Glucan-Binding Activity of Silkworm 30-kDa Apolipoprotein and Its Involvement in Defense against Fungal Infection

Minoru Ujita,1,2*, Yosuke Katsuno,1 Ichiro Kawachi,1 Yoshinori Ueno,3 Yutaka Banno,3 Hiroshi Fuhin,3 and Akira Hara1,2

1Laboratory of Biological Chemistry, Department of Applied Biological Chemistry, Faculty of Agriculture, Meijo University, Tempaku-ku, Nagoya 468-8502, Japan
2Agricultural High-Tech Research Center, Meijo University, Tempaku-ku, Nagoya 468-8502, Japan
3Institute of Genetic Resources, Kyushu University, Higashi-ku, Fukuoka 812-8581, Japan

Received February 22, 2005; Accepted March 15, 2005

The silkworm Bombyx mori 30-kDa lipoproteins (6G1 and 19G1), major components of the hemolymph, were shown to bind to glucans. 6G1 apolipoprotein was expressed as a fusion protein with glutathione S-transferase in Escherichia coli and assayed for its binding activity. The purified recombinant 6G1 apolipoprotein specifically bound to β-glucan, but not to chitin, mannann, peptidoglycan, or oligosaccharide chains on glycoproteins. The β-glucan binding of the recombinant 6G1 was inhibited by laminaribiose and laminarin, a soluble glucan, but not by lipopolysaccharide or insect blood sugar, trehalose at physiological concentration. Furthermore, the recombinant 6G1 was shown to participate in the activation of prophenoloxidase cascade and to interfere with hyphal growth of the entomopathogenic fungus Paecilomyces tenuipes, injected into pupae of B. mori. These results suggest that 6G1 lipoprotein plays a role in the protection of B. mori against invading fungi.

Key words: Bombyx mori; apolipoprotein; β-glucan; phenoloxidase; entomopathogenic fungi

Pattern recognition molecules serve as biosensors for detection of invading microorganisms in vertebrate and invertebrate animals. The major structural components of the fungal cell wall are β-1,3- and β-1,6-glucans, chitin, mannann, and glycoproteins, and insect hemolymph β-glucan-binding proteins function in innate immune responses by binding to β-glucans on the cell surface of invading fungi and initiating host defense reactions.1-3 The silkworm Bombyx mori β-1,3-glucan recognition protein has been shown to trigger the prophenoloxidase (proPO) cascade, a pathway in insects for defense against microbial infection, via binding to β-1,3-glucan on the surface of invading pathogens.4,5 Recently we found that B. mori 30-kDa lipoproteins (6G1 and 19G1), major plasma proteins of the fifth instar larvae and of pupae,6-8 bind to β-glucan, a fungal immune elicitor.9 These lipoproteins are synthesized in fat body of both sexes and are secreted into the hemolymph. Biosynthesis of the 30-kDa lipoproteins was shown to be developmentally regulated in a stage-dependent manner at the transcriptional level in fat body, although their biological functions remain unclear. Furthermore, it was found that expression of these lipoproteins is regulated by juvenile hormone and that large amounts of these proteins are also present in the oocytes of sexually mature females. These two proteins are structurally similar to each other and belong to the microvitellogenin superfamily. It was shown previously that hemagglutinins are identical to the vitellogenins in the Colorado potato beetle, Leptinotarsa decemlineata.10 These results suggest that B. mori 30-kDa lipoproteins agglutinate invading fungi through binding to β-glucans on the cell surface and trigger immune reactions for fungal infection in the hemolymph. However, the interaction of B. mori 30-kDa lipoproteins with other microbial cell wall components, such as chitin, mannann, peptidoglycan, lipopolysaccharide, or glycoproteins, has not been investigated, and it remains to be elucidated whether the β-glucan-binding activity is due to the polypeptide of the 30-kDa lipoproteins. Moreover, it is also unclear whether B. mori 30-kDa lipoproteins are involved in activation of the proPO cascade via recognition of β-glucan and interact with fungi in vivo.

