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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 bind 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 (PGRPs), 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, *Pacifastacus leniusculus* (8), the mealworm, *Tenebrio molitor* (9), and the moth, *Plodia interpuncella* (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 electroelution (Fig. 1B, lane 3). The purified denatured 72-kDa was injected into a rabbit to raise antiserum. The antiserum 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* hemolumph 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, IYFWTYVIK, VCAGSLVFSEEFDK, DMPDWTAEIK, QASGAQILPPVLSAK, YESGLMR, GNAVFAK, LYGGPVLSDTEPFR, IGINNWNK, VGGVNDFADGTDKPWR, AMLSFWNDR, WLPTWYDANLK. These amino acid sequences were found to be identical

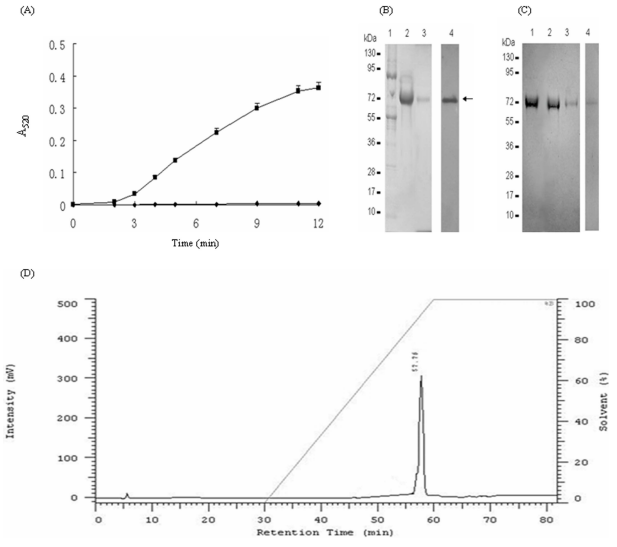

Fig. 1. Purification of *Ap*-βGRP. (A) PO activity in the hemolymph of *A. pernyi* larvae. 10 μl hemolymph was incubated with 100 mM 1,3-β-D-glucan (*closed squares*), control (*closed diamonds*). PO activity was measured as described in "Materials and Methods". (B) SDS-PAGE and Western blotting analysis of *Ap*-βGRP purified by affinity (curdlan) method. *Lanes 1 to 3* were stained with Coomassie Blue; *lane 4* was detected by Western blotting with antibody to *Ap*-βGRP. *Lane 1, 4*, Hemolymph; *lane 2*, *Ap*-βGRP eluted by sample buffer; *lane 3*, *Ap*-βGRP purified by the electroelution. (C) SDS-PAGE analysis of purified native *Ap*-βGRP, *lane 1 to 3* was under reducing conditions; *lane 4* was under nonreducing conditions. *Lane 1*, 30 μg; *lane 2, 3* μg; *lane 3, 4*, 0.3 μg. (D) The purified *Ap*-βGRP was analyzed using a C18 reversed phase column.

