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NOTE

Heterogeneous expression and emulsifying activity of class I hydrophobin from *Pholiota nameko*

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Abstract Hydrophobins secreted by filamentous fungi self-assemble into an amphipathic film at hydrophilic/hydrophobic interfaces. This unique property suggests that the hydrophobins have a high potential for industrial applications. However, the assemblages of class I hydrophobins are highly insoluble, making such commercial applications difficult. To enhance the solubility of class I hydrophobins, we have attempted to express class I hydrophobin PNH1 from *Pholiota nameko* fused with glutathione *S*-transferase (GST) in *Escherichia coli*. The GST–PNH1 was effectively isolated from the soluble fraction of transformed *E. coli*, and subsequent analysis revealed that the purified GST–PNH1 had almost the same emulsifying activity as PNH1.

Keywords Basidiomycete · Emulsion · Fusion protein · Glutathione *S*-transferase

Hydrophobins are small secreted proteins characterized by eight conserved cysteine residues that are produced by filamentous fungi belonging to the phyla Ascomycota and Basidiomycota. When secreted to extracellular space, they self-assemble into an amphipathic film at hydrophilic/hydrophobic (e.g., water/air) interfaces (Linder et al. 2005) and where they have significant roles in various processes, such as fungal development and adhesion (Wösten and Wessels 1997). Wessels (1994) classified hydrophobins into two classes on the basis of the hydropathy profiles and biophysical properties: class I and class II. Assemblages of class I hydrophobins are highly insoluble [resistance to 2% sodium dodecyl sulfate (SDS) at 100°C] and can be dissociated only in strong acid. In contrast, assemblages of class II hydrophobins can be dissolved in 60% ethanol or 2% SDS easily (Wessels 1997). In addition, a mosaic of amyloid fibrils called "rodlets" is observed on the surface of class I hydrophobin assemblages but not on that of their class II counterparts (Linder 2009). Hydrophobins that exhibit these interesting properties are expected to have applications in a variety of fields, such as medical and technical coatings, proteinaceous glues, cosmetics, and emulsifying agents (Hektor and Scholtmeijer 2005). For example, it has been reported that HFBII, a class II hydrophobin from *Trichoderma reesei*, can be used as the foam stabilizer of a chocolate milk shake because it has high foam stability (Cox et al. 2007, 2009). In contrast, class I hydrophobins form a highly insoluble film, and this property represents a serious obstacle to its general applicability. One approach that has been used with success to improve the solubility of a protein of interest is to fuse that protein with a highly soluble protein. However, the heterologous fusion protein may greatly lose its original properties, and the effect of the fusion is not yet evident.

We previously reported that three hydrophobin genes, *pnh1*, *pnh2*, and *pnh3*, in the mycelia of *Pholiota nameko* (T. Ito) S. Ito et Imai in Imai strain N2 were specifically expressed under conditions of inorganic phosphate

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deficiency (P^-) (Tasaki et al. 2004). To enable the industrial utilization of class I hydrophobins by increasing their solubility, we produced the class I hydrophobin proteins PNH1, PNH2, and PNH3 in *Escherichia coli* as a fusion protein with a glutathione *S*-transferase (GST), which is a highly soluble protein, and selected GST–PNH1 to test among the three fusion proteins constructed. In the study present here, we report the heterogeneous expression and the emulsifying activity of GST–PNH1.

Mycelia of the dikaryotic strain N2 of *P. nameko* (Onuki Kinjin, Utsunomiya, Japan) were cultured for 20 days in P^- media containing inorganic phosphate as KH_2PO_4 at a concentration of 50 mg/L, as described by Tasaki et al. (2001). Total RNA was isolated from the mycelium by the phenol-SDS method of Shirzadegan et al. (1991). The cDNA was synthesized from total RNA using a PrimeScript II First Strand cDNA Synthesis kit (Takara Bio, Otsu, Japan). The open reading frame of *pnh1* (accession number: AB079128), excluding the putative signal peptide sequence, was amplified by PCR using the sense primer 5′-AGATGAATTCACGCCCACTCGTCGCAAC-3′ (*Eco*RI site underlined) and the antisense primer 5′-CAGGCTCGAGGTCTAGAGGTTGATATTG-3′ (*Xho*I site underlined). The PCR product was digested with *Eco*RI (Takara Bio) and *Xho*I (Toyobo, Osaka, Japan) and inserted downstream of the GST gene in expression vector pGEX-4T-1 (http://www.gelifesciences.co.jp/tech_support/manual/pdf/28918444.pdf; GE Healthcare, Little Chalfont, UK) linearized with the same restriction enzymes. *Escherichia coli* Origami B (DE3) [F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1 aphC* (DE3) *gor522*::Tn*10 trxB* (Kan^R, Tet^R); Novagen, Madison, WI] was transformed with the resulting plasmid. The transformants were selected on media containing 50 μg/mL ampicillin, 20 μg/mL kanamycin, and 20 μg/mL tetracycline.

