Purification and characterization of a 1,3-β-D-glucan recognition protein from *Antheraea pernyi* larve that is regulated after a specific immune challenge

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Pattern recognition receptors are known to participate in the activation of Prophenoloxidase system. In this study, a 1,3-β-D-glucan recognition protein was detected for the first time in *Antheraea pernyi* larvae (*Ap-βGRP*). *Ap-βGRP* was purified to 99.9% homogeneity from the hemolymph using traditional chromatographic methods. *Ap-βGRP* specifically binds 1,3-β-D-glucan and yeast, but not *E. coli* or *M. luteus*. The 1,3-β-D-glucan dependent phenoloxidase (PO) activity of the hemolymph inhibited by anti-*Ap-βGRP* antibody could be recovered by addition of purified *Ap-βGRP*. These results demonstrate that *Ap-βGRP* acts as a biosensor of 1,3-β-D-glucan to trigger the Prophenoloxidase system. A trace mount of 1,3-β-D-glucan or *Ap-βGRP* alone was unable to trigger the proPO system, but they both did. *Ap-βGRP* was specifically degraded following the activation of proPO with 1,3-β-D-glucan. These results indicate the variation in the amount of *Ap-βGRP* after specific immune challenge in *A. pernyi* hemolymph is an important regulation mechanism to immune response. [BMB Reports 2013; 46(5): 264-269]

INTRODUCTION

Infection by a variety of pathogens, such as Gram-positive and Gram-negative bacteria, fungi, and parasites, evokes a host-defense system termed innate immunity (1, 2). The innate immune response requires an initial recognition event that signals the host organism of an impending threat. Pattern recognition receptors (PRRs) function to initiate the host immune response upon binding to non-self pathogen-associated molecular patterns (PAMPs). PAMPs are the conserved surface determinants of microorganisms, such as lipopolysaccharide, lipoteichoic acid and peptidoglycan from bacteria and 1,3-β-D-glucan from fungi. PRRs characterized in insects include C-type lectins, peptidoglycan recognition proteins (PGRs), 1,3-β-D-glucan recognition proteins (βGRPs), Gram-negative bacteria-binding proteins (GNBPs) (3-5).

βGRPs have high specific affinity for 1,3-β-D-glucan, and serve as a biosensor against fungi. To date, βGRPs have been found and identified in several kinds of arthropods, such as the silkworm, *Bombyx mori* (6), the tobacco hornworm, *Manduca sexta* (7), the crayfish, *Pacificaotus leniusculus* (8), the mealworm, *Tenebrio molitor* (9), and the moth, *Plodia interpunctella* (10). All identified βGRPs contained a conserved C-terminal region with high sequence similarity to the catalytic regions of glycosyl hydrolase found in bacteria, but lack glucanases activity due to amino acid substitutions in key residues of the catalytic sites (11, 12). Further studies have demonstrated that the unique N-terminal domain of βGRPs was responsible for the high affinity for 1,3-β-D-glucan and stimulation of the prophenoloxidase system (13).

The interaction between 1,3-β-D-glucan and βGRP will induce activation of several serine proteases within the proPO system, subsequently producing quinones and other reactive compounds for melanin formation, protein crosslinking, and microbe killing (14, 15).

Although the mechanism of proPO system has been determined, the precise contribution of βGRPs interaction with 1,3-β-D-glucan for proPO activation remains to be fully elucidated. Here, we describe the purification of a new 1,3-β-D-glucan recognition protein in the Chinese oak silkmoth, *A. pernyi*, which belongs to Saturniidae, Bombycoidea and is one of the main silk producing species. Our research aimed to investigate the function of *Ap-βGRP* in the proPO system. Moreover, the variation in *Ap-βGRP* at a protein level after immune challenge was studied along with its biological function.

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RESULTS

Purification and preliminary characterization of native Ap-βGRP

As shown in Fig. 1A, A. pernyi hemolymph exhibited a rapid increase in PO activity in the presence of 1,3-β-D-glucan. This result suggests that A. pernyi hemolymph contains all factors involved in the 1,3-β-D-glucan-dependent proPO system, such as proPO-activating enzymes, proPO(s), and 1,3-β-D-glucan recognition protein(s).

To identify proteins that can bind to 1,3-β-D-glucan, curdlan (an insoluble 1,3-β-D-glucan preparation) was used as an affinity matrix to purify proteins from A. pernyi hemolymph. Sample was analyzed by SDS-PAGE, a major protein with a molecular mass of approximately 72 kDa (Fig. 1B, lane 2) was found to be enriched compared with the hemolymph (Fig. 1B, lane 1). The 72-kDa protein accounted for more than 80% of the total protein eluted from curdlan, and was further purified to homogeneity by electrophoresis (Fig. 1B, lane 3). The purified denatured 72-kDa was injected into a rabbit to raise antiseraum. The antiseraum recognized the single protein band of 72-kDa in western blotting analysis of A. pernyi hemolymph (Fig. 1B, lane 4).

