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Factors functioning in nodule melanization of insects and their mechanisms of accumulation in nodules

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ABSTRACT

Nodules consisting of hemocytes and trapped microorganisms are important targets for melanization, which is best known in the insect immune system. We investigated factors functioning in nodule melanization and the mechanism by which these factors congregate in the nodule. BmHP21, BmSPH1 and BmSPH2, *Bombyx mori* orthologs of *Manduca sexta* serine protease HP21, serine protease homologs (SPH1 and SPH2), and a prophenoloxidase, BmPO1 were observed as inactive forms in the plasma, but as putatively active forms in the nodule. Production of prophenoloxidase-activating proteinases, BmPAP1 and BmPAP3/PPAE and BmPO1 were confirmed in hemocytes. BmSPH1 and BmSPH2 were observed on trapped bacterial cells in the nodule and were isolated from the surface of bacterial cells incubated with plasma. BmSPH1 and BmSPH2 were found in plasma in complex with a pattern recognition receptor, BmLBP. These data suggest that melanization-regulating factors congregate in nodules through a combination of microorganism-dependent and hemocyte-dependent routes.

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1. Introduction

Innate immunity plays a key role in the defense against microbial infection in insects, which lack an acquired immune system. In insects, pathogens recognized as "nonself" are eliminated by both humoral and cellular responses (Vilmos and Kurucz, 1998). The cellular responses, which are mediated by hemocytes, include phagocytosis, encapsulation, and nodule formation (Marmaras and Lampropoulou, 2009). The humoral responses include production of antimicrobial peptides and melanization, a process induced by rapid activation of the prophenoloxidase (proPO) cascade (Hoffmann and Reichhart, 2002; Cerenius and Soderhall, 2004).

The molecular mechanism of proPO cascade-dependent melanization in hemolymph has been studied for more than 40 years. In this system, pattern-recognition molecules such as beta-1,3-glucan recognition protein (βGRP) elicit a cascade reaction consisting of serine proteases (SPs) in response to microbial invasion (Wang and Jiang, 2006). The regulation of this cascade also involves serine proteases homologs (SPHs) in which the catalytic serine residue of SP domain is substituted basically with glycine (Yu et al., 2003; Wang and Jiang, 2004; Gupta et al., 2005). By the end of 2009, most components of the proPO cascade had been elucidated in *Manduca sexta* (An et al., 2009) and *Tenebrio molitor* (Kan et al., 2008).

Nodule formation is an expeditious cellular response in which hemocytes surround and isolate invading pathogens. Nodule formation was divided into two distinct stages (Ratcliffe and Rowley, 1979). In the silkworm *Bombyx mori*, the first stage of nodule formation is nearly accomplished within 30 s after bacterial injection (Arai et al., 2013). Hemolymph can be cleared of 10^6 bacterial cells by a highly efficient process that depends on nodule formation (Koizumi et al., 1997). The melanization response that follows the first stage of nodule formation is thought to reinforce the nodules and provide microbicidal reactive oxygen species (ROS) that, together with antimicrobial peptides, contribute to immune defense (Kan et al., 2008). Because ROS are also harmful to insect tissues, it is plausible that insects have an ingenious system that masks the harmful effects of melanization. In fact, when *Escherichia coli* cells are injected into the hemocoel of insect larvae, melanization of the nodules progresses faster than the observed activation of proPO in plasma (Sakamoto et al., 2011). Thus, it is plausible that insects both kill microbes and protect themselves via nodule-specific melanization. Furthermore, it is possible that melanization of hemolymph is merely a byproduct of nodule melanization. In a

Abbreviations: SP, serine protease; SPH, serine protease homolog; proPO, prophenoloxidase; PO, phenoloxidase; PAP, prophenoloxidase-activating proteinase; PPAE, proPO-activating enzyme; ROS, reactive oxygen species; BmLBP, *Bombyx mori* lipopolysaccharide binding protein; BmMBP, *B. mori* multibinding protein; DAPI, 4′,6-diamidino-2-phenylindole.