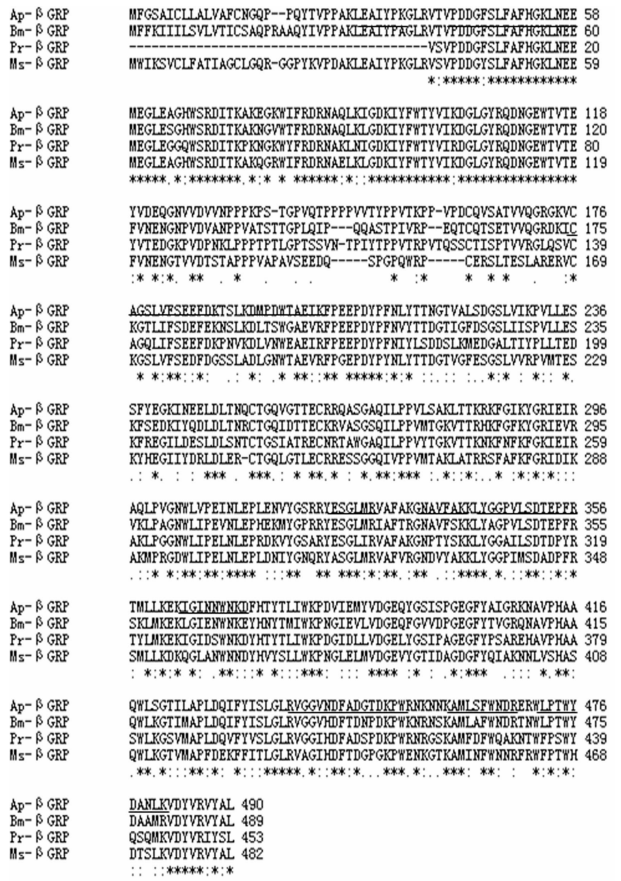

Fig. 2. Alignment of partial amino acid sequences of *Ap*-βGRP with those of other similar insect βGRPs. Multiple sequence alignment of amino acid sequences: *Ap*-βGRP, *A. pernyi* βGRP (AFC35297.1); *Bm*-βGRP, *B. mori* βGRP3 precursor (NP_001128672.1); *Pr*-βGRP, *P. rapae* βGRP2 (ACI32822.1); *Ms*-βGBP, *M. sexta* βGBP2 (Q8ISB6.1). The determined partial amino acid sequences are indicated with *under-lines*. The *asterisks* indicate that the residues are identical. The *dots* indicate that the amino acids have similar properties.

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

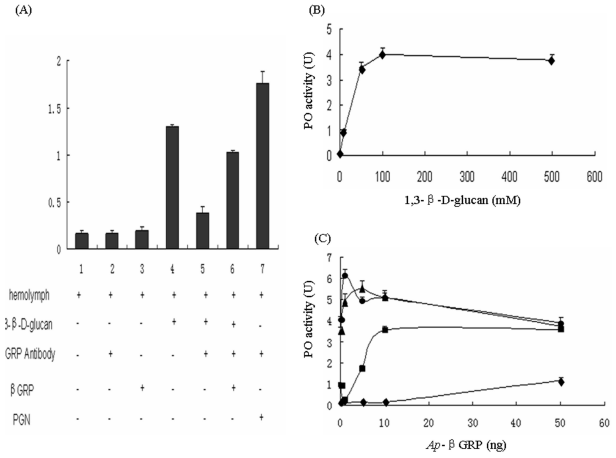

Fig. 3. Effects of *Ap*-βGRP on PO activity. (A) Involvement of *Ap*-βGRP in the proPO system. 50 μg *Ap*-βGRP antibody was preincubated with 10 μl hemolymph to block the endogenous *Ap*-βGRP. Purified *Ap*-βGRP (10 ng), 1,3-β-D-glucan (100 mM) or PGN was incubated with the hemolymph with or without *Ap*-βGRP antibody. (B) Effects of 1,3-β-D-glucan on hemolymph PO activity. (C) Effects of exogenous *Ap*-βGRP on 1,3-β-D-glucan dependent PO activity. Each reaction mixture consisted of 10 μl serially diluted *Ap*-βGRP sample, 10 μl hemolymph and 10 μl 1,3-β-D-glucan. 1,3-β-D-glucan in the reaction mixtures: 0 mM (*closed diamonds*); 10 mM (*closed squares*); 50 mM (*closed triangles*); 100 mM (*closed circles*).

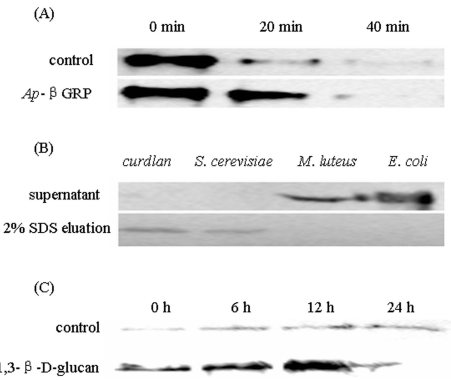

Fig. 4. Binding specificity and *Ap*-βGRP variation in protein level. (A) After incubation of 10 μl hemolymph with purified *Ap*-βGRP in the presence of 1,3-β-D-glucan at different time intervals, *Ap*-βGRP was detected by Western blotting against *Ap*-βGRP antibody. anti-coagulation buffer instead of *Ap*-βGRP as the control (B) purified *Ap*-βGRP was incubated with microbial cells or curdlan. Supernatant and 2% SDS eluation were analyzed using Western blotting against *Ap*-βGRP antibody. (C) Western blotting analysis of hemolymph samples with *Ap*-βGRP antibody 0, 6, 12, 24 h after injection with 1,3-β-D-glucan in anti-coagulation buffer. Sterilized anti-coagulation buffer was injected as a control.

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, out 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

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 bookle 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 was analyzed by SDS-PAGE and Western blotting using antibody against *Ap*-βGRP. 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 with buffer D. The column was then eluated with a 350 ml gradient from 0 to 0.5 M NaCl in buffer D. Finally, 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 eluated 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.

The amino acid sequence of *Ap*-βGRP was analyzed by the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple amino acid sequence alignment was carried out using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/).

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 (1×10^8 cells/ml), *E. coli* (1×10^9 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 eluated with 8 M urea, then 2% SDS. 2% SDS eluation 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 ethylester 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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