The native target protein was purified from A. pernyi hemolymph by traditional chromatographic methods using western blotting as tracking strategy. Purified protein (serial diluted) migrated as a single band with an apparent molecular mass of 72 kDa in SDS-PAGE (Fig. 1C), emerged as a single prominent peak (Fig. 1D) using reverse phase HPLC with a C18 column. These results suggest that 72 kDa protein exists as a monomer in solution. Partial amino acid sequences of this protein were determined by mass spectrometry and the results are as follow: LEAIYPK, VSIPDDGFSLFAFHGK, LNEEMEGLEAGHWSR, IYFWTVYIK, VCAEGSVFSEEDK, DMPDWTAEIK, QASGAQLPPVLSAK, YESGLMR, GNAVFAK, LGYPGLSDTEPF, IGGINWLNK, VGGVNDADGTDPKWR, AMLSWNDR, WLPTWYDANLK. These amino acid sequences were found to be identical to the sequences determined by mass spectrometry.

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to the reported sequences of *A. pernyi* βGRP (GenBank: AFC35297.1) using NCBI data. This result suggests that the purified 72-kDa protein is likely to be a *A. pernyi* βGRP, so it was termed Ap-βGRP.

The internal amino acid sequence of Ap-βGRP purified as described above was identical to that obtained from the protein eluted from curdlan (data not shown). A database search with the Blast algorithm revealed that Ap-βGRP (AFC35297.1) displayed the closest match to *B. mori* βGRP3 precursor (16), *M. sexta* βGRP2 (17) and *P. rapae* βGRP (18), and the highest amino acid sequence similarity (51%) to *B. mori* βGRP3 precursor (NP_001128672.1) (Fig. 2). The calculated molecular mass of the 490-residue mature protein was 54871 Da, which is 17129 Da less than the mass determined by SDS-PAGE.

**Activation of the proPO system**

As shown in Fig. 3A, when anti-Ap βGRP antibody was incubated with hemolymph, PO activity increased by 1,3-β-D-glucan was significantly repressed. However, this inhibition was not observed in PGN-dependent proPO activation. Also, 1,3-β-D-glucan dependent PO activity of hemolymph inhibited by anti-Ap βGRP antibody could be recovered by the addition of purified Ap βGRP. These results indicated that Ap βGRP directly involved in the proPO system and played a role as an initiator of the proPO activation of the *A. pernyi*.

We tested the effects of purified Ap βGRP on proPO activation of hemolymph. Ap βGRP was serially diluted and added to hemolymph with or without 1,3-β-D-glucan. Under these conditions, 1,3-β-D-glucan (10 to 100 mM) interacted with endogenous Ap βGRP in hemolymph causing a concentration-dependent increase in PO activity, but no further growth with an excess of 1,3-β-D-glucan (Fig. 3B). Exogenous Ap βGRP (1-10 ng) alone did not cause any substantial enhancement of proPO activation (Fig. 3C). When purified Ap βGRP and a small amount of 1,3-β-D-glucan (10 mM) were added in hemolymph, significant enhancement of PO activity was detected as more exogenous Ap βGRP added (Fig. 3C). When the proPO was already activated by more 1,3-β-D-glucan (50, 100 mM), the concentration increase of Ap βGRP in the system caused a instant enhancement of PO activity that reached a maximum and then decreased until it reached a stable level of about 3.75 U.

Similar results were also observed with higher concentrations of 1,3-β-D-glucan (400, 600 mM, data not shown).

We further monitored Ap βGRP at different times during proPO activation with an antibody against Ap βGRP. As shown in Fig. 4A, Ap βGRP in hemolymph gradually decreased, when proPO was already activated by 1,3-β-D-glucan. At the same time, as exogenous Ap βGRP was added to hemolymph, a relatively slow degradation of Ap βGRP was observed compared with that occurring in the presence of hemolymph alone.

**Binding specificity of the Ap βGRP**

Western blotting analysis showed that (Fig. 4B) Ap βGRP bound to curdlan, and yeast specifically and strongly but not *E. coli* or *M. luteus*. 1,3-β-D-glucan appeared to be responsible bind to curdlan, and yeast specifically and strongly but not *E. coli* or *M. luteus*.

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for the specific binding between Ap-βGRP and yeast.