In this report, we describe the carbohydrate-binding specificity of a recombinant B. mori 30-kDa 6G1 apolipoprotein expressed in Escherichia coli and the involvement of 6G1 lipoprotein in host defense against invading β-glucan-containing microorganisms.

Abbreviations: BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; Con A, concanavalin A; DOPA, 3,4-dihydroxy-L-phenylalanine; GST, glutathione S-transferase; HRP, horseradish peroxidase; PCR, polymerase chain reaction; PO, phenoloxidase; proPO, prophenoloxidase; PVDF, polyvinylidene difluoride; RCA, Ricinus communis agglutinin; TBS, Tris-buffered saline

* To whom correspondence should be addressed. Fax: +81-52-835-7450; E-mail: ujita@ccmfs.meijo-u.ac.jp
Materials and Methods

Insects. The p50 strain of B. mori, maintained in the Silkworm Genetic Division, Institute of Genetic Resources, Kyushu University, was used.

Construction of expression plasmid. To produce a glutathione S-transferase (GST)–6G1 fusion protein, an E. coli expression plasmid that encodes the mature protein of B. mori 6G1 apolipoprotein was constructed. The DNA encoding the mature protein of B. mori 6G1 was prepared by polymerase chain reaction (PCR) using B. mori genomic DNA as a template based on the published nucleotide sequence.3) The mature region of the B. mori 6G1 apolipoprotein gene contains no intron sequence. Sense and antisense primers for this PCR were 5’-CCGGATCCCCACCTTGACCAAGAACTGTGAC and 5’-CCGAATTCGTAGGGACCATGTCACATGC (BamHI and EcoRI sites are underlined). The PCR product encoding amino acid residues 20–256 of 6G1 apolipoprotein was digested with BamHI and EcoRI, and then cloned in the BamHI and EcoRI sites of pGEX-3X (Amersham Biosciences), yielding pGEX-3X/6G1. The recombinant pGEX-3X/6G1 was introduced into competent E. coli JM109 cells. The nucleotide sequence of the expression construct was analyzed by dideoxy nucleotide sequencing to ensure lack of errors.

Expression and purification of fusion protein. The GST-6G1 fusion protein or GST alone was expressed in E. coli and purified as described previously.4) The purified fusion protein was dialyzed against distilled water or 10 mM Tris–HCl, pH 7.5, and then analyzed by 10% SDS–PAGE and immunoblotting. Proteins in the gel were stained with Coomassie Brilliant Blue (CBB) or transferred to a nitrocellulose membrane. The membrane was then stained with horseradish peroxidase (HRP)-conjugated anti-GST antibody (Amersham Biosciences) to detect GST fusion proteins. Positive bands were visualized using the enhanced chemiluminescence detection system (Amersham Biosciences).

Affinity chromatography. The binding specificity of 6G1 apolipoprotein was identified by affinity chromatography on various insoluble polysaccharides.3,4) The purified GST-6G1 fusion protein was dissolved in a loading buffer containing 125 mM NaCl, 10 mM CaCl$_2$, and 25 mM Tris–HCl, pH 7.5. The fusion protein solution (0.5 ml) was put on a 1-ml column of β-glucan (from Saccharomyces cerevisiae, Sigma), chitin beads (New England Biolabs), mannan (Sigma), or peptidoglycan (from Staphylococcus aureus, Wako) equilibrated with the loading buffer, and then washed with 10 ml of the loading buffer. In some cases, competing carbohydrates (100 mM laminaribiose, 15 mM trehalose, and 1 mg/ml laminarin) or 1 mg/ml lipopolysaccharide (from E. coli, Sigma) was added to the loading buffer and samples. The column was eluted with 1% SDS. Fractions were collected, and aliquots were analyzed by 10% SDS–PAGE and immunoblotting. Fusion proteins were detected by silver staining or anti-GST antibody. As a control, the binding activity of GST alone was tested under the same conditions.