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previous study (Sakamoto et al., 2011), we indicated that SPH1, which may regulate proPO activation in the plasma (Yu et al., 2003; Lu and Jiang, 2008), is present in nodules and is responsible for their melanization. However, there is no information on other factors involved in the proPO cascade in nodules. Therefore, in the present study, we investigated whether factors involved in the proPO cascade that are present in the hemolymph, where they function in melanization regulation, also exist in the nodule. We also studied the mechanism of efficient congregation and concentrations of factors in nodules.

2. Materials and methods

2.1. Laboratory animals

Silkworm, *Bombyx mori* (Kinsyu × Showa), were reared on an artificial diet (Silkmate, Nihon-Nosanko) containing chloramphenicol at 25 °C. Fifth-instar larvae 3–4 days after molting were sterilized by dipping in 70% ethanol and used for experiments.

2.2. Preparation and cultivation of bacteria and yeast cells

Gram-positive bacteria *Micrococcus luteus* (IAM1056) and Gram-negtive bacteria *E. coli* K-12W3110 were cultured in Luria–Bertani (LB) medium (peptone 10 g, yeast extract 5 g, NaCl 5 g, distilled water 1 l). *Saccharomyces cerevisiae* (IAM4125) was cultured in yeast and malt (YM) medium (glucose 10 g, peptone 5 g, yeast extract 3 g, malt extract 5 g, distilled water 1 l). Microorganisms grown in the logarithmic phase were collected by centrifugation at 8000 rpm for 20 min at 4 °C, washed twice with insect physiological saline (IPS, 150 mM NaCl, 5 mM KCl, pH 6.8), and fixed in 4% formaldehyde with gentle shaking for 1 h. The fixed cells were collected by centrifugation at 3300 rpm for 20 min at 4 °C and washed 5 times with IPS. The density of prepared suspension containing formaldehyde fixed cells was adjusted to $OD_{600} = 0.8$ (approximately equivalent to 8×10^8, 4×10^8, and 1×10^7 cells/ml, respectively for *E. coli*, *M. luteus*, and *S. cerevisiae* cells). Cells were stained with Coomassie Brilliant Blue R250 (CBB) and washed 5 times with IPS. Cells of 1.0×10^7 and 1.0×10^6 were suspended in 100 μl of IPS respectively for *E. coli* and *M. luteus*, and *S. cerevisiae* cells. For the challenge with bacterial and yeast cells, 10 μl of CBB-stained cells were injected into the hemocoel of *B. mori* using a MICROLITER syringes (Hamilton).

2.3. Determination of B. mori ortholog factors

B. mori orthologs of *M. sexta* SPH2 (serine proteinase homolog 2, AF518768.1), HP21 (hemolymph proteinase 21, AAV91019.1), PAP1 (prophenoloxidase-activating proteinase-1, AAX18636.1), and PAP3 (prophenoloxidase-activating proteinase-3, AAX18637.1) belonging to proPO activating cascade were searched using TBLASTN search system of the SILKWORM GENOME RESEARCH PROGRAM (http://sgp.dna.affrc.go.jp). Sequences with the highest identity were named BmSPH1 (AF513368), BmSPH2 (BGIBMGA009551), BmPAP1 (BGIBMGA010546), BmPAP3 (BGIBMGA013746) which was previously reported by Satoh et al. (1999) as PPAE, and BmHP21 (BGIBMGA009610), respectively. *B. mori* proPO1 (BmPO1) (AF178462), a subunit of a heterodimeric protein previously reported by Kawabata et al. as proPO-P1 (Kawabata et al., 1995) was reconfirmed to be an ortholog of M. sexta proPO.