The bacterial cells were grown at 37°C in LB medium containing ampicillin. When the OD_{600} reached 0.3–0.6, isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce the expression of the fusion protein. After incubation for 6 h at 30°C, cells were harvested by centrifugation and suspended in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). The cell suspension was sonicated and the supernatant was collected by centrifugation as a soluble fraction. The amount of GST in the soluble fraction was assayed using a GST·Tag Assay kit (Novagen). The protein concentration was estimated by the method of Bradford (1976) using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard protein. The soluble fractions adjusted to the same protein concentration were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 16% (w/v) polyacrylamide gels according to the method of Laemmli (1970). The proteins were visualized by staining with Brilliant Blue R (Sigma-Aldrich, St Louis, MO).

Quantitative assays of GST revealed that the addition of IPTG increased the expression of GST and GST–PNH1 in soluble fractions from transformants (data not shown). The SDS-PAGE analysis revealed that the amount of some of the proteins in the soluble fractions increased following the addition of IPTG (Fig. 1, left) but that there was no distinct increase in the amount of protein in the insoluble fractions (data not shown). The putative molecular weight of GST and GST–PNH1 is 26,000 and 35,000, respectively. Although a band with molecular weight 28,000 appeared in both the GST and GST–PNH1 transformants following the addition of IPTG, we judged this not to be GST or GST–PNH1 based on the difference in molecular weight. The band corresponding to GST (M_r 26,000) could be clearly detected in the GST transformant, whereas the band corresponding to GST–PNH1 (M_r 35,000) was not clearly detectable in the GST–PNH1 transformant. Western blot analysis was therefore attempted using an anti-GST antibody to specifically detect GST fusion protein.

Following SDS-PAGE, proteins were electroblotted onto a polyvinylidene difluoride membrane using semi-dry blotting apparatus. The membrane was subjected to immunodetection using GST·Tag Monoclonal Antibody (Novagen) and goat anti-mouse IgG–HRP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as primary and secondary antibodies, respectively. Bound antibody was detected using ECL Western Blotting Detection reagents (GE Healthcare) and visualized by LAS-3000 (Fujifilm, Tokyo, Japan).

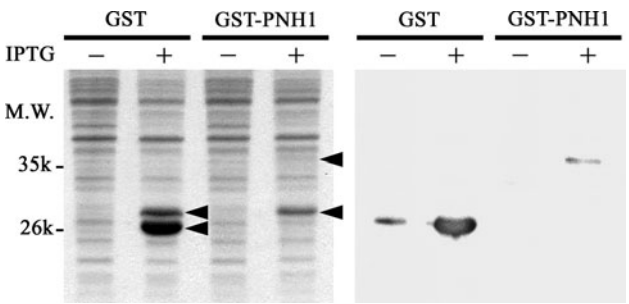

Fig. 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis of soluble fractions from *Escherichia coli* transformed with pGEX-4T-1 and pGEX-4T-1-*pnh1*. Transformants were cultured in the presence or absence of IPTG (isopropyl-*β*-D-thiogalactopyranoside). *Left* Proteins containing glutathione *S*-transferase (GST) or GST–PNH1 were visualized with Brilliant Blue R after SDS-PAGE. The proteins induced by adding IPTG are indicated by *arrowheads*. *Right* After blotting, GST proteins were detected using an anti-GST antibody. *M.W.* Molecular weight