Specific immune challenge
To determine the quantitative change of endogenous Ap-βGRP at a protein level after an immune challenge with 1,3-β-D-glucan, Western blot analysis was performed using anti-Ap-βGRP antibody by loading the same amount of hemolymph after different challenge times. Low levels of Ap-βGRP protein were detected in hemolymph samples from larvae injected with anti-coagulation buffer and Ap-βGRP concentration dramatically increased 12 h after the injection of 1,3-β-D-glucan (Fig. 4C). This increased generation of Ap-βGRP in hemolymph appears to be in response to a specific immune challenge of 1,3-β-D-glucan. At the same time, Ap-βGRP significantly decreased 24h after induction, which appeared to be due to degradation of Ap-βGRP (Fig. 4A).

DISCUSSION
It is well known that pathogenic microbial infections in insects and other invertebrates trigger the activation of the proPO system (7, 8, 10), and several pattern recognition proteins are constitutively expressed and then increased dramatically in response to a specific immune challenge (19-21). A reasonable explanation for this increased generation of pattern recognition proteins caused by immune challenge is to maintain a high level of pattern recognition protein for rapid pathogen recognition in the host hemolymph and act as a protection against future reinfection or to control latent infections (20). In this study, we found that Ap-βGRP is present in the hemolymph of A. pernyi and dramatically increased 12 h at protein level after the injection of 1,3-β-D-glucan for 12 h. Small quantities of Ap-βGRP were safely stored in hemolymph (Fig. 4C, control), which could trigger the proPO system in the presence of 1,3-β-D-glucan (Fig. 1A), and the strong upregulation of Ap-βGRP after immune challenge is proposed to be a mechanism of regulating the proPO system. To test if increasing the amount of Ap-βGRP had an effect on the proPO system, we incubated hemolymph with 1,3-β-D-glucan in the absence or presence of purified Ap-βGRP. Interestingly, it was found that a trace amount of 1,3-β-D-glucan alone failed to trigger the proPO system, while a small amount of exogenous Ap-βGRP rapidly led to a synergistic enhancement of PO activity (Fig. 3D). Thus, our research further supported the belief that an increased concentration of Ap-βGRP in hemolymph after immune challenge produces rapid and sensitive pathogen recognition.

However, a continuous activation of the proPO system leads to oxidative stress and immunopathological effects (21-23) that may eventually reduce the lifespan (24), might be well regulated by pattern recognition receptors or proPO activating factors (9, 25, 26). Not surprisingly, the protein level of Ap-βGRP dramatically increased in response to immune challenge and then finally fell to a low level at 24 h (Fig. 4C). Zhang et al. previously reported degradation of βGRP by the PO activity in Tenebrio molitor (9), and we make the similar observation with Ap-βGRP (Fig. 4A). It appeared that removal of Ap-βGRP from the organism is an important regulatory mechanism to prevent oxidative stress. We also found that enhancement of PO activity finally reached to a stable level (3.75 U) as excessive exogenous Ap-βGRP was added to the hemolymph in the presence of 1,3-β-D-glucan. We propose that the large amount of Ap-βGRP present in hemolymph caused excessive activation of the proPO system and then accompanied by negative regulation of the proPO system.

In conclusion, A native Ap-βGRP was purified from A. pernyi to homogeneity. The phenoloxidase activity of the hemolymph with endogenous Ap-βGRP blocked by anti-Ap-βGRP antibody could be recovered by addition of purified Ap-βGRP. A trace mount of 1,3-β-D-glucan or Ap-βGRP alone was unable to trigger the proPO system, but they both did. These results demonstrate that Ap-βGRP is a component of the A. pernyi proPO system and acts as a biosensor of 1,3-β-D-glucan to trigger the proPO system. Exogenous Ap-βGRP involvement could cause a significant enhancement of PO activity in the presence of 1,3-β-D-glucan. Ap-βGRP was specifically degraded by the activation of proPO system. These results indicating that the protein level of Ap-βGRP variation in A. pernyi hemolymph after a specific immune challenge is to recognize the pathogen rapidly and sensitively and prevent damage caused by excessive melanization. However, the molecular mechanism of Ap-βGRP and how it interacts with other components of the proPO system and how it triggers and regulates the proPO activation are interesting questions that should be addressed in the future to give us a better understanding of the immune system of this insect.

MATERIALS AND METHODS

Hemolymph collection
A. pernyi larvae were purchased from Shenyang Agricultural University. On day 3, 5th instar larvae were surface sterilized in 95% ethanol, placed on ice, and hemolymph was collected by cutting the third proleg with sterile scissors, and transferring it to a test-tube containing anti-coagulation buffer (30 mM trisodium citrate, 26 mM citric, 20 mM EDTA, and 15 mM sodium chloride, pH 5.0, buffer A) on ice. The collected hemolymph was centrifuged at 12,000 g for 15 min at 4°C and the supernatant was stored at −80°C.

In the experiment involving immune challenge of the insect, larvae were injected with 10 μl anti-coagulation buffer containing 0.1 μg 1,3-β-D-glucan and the hemolymph was collected from the challenged larvae 6, 12 and 24 h later.