Solid-phase binding assay. To analyze the oligosaccharide-binding activity of 6G1 apolipoprotein, a solid-phase binding assay was done using glycoproteins having different types of oligosaccharide chains: ovalbumin, fetuin, and asialofetuin (all from Sigma). Ovalbumin has heterogeneous high mannose-type N-linked chains and also has heterogeneous hybrid-type N-linked chains.12,13) Fetuin has three equal triantennary complex-type N-linked chains and three equal O-linked chains.14) Asialofetuin is obtained by removing sialic acids from fetuin. Polystyrene wells coated with various glycoproteins were blocked with a solution of 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) (150 mM NaCl, 10 mM Tris–HCl, pH 7.5) for 2 h at room temperature. The binding assay was done by the addition of 100 μl of the purified GST-6G1 fusion protein or GST in TBS containing 0.05% (v/v) Tween 20, 1% BSA, and 10 mM CaCl$_2$ to the wells. The wells were incubated for 2 h and then washed three times with TBS containing 0.05% (v/v) Tween 20 and 10 mM CaCl$_2$. After HRP-conjugated anti-GST antibody was added, the wells were incubated for 2 h and washed. Finally, the wells were incubated with o-phenylenediamine and H$_2$O$_2$, and the absorbance at 490 nm was measured using a microplate reader. As a control experiment, coated wells were incubated successively with lectin, anti-lectin antibody, and HRP-conjugated anti-IgG antibody (Amersham Biosciences), as described above. Concanavalin A (Con A), Ricinus communis agglutinin (RCA)-I, and anti-Con A antibody were obtained from Sigma, and anti-RCA-I antibody was purchased from E-Y Laboratories. Each assay was done in triplicate.

To analyze the carbohydrate-binding specificity of 6G1 apolipoprotein, a solid-phase binding assay was done using polyvinyl (PV)-sugars.15,16) PV-sugar is a water-soluble polystyrene derivative bearing carbohydrate residues and adsorbs to polystyrene plates. Wells of microplates were coated with PV-sugars (PV-laminaribiose, PV-lactose, PV-mannobiose, or PV-di-N-acetylchitobiose) (Seikagaku, Tokyo) and blocked as described above. The binding assay was done by the addition of 100 μl of the purified GST-6G1 fusion protein or GST in TBS containing 0.05% (v/v) Tween 20, 1% BSA, and 10 mM CaCl$_2$ to the wells. In some cases, 100 mM competing carbohydrates were added to the reaction mixtures. The wells were incubated and washed as described above. Finally, absorbance at 490 nm was measured as described above. Each assay was done in triplicate.
Injection of β-glucan into B. mori. To test whether β-glucan affects the amount of 6G1 lipoprotein in the hemolymph, B. mori fifth instar larvae and pupae were injected with 50 μl of 0.3% β-glucan. Hemolymph was collected at different times after injection and analyzed by 10% SDS–PAGE.

N-Terminal amino acid sequencing. The proteins in B. mori hemolymph were put through 10% SDS–PAGE, and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Sequi-Blot, Bio-Rad).17) The proteins were cut from the PVDF membranes and sequenced by automated Edman degradation on a gas-phase protein sequencer (PPSQ-21A, Shimadzu, Kyoto, Japan).

Activation of the proPO pathway by GST-6G1 in the presence of laminarin. Hemolymph was collected from B. mori fifth instar larvae. Cell-free hemolymph (plasma) was prepared according to the method of Ashida.4,18–20) The B. mori plasma was passed through a column of curdlan (Sigma) to remove β-glucan recognition proteins, and the effluent (78 μl) was mixed with laminarin (10 μl, 1 mg/ml) and CaCl2 (2 μl, 250 mM).5,20) Control samples lacked laminarin, which was replaced with distilled water. Purified GST-6G1 (10 μl, 2 mg/ml), GST (10 μl, 2 mg/ml), or distilled water as a control was added to the above mixture (90 μl), incubated at 25 °C, and used as an enzyme source for phenoloxidase (PO) activity.20)

PO activity was assayed using 3,4-dihydroxy-L-phenylalanine (DOPA) as a substrate by the method of Horowitz and Shen.18,21,22) The enzyme activity was expressed as an increase of absorbance at 490 nm.