Western blot analysis of the soluble GST and GST–PNH1 fractions to which IPTG had been added with an anti-GST antibody revealed the presence of one band at the position corresponding to a molecular weight of 26,000 or 35,000, respectively (Fig. 1, right). This result shows that the fusion protein GST–PNH1 was included in the soluble fraction obtained from the transformant. Tagu et al. (2001) attempted to express the class I hydrophobin HYDPt-1 from *Pisolithus tinctorius* as a His-tagged fusion protein in *E. coli*. However, the produced fusion protein was insoluble and required highly denaturing extraction conditions using 6 M guanidinium hydrochloride to be solubilized, suggesting that the fusion with a highly soluble protein, such as the GST used in this study, is an efficient procedure for the solubilization of the class I hydrophobin. Albuquerque et al. (2004) expressed class I hydrophobin PbHYD1 from *Paracoccidioides brasiliensis* as a fusion protein with GST in *E. coli* using much the same method as the one described here. However, the expression level of the fusion protein GST–PbHYD1 was low, and most of the expressed protein was present in an insoluble protein pellet, which was solubilized in 8 M urea. In our study and that Albuquerque et al. (2004), GST–PNH1 and GST–PbHYD1 were expressed using the same pGEX-4T expression vector and *E. coli* host. The notable difference between the two expressed proteins was the amino acid sequence of each hydrophobin and whether there was a signal peptide or not. The GST–PbHYD1 expression vector included a signal peptide between GST and the mature PbHYD1, whereas the signal peptide in the GST–PNH1 expression vector used in our study was absent. The signal peptide is composed of hydrophobic amino acid residues. Excluding the signal peptide may be effective for the expression of class I hydrophobin proteins in soluble form in *E. coli*.

The soluble fraction containing the GST–PNH1 fusion protein was filtered through a cellulose acetate filter (pore size 0.45 μm; Advantec, Tokyo, Japan) and loaded onto a Glutathione Sepharose 4B column (GE Healthcare). After the column was washed with PBS, the adsorbed protein was eluted with 50 mM Tris-HCl buffer, pH 8.0 containing 20 mM reduced glutathione.

Following SDS-PAGE of the adsorbed and non-adsorbed fractions, we were able to detect a protein of molecular weight 35,000 in the adsorbed fraction (Fig. 2). This protein was also detected by western blot analysis using anti-GST antibody (data not shown), showing that it was the target GST–PNH1. Based on the amount of GST in the absorbed fraction, the yield of the GST–PNH1 protein was estimated to be 10 mg/L culture. The expression level of class I and class II hydrophobins in *E. coli* has been generally low (Scholtmeijer et al. 2001). Tagu et al. (2001) reported that the yield of His-tagged class I HYDPt-1,

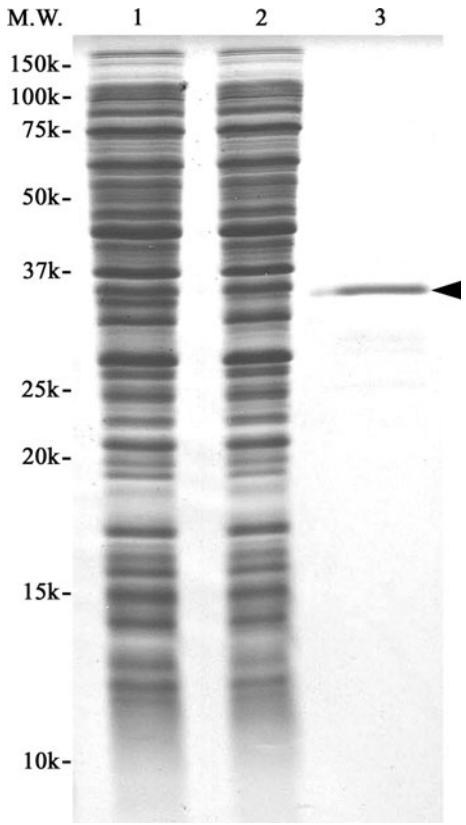

Fig. 2 Purification of GST–PNH1 by affinity chromatography. Proteins were separated by SDS-PAGE and visualized with Brilliant Blue R. *Lanes*: *1* Unpurified soluble fraction, *2* non-adsorbed fraction, *3* adsorbed fraction. GST–PNH1 is indicated by an *arrowhead*

which was expressed in the insoluble fraction of *E. coli* and solubilized using 6 M guanidinium hydrochloride, was only 1–2 mg/L culture. In comparison, therefore, in our study, the yield of the class I hydrophobin as a soluble protein was relatively higher. However, such proteins need to be produced at the gram per liter level for commercial applications. Lahtinen et al. (2008) have succeeded in expressing class II hydrophobin HFBI from *T. reesei* efficiently using an insect cell expression system. It is therefore possible that class I hydrophobins also could be produced at a high level using an insect cell, yeast, or cell-free protein expression system. We expect that the use of a cell-free expression system would improve the expression efficiency because hydrophobins, with their surfactant potency, may prevent the growth and the function of host cells.