2.4. Preparation of antisera

The regions encoding mature peptides of predicted BmSPH2, BmPAP1, BmPAP3, BmPO1 and BmHP21 were amplified by PCR using the PrimeSTAR® HS DNA Polymerase (Takara). The amplified cDNA fragments were cloned in the GST fusion protein expression vector pGEX-4T-3 (GE Healthcare) and used to transform *E. coli* BL 21 cells. Using transformed-BL21 cells, recombinant BmSPH2, BmPAP1, BmPAP3, BmPO1 and BmHP21 proteins (GST fusion proteins) were obtained and purified as described previously (Arai et al., 2013). Briefly, overnight cultures of transformed-BL21 cells were diluted with fresh LB medium containing 100 mg/ml ampicillin, and the cells were grown at 37 °C. Production of the fusion proteins was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 1 mM. The bacterial cells were harvested by centrifugation and sonicated to release the inclusion bodies, which were denatured with 10 mM dithiothreitol and 25 mM NaOH and dialyzed in phosphate buffered saline (10 mM sodium phosphate buffer, 145 mM NaCl, pH 7.4). The fusion proteins were prepared by the preparative electrophoresis. To raise antisera, the fusion proteins were first injected to female mice with complete Freund's adjuvant and subsequently with incomplete Freund's adjuvant as booster shots (Sigma). Anti-BmLBP, Anti-BmMBP, and Anti-BmSPH1 antisera were raised and specificities of these antisera were evaluated as previously reported (Koizumi et al., 1997; Watanabe et al., 2006; Sakamoto et al., 2011).

2.5. Western blot analysis of hemolymph and tissues

Fixed and CBB-stained *E. coli* cells (10^6 cells in 10 μl) were injected into the hemocoel of *B. mori* fifth-instar larvae. Hemolymph from no-injected larvae or larvae 15, 30 or 60 min post-injection were collected in IPS mixed with 10 mM benzamidine and 4 mM dithithreitol (DTT) and centrifuged at 800*g* for 15 min at 4 °C. The supernatant was collected as a plasma fraction. The precipitate was washed with IPS and centrifuged as above. After centrifugation, hemocytes were collected by removal of the supernatant. Nodules were collected from larvae in IPS 15, 30 or 60 min post injection with 1.0×10^6 CBB-stained *E. coli* cells. And Nodules were also collected from larvae 15 min post injection with CBB-stained *M. luteus* and *S. cerevisiae* cells.

Plasma (10 μl) and hemocytes (10 μl as a precipitate) were mixed with 2× SDS–PAGE sample buffer (250 mM Tris–Cl (pH 6.8), 5% SDS, 0.25% bromophenol blue, 25% glycerol) (40 μl) and nodules (10 μl as a precipitate) were mixed with 5× SDS–PAGE sample buffer (40 μl) after homogenization. Samples (10 μl each) were loaded and separated by SDS–PAGE (Laemmli, 1970) using 12.5% polyacrylamide. For Western blot analysis, the proteins were transferred onto PVDF membrane. After blocking with 2% BSA, the membrane was incubated with 10,000-fold diluted mouse antiserum against BmSPH2, BmPO1 or BmHP21, or 15,000-fold diluted rabbit antiserum against BmSPH1, BmLBP or BmMBP for 1 h. After washing, the membrane was incubated with 10000-fold diluted peroxidase-conjugated goat anti-mouse IgG (Wako Pure Chemical) or 15,000-fold diluted peroxidase-conjugated goat anti-rabbit IgG (Wako Pure Chemical) for 1 h, and then stained using ECL Western bloting detection system (GE Healthcare).

2.6. Immunofluorescent staining of the nodules

Fifth-instar larvae were injected with formaldehyde fixed 1.0×10^6 *E. coli* or *M. luteus* cells and nodules were collected. Nodules were fixed in 4% paraformaldehyde in PBS for 2 h, embedded in paraffin, and sectioned at 6 μm using a Leica RM2125 microtome. After blocking with 2% BSA in Tris–buffered saline with Tween 20® (TBST), the sections were incubated for 60 min with 1000-fold diluted rabbit antiserum against BmSPH1, BmLBP or BmMBP, or 1000-fold diluted mouse antiserum against BmSPH2 or BmPO1 in TBST containing 1% BSA for 1 h. After washing, the sections were incubated with 1000-fold diluted Alexa Fluor®

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