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A low-cost affinity purification system using β -1,3-glucan recognition protein and curdlan beads

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Silkworm β -1,3-glucan recognition protein (β GRP) tightly and specifically associates with β -1,3-glucan. We report here an affinity purification system named the 'GRP system', which uses the association between the β -1,3-glucan recognition domain of β GRP (GRP-tag), as an affinity tag, and curdlan beads. Curdlan is a water-insoluble β -1,3-glucan reagent, the low cost of which (about 100 JPY/g) allows the economical preparation of beads. Curdlan beads can be readily prepared by solubilization in an alkaline solution, followed by neutralization, sonication and centrifugation. We applied the GRP system to preparation of several proteins and revealed that the expression levels of the GRP-tagged proteins in soluble fractions were two or three times higher than those of the glutathione S-transferase (GST)-tagged proteins. The purity of the GRP-tagged proteins on the curdlan beads was comparable to that of the GST-tagged proteins on glutathione beads. The chemical stability of the GRP system was more robust than conventional affinity systems under various conditions, including low pH (4–6). Biochemical and structural analyses