To examine the emulsifying activity of the proteins, GST–PNH1, PNH1, and GST were prepared. PNH1 was obtained by digesting 100 μg purified GST–PNH1 with 1 U thrombin protease (GE Healthcare) in PBS for 24 h at 22°C, because there was a thrombin recognition sequence between GST and PNH1. The digestion was confirmed by SDS-PAGE and western blot analysis (data not shown).

GST was prepared from Origami B (DE3) transformed with empty pGEX-4T-1. Each 2 mL of liquid containing 100 μg/mL protein and 8% (v/v) soy oil was thoroughly mixed using a vortex mixer. After 1 h, the lower water phase was observed visually and with an optical microscope (BH-2; Olympus, Tokyo, Japan).

Emulsification was not observed in water and GST (Fig. 3a). On the other hands, PNH1 prepared by thrombin digestion and GST–PNH1 clearly showed emulsifying activity. After digestion by thrombin, PNH1 liberated from GST–PNH1 could be presumed to form an insoluble film. Because thrombin alone did not show any activity (data not shown), the emulsifying activity of PNH1 was confirmed to originate from the property of the hydrophobin. Observation with an optical microscope also showed the formation of oil-in-water micelles in the lower phase of GST–PNH1 (Fig. 3b). It has been reported that the class I hydrophobins SC3p purified from culture medium of *Schizophyllum commune* (Wösten et al. 1994) or Po.HYD1 purified from aerial hyphae of *Pleurotus ostreatus* (Ma et al. 2008) showed emulsifying activity. In our study, we demonstrated that class I hydrophobins PNH1 and GST–PNH1 prepared from transformed *E. coli* also have emulsification activity. de Vocht et al. (2000) reported that the formation of disulfide bridges formed by the eight conserved cysteine residues was essential for the stabilization and function of hydrophobin. Thus, the use of *E. coli* Origami B (DE3) as the host in our study may greatly facilitate cytoplasmic disulfide bond formation. The comparison between native and recombinant hydrophobins by circular dichroism and other spectroscopical methods will provide additional information on their three-dimensional structures, which was beyond the scope of the present investigation. It is possible that the expression in this *E. coli* strain allows the hydrophobin to retain its native properties. We did not performed a quantitative assay of the emulsifying activity, but our microscopic observation determined that GST–PNH1 and PNH1 showed almost the same emulsifying activities based on the extent of emulsification. Therefore, we suggest that the fusion with GST had little effect on the native emulsifying activity of PNH1. Class I hydrophobin is difficult to purify and is currently not well utilized as an emulsifying agent in industrial applications because the monomer forms a highly insoluble film through self-assembly. We propose that a fusion of class I hydrophobin and a highly soluble protein, such as the GST used in our study, will be a useful approach for preparing class I hydrophobins for various industrial purposes.

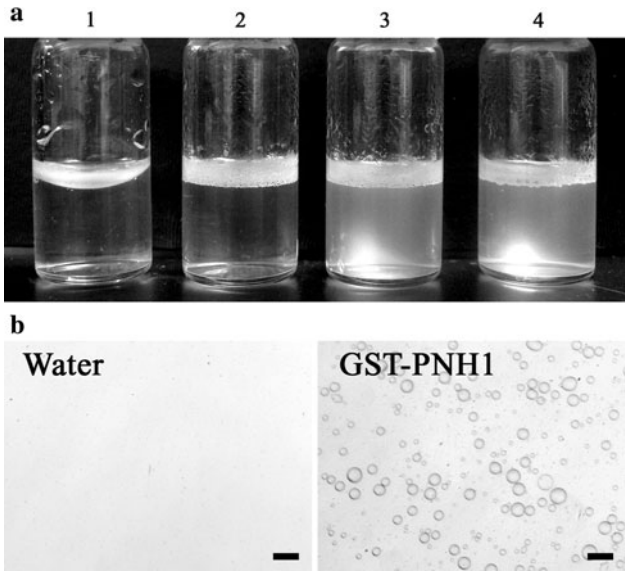

Fig. 3 Emulsifying activities of recombinant proteins. Each solution containing proteins was thoroughly mixed with soy oil and observed after 1 h. **a** Picture of emulsified solutions: *1* water, *2* GST, *3* GST–PNH1, *4* PNH1. **b** Optical microscopy images of the water phase. *Scale bar* 100 μm

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