)-RIL with extra copies of *E. coli* argU, ileY, and leuW tRNA genes (*18*). The Bac-to-Bac/BmNPV BES, as an eukaryotic expression system, has advantages such as glycosylation, phosphorylation, and post-translational modification, which make the expressed product similar to their natural types (*29*). The SDS-PAGE, Western blotting analysis, and purification indicated that the purified r*Acc*apalbumin1 was around 55 kDa. Most of the r*Acc*apalbumin1 was found in the supernatant of the hemolymph. The yield of r*Acc*apalbumin1 was estimated at 486 μg per larva, which constitutes a much higher yield than that obtained in *E. coli*. Further work will be required to clarify the function of the r*Acc*apalbumin1.

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