Inoculation of Paecilomyces tenuipes to pupae of B. mori. The entomopathogenic fungus P. tenuipes was grown in a Potato-Dextrose broth medium (Difco) at 25 °C. At the late logarithmic phase of growth, the hyphal bodies were collected and suspended in sterilized distilled water. Hyphal bodies were prepared according to the method of Sato and Shimazu.23) To inoculate P. tenuipes to B. mori, the pupae on day 4 were injected with 50 μl of the hyphal body suspension (2.0 × 106 cells/ml, untreated P. tenuipes), and incubated at 25 °C.24,25) To investigate the influence of 6G1 apolipoprotein on immune-related reactions, we performed in vivo studies with the GST-6G1 fusion protein. The hyphal body suspension (2.0 × 106 cells/ml) of P. tenuipes was incubated with GST-6G1 (1 mg/ml) at room temperature for 30 min (GST-6G1-treated P. tenuipes) and injected into pupae of B. mori, as described above. GST at the same concentration was used as a control (GST-treated P. tenuipes).

Results and Discussion

Production of GST fusion protein containing B. mori 30-kDa 6G1 apolipoprotein

The GST-6G1 fusion protein containing the mature protein of B. mori 6G1 apolipoprotein was purified and used to test its binding activity. SDS–PAGE and immunoblot analysis showed that the purified fusion protein had the expected molecular size of 54 kDa and was detectable with anti-GST antibody (Fig. 1).

β-Glucan-binding activity of GST-6G1 fusion protein

First we examined the interaction of GST-6G1 fusion protein with Saccharomyces cerevisiae β-glucan as a model of pathogenic fungi. GST-6G1 bound to β-glucan, but did not bind chitin, mannan, or peptidoglycan (Fig. 2). Furthermore, GST-6G1 did not bind to chito-oligo-agarose, lactose-agarose, mannose-agarose, or fucose-agarose (Ujita et al., unpublished results). GST, the other portion of the fusion protein, did not bind to β-glucan (data not shown), indicating that the observed β-glucan-binding activity of the fusion protein

Fig. 1. Expression and Purification of GST-6G1 Fusion Protein.

The glutathione-Sepharose-purified GST-6G1 fusion protein was electrophoresed on a 10% SDS–polyacrylamide gel and then stained with CBB (lane 1) or transferred to a nitrocellulose membrane. The membrane was stained with anti-GST antibody (lane 2). Molecular mass markers are shown to the left.

Fig. 2. β-Glucan-Binding Activity of GST-6G1.