Purification of the native Ap-βGRP
Curdlan was used as an affinity matrix to purify Ap-βGRP from A. pernyi hemolymph. The method was according to Ochiai and Ashida (6), and Fabrick et al. (10). The sample was analyzed by SDS-PAGE under reducing conditions, and the major
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72-kDa band was cut from the gel and extracted by electroelution.

An antibody against 72-kDa protein was raised and purified according to McCauley and Racker (27) and Cho et al. (28). The method as described in the technical booklet for the ECL Plus Western Blotting system (Amersham, UK) is used for Western Blotting.

280 ml hemolymph was fractionated by ammonium sulfate precipitation at 4°C. The proteins of the 30-50% saturated ammonium sulfate fraction were dissolved in buffer C (50 mM citrate buffer, 5 mM EDTA, pH 5.0) and dialyzed against the same buffer overnight at 4°C. Then the sample was applied to a SP sepharose Fast Flow ion exchange column (Amersham Pharmacia Biotech, 3×10 cm). The column was eluted at 2 ml/min with a gradient of 0 to 1 M NaCl in buffer C. Fractions containing Ap-βGRP were dialyzed against buffer D (20 mM Tris-HCl buffer, pH 9.0). The dialyzed solution was loaded onto a Q sepharose Fast Flow column (Amersham Pharmacia Biotech, 3×10 cm) equilibrated in buffer D (20 mM Tris-HCl buffer, pH 9.0). The column was eluted with 200 ml 1 M NaCl in buffer D. Fractions containing Ap-βGRP were dialyzed against buffer E (20 mM sodium phosphate buffer, pH 7.6) and loaded onto a Hydroxylapatite FPLC column (5×50 mm, Bio-Rad) equilibrated in buffer E. The column was eluted with a 25 ml gradient from 0 to 0.5 M NaCl in buffer D. Finally, the column was eluted with 200 ml 0.5 M NaCl in buffer D. Fractions containing Ap-βGRP were dialyzed against buffer E (20 mM sodium phosphate buffer, pH 7.6) and loaded onto a Hydroxylapatite FPLC column (5×50 mm, Bio-Rad) equilibrated in buffer E. The column was eluted with a 25 ml gradient from 20 mM to 400 mM sodium phosphate in the same buffer. A major peak eluted at 240 mM sodium phosphate was used as the Ap-βGRP preparation for further study.

Internal amino acid sequence determination
The purified native Ap-βGRP (5 μg) was reduced, alkylated, and digested with Sequencing Grade Modified Trypsinase (Promega) at 37°C for 12 h. The digested peptides were separated by HPLC on a C18 reverse phase column, and the most prominent peaks were sequenced by ESI-TOF.

Electrophoresis and HPLC analysis
SDS-PAGE was carried out by the method of Laemmli (29). A reverse phase C18 column (0.46×25 cm) was used to check the homogeneity of Ap-βGRP. Protein was eluted with a linear gradient of 0-80% methanol in 10 mM sodium phosphate buffer, pH 5.0 at 0.8 ml/min for 30 min.

Binding of Ap-βGRP to microorganisms and curdlan
M. luteus (2×10^8 cells/ml), E. coli (1×10^8 cells/ml), and S. cerevisiae (2×10^8 cells/ml) were treated with 4% formaldehyde at 25°C for 2 h. Purified Ap-βGRP (5 μg) was mixed with the 200 μl formaldehyde-treated cells or curdlan at 4°C for 2 h. After centrifugation at 5,000 g for 15 min, the supernatant was analyzed as the unbound fraction. The pellet was eluted with 8 M urea, then 2% SDS. 2% SDS elution was analyzed as the bound fraction. As controls, the microbial cells or curdlan only underwent similar treatment. All the fractions were subjected to 10% SDS-PAGE and Western blotting analysis.

Activation of the proPO system by Ap-βGRP
10 μl A. pernyi hemolymph was incubated with 10 μl 1,3-β-D-glucan (serial diluted) or PGN and then 450 μl substrate solution (1 mM 4-methylcatechol, 2 mM 4-hydroxyproline ethyl-ester in 20 mM Tris-HCl buffer, pH 8.0) was added to the reaction mixture. The absorbance at 520 nm was monitored in the kinetic mode, and one unit of PO activity was equal to the amount of enzyme giving an increase of 0.1 absorbance units per min. For reconstitution of PO activity, 10 μl hemolymph was pre-incubated with 10 μl purified Ap-βGRP or 10 μl anti-Ap-βGRP antibody or both for 20 min at 4°C, then incubated with 1,3-β-D-glucan. The recovered PO activity was measured after addition of substrate by spectrophotometry at 520 nm. PGN was used as the control instead of 1,3-β-D-glucan.

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