The purified GST-6G1 fusion protein was put onto a column of β-glucan (lane 1), chitin beads (lane 2), mannan (lane 3), or peptidoglycan (lane 4), and then eluted with 1% SDS. Competition experiments with free carbohydrates or lipopolysaccharide for the binding of GST-6G1 to β-glucan were done to assess binding specificity. Laminaribiose (lane 5), trehalose (lane 6), laminarin (lane 7), or lipopolysaccharide (lane 8) was present in the sample and buffer. The bound proteins were put through 10% SDS–PAGE, and detected by silver staining (lanes 1–6) or anti-GST antibody (lanes 7 and 8). Molecular mass markers are shown to the left.
was due to the portion corresponding to the mature protein of B. mori 6G1 apolipoprotein. The mature protein of 6G1 apolipoprotein contains no potential N-glycosylation site, suggesting that N-linked oligosaccharide chains are not required for β-glucan-binding activity. These results also indicate that the lipid moiety of B. mori 6G1 lipoprotein is not essential for β-glucan-binding activity. The ability of GST-6G1 to bind to β-glucan suggests that 6G1 apolipoprotein can function for detection of fungal pathogens. The binding of GST-6G1 to β-glucan was inhibited by the addition of 100 mM laminaribiose (Glcβ1–3Glc) or 1 mg/ml laminarin, a soluble β-glucan (Fig. 2), indicating that 6G1 apolipoprotein specifically recognizes β-glucan. But, the β-glucan binding of GST-6G1 was not inhibited by insect blood sugar, trehalose at physiological concentration (15 mM) (Fig. 2). These results show that 6G1 lipoprotein can bind to β-glucans on the cell surface of invading fungi in the presence of trehalose in B. mori hemolymph. In addition, the binding of GST-6G1 to β-glucan was barely inhibited by 1 mg/ml lipopolysaccharide (Fig. 2), although lipopolysaccharide contains glucose as well as mannose, galactose, rhamnose, glucosamine, N-acetylgalactosamine, arabinose, 2-keto-3-deoxyoctonate (KDO), heptose, etc. Therefore, it is unlikely that 6G1 apolipoprotein strongly interacts with glucose residues in heteropolysaccharides such as the carbohydrate moiety of lipopolysaccharide. Peptidoglycan, a polysaccharide covalently linked to short peptides, contains N-acetylgalactosamine and N-acetylmuramic acid, and GST-6G1 did not bind peptidoglycan (Fig. 2). Since peptidoglycan and lipopolysaccharide are major constituents of the cell wall of Gram-positive and Gram-negative bacteria respectively, 6G1 lipoprotein might not be a self-defense factor for bacteria. The periplasm in some Gram-negative bacteria contains small β-glucans, but 6G1 lipoprotein should not bind to components of the periplasm inside the outer membrane. It has been found that lipophorin forms a complex with lipopolysaccharide of bacteria in B. mori hemolymph. Furthermore, a Gram-negative bacteria-binding protein has been purified from the hemolymph of B. mori and the cDNA cloned. B. mori peptidoglycan recognition protein, a member of the proPO cascade, has also been purified from the hemolymph and cloned. This protein specifically binds to peptidoglycan and is considered to be one of the pattern recognition proteins in innate immunity.

Carbohydrate-binding specificity of GST-6G1 fusion protein

The solid-phase binding assay showed that the GST-6G1 fusion protein bound to PV-laminaribiose, but not to PV-lactose, PV-mannobiose, or PV-di-N-acetyltetratosamine (Fig. 3A). GST showed no binding activity to PV-sugars. The binding to PV-laminaribiose was inhibited by laminaribiose and glucose, but not by the other carbohydrates such as lactose, mannobiose, di-N-acetyltetratosamine, N-acetylgalactosamine, galactose, N-acetyltetrasamin, mannose, or fucose (Fig. 3B). These results indicate that 6G1 apolipoprotein specifically recognizes the glucose residues in β-glucan. The solid-phase binding assay also showed that the GST-6G1 fusion protein did not bind to glycoproteins having different types of oligosaccharide chains (Fig. 4A). Con A, a lectin that binds mainly to high mannose-type and hybrid-type N-linked oligosaccharides, bound to ovalbumin, having high mannose-type and hybrid-type chains, and RCA-I, a lectin that reacts with the Galβ1–4GlcNAc-structure, bound to fetuin and asialofetuin, having triantennary complex-type N-linked chains, although the presence of sialic acids inhibited the RCA-I reactivity (Fig. 4A). The binding of Con A or RCA-I was dependent on the concentration of glycoproteins coated onto the wells, and maximal binding was seen at a concentration of 10 μg/ml (Fig. 4B). However, GST-6G1 did not bind to glycoproteins even though they were coated onto the wells at a concentration of 100 μg/ml (Fig. 4B). Therefore, it is unlikely that no binding of GST-6G1 to glycoproteins tested in this assay is due to the low concentration of glycoproteins used to coat the wells. These results suggest that 6G1 apolipoprotein cannot bind to oligosaccharides on glycoproteins.

Fungi produce a variety of glycoconjugates and polysaccharides as part of their cell walls. The GST-6G1 fusion protein showed no binding activity to chitin, mannann, or glycoproteins (Figs. 2 and 4). Therefore, it is probable that β-glucan is a target for 6G1 lipoprotein in B. mori antifungal mechanisms, although the fungal cell wall contains β-glucan, chitin, mannann, and glycoproteins having high mannose-type glycans as major structural components. The biological roles of 6G1
lipoprotein are ill-defined, but presumably this molecule contributes to a specific defense against invading glucan-containing microorganisms such as fungi through its strict binding specificity. Recently, certain species of bacteria were shown to glycosylate proteins.\textsuperscript{25) 6G1 lipoprotein should not bind to these bacterial glycoproteins, although the oligosaccharides vary in core structure to a much greater extent than seen in eukaryotes.

**Protein composition in B. mori hemolymph after injection of β-glucan**

After *B. mori* fifth instar larvae and pupae were injected with β-glucan, the protein composition of the hemolymph was investigated by SDS–PAGE in a time course experiment, and it was found that β-glucan did not affect the amount of 6G1 lipoprotein in the hemolymph (Fig. 5). To identify 6G1 lipoprotein, the N-terminal amino acid sequence of 30-kDa proteins was analyzed (Fig. 6). No change in the 6G1 lipoprotein concentration was seen until 48 h after injection of β-glucan, although injection of heat-killed bacteria into larvae of the greater wax moth, *Galleria mellonella*, is followed by changes in lipoprotein composition in the hemolymph.\textsuperscript{31) The mRNA of *B. mori* β-1,3-glucan recognition protein has been shown to be constitutively expressed in hemocytes, fat body, and epithelial cells, and a bacterial or yeast challenge induced the transcription.\textsuperscript{31} Furthermore, it has been found that *B. mori* Gram-negative bacteria-binding protein is constitutively expressed in fat body and cuticular epithelial cells, and that expression is rapidly induced following a bacterial
challenge. It has also been found that *B. mori* peptidoglycan recognition protein is constitutively expressed in fat body, epithelial cells, and hemocytes, and that a bacterial challenge induces the gene expression. The lack of inducibility distinguishes 6G1 expression from that of these pattern recognition proteins from *B. mori*, which are synthesized in response to exposure to microbial elicitors. A 1,3-glucan recognition protein from the tobacco hornworm, *Manduca sexta*, has been shown to be constitutively expressed in fat body and secreted into hemolymph. The mRNA level of *G. mellonella* apolipophorin III, an immune-activator, did not vary after larvae were injected with bacteria and yeast. The mRNA level of *G. mellonella* apolipophorin III, an immune-activator, did not vary after larvae were injected with lipopolysaccharide, in comparison to untreated larvae. Since *B. mori* 6G1 lipoprotein is one of the most abundant protein components in the hemolymph at late larval to early pupal stages of both sexes, this protein might be a self-defense factor against infection of fungi through 1,3-glucan-binding activity.

**Role of 6G1 apolipoprotein in proPO activation in *B. mori* hemolymph**

Activation of proPO in *B. mori* hemolymph can be triggered by 1,3-glucan. Because GST-6G1 was able to bind specifically to 1,3-glucan, we tested whether a complex of 6G1 apolipoprotein with 1,3-glucan might stimulate activation of the proPO cascade. *B. mori* larval plasma was passed through a column of curdlan to decrease the concentration of endogenous 1,3-glucan-binding proteins. This plasma was incubated with the soluble 1,3-glucan laminarin and GST-6G1, and then PO activity was measured (Fig. 7). GST-6G1 combined with laminarin increased PO activity significantly. But, laminarin alone, GST-6G1 alone, or GST combined with laminarin stimulated a much smaller degree of proPO activation (Fig. 7). PO generates quinones that are intermediates for melanization. These results suggest that 6G1 lipoprotein is involved in the activation of the proPO pathway, which involves a serine proteinase cascade, via recognition of fungal cell wall 1,3-glucans, and functions as a biosensor for 1,3-glucans in defense. Similar results have been observed with *B. mori* 1,3-glucan recognition protein. The tobacco hornworm *M. sexta* 1,3-glucan recognition proteins have been shown to agglutinate microorganisms and activate the PO cascade. Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and 1,3-glucan, has been found to mediate the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells. Overexpression of Gram-negative bacteria-binding protein-1 in *Drosophila* immunocompetent cells enhanced expression of the NF-κB-dependent antimicrobial peptide gene. 6G1 lipoprotein is thought to be a circulating 1,3-glucan-binding protein that transduces signals and stimulates a defensive response. After binding to 1,3-glucans on the surface of invading fungi, 6G1 lipoprotein might change the conformation and interact with a serine protease, leading to activation of the proPO cascade. *B. mori* plasma proteins and 6G1 lipophorin have been reported to activate the proPO cascade, but it is not yet known whether proteins other than lipophorin act as an elicitor. In this study, we found that 6G1 lipoprotein participates in activation of the proPO cascade. Furthermore, it has been reported that lipophorin forms a complex with melanin to encapsulate invading microorganisms in insects. These results indicate that different recognition molecules have overlapping binding specificity and functions.

**Inoculation of *P. tenuipes* to *B. mori* pupae**

The entomopathogenic fungus *P. tenuipes* infects larvae and pupae of moths, and proliferates in the host insect. We examined the effect of 6G1 apolipoprotein on the ability of injected *P. tenuipes* to colonize...
hemolymph and cause mortality in B. mori pupae. The hyphal body suspension of P. tenuipes was injected into B. mori pupae with or without the GST-6G1 fusion protein. Hyphal bodies are formed by the budding of hyphae and proliferate in the body cavity of insects. At 4 d after injection of untreated P. tenuipes, white hyphal masses appeared on the surfaces of the pupae, particularly on the area of spiracles (Fig. 8). Within 2 weeks, the pupae were covered with mycelia (Fig. 8). GST-6G1-treated P. tenuipes proliferated more slowly in the hemolymph than untreated P. tenuipes. In B. mori pupae, percentage mortality was lower among those injected with GST-6G1-treated P. tenuipes than among those injected with untreated P. tenuipes. Survival times were higher for GST-6G1-treated P. tenuipes than for untreated P. tenuipes or P. tenuipes treated with GST used as a control at the same concentration (GST-treated P. tenuipes). The relative pathogenicity of GST-treated P. tenuipes against B. mori pupae was the same as that of untreated P. tenuipes. The pupae died within 4 d after injection when untreated P. tenuipes was inoculated (Fig. 8), but incubation of P. tenuipes with GST-6G1 resulted in eclosion over 70% of pupae treated (30 pupae were treated) (Fig. 8). These results indicate that GST-6G1 interferes with hyphal growth of injected fungi and exerts a fungistatic activity. These results also suggest that 6G1 apolipoprotein limits the spread of invading fungi in B. mori to ensure a localized defense reaction, and binds to the surface of invading pathogens, which contain β-glucans in their cell walls, leading to more efficient clearance of the microorganisms by hemocytes through encapsulation or nodule formation. It was shown previously that intrahemocoelic injection of G. mellonella apolipopophorin III into G. mellonella larvae is followed by a dose-dependent increase in antibacterial activity in the hemolymph. Furthermore, G. mellonella apolipopophorin III enhanced the phagocytic activity of hemocytes in vitro.32,34

6G1 lipoprotein appears to be multifunctional and to be involved in both lipid transport and defense. This study suggests a novel function of insect lipoproteins. Recently, several anti-apoptotic fractions were obtained from B. mori hemolymph, and the anti-apoptotic protein exhibiting the highest activity was shown to be one of the 30-kDa lipoproteins in B. mori.35–38 Information from research on insects might lead to the identification of novel biological functions of lipoproteins in higher organisms, including humans.

Acknowledgments

We thank Dr. Eiji Yokoyama of Meijo University for useful discussion. This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Agricultural High-Tech Research Center of Meijo University, under the project Environmental Control through the Functions of Microorganisms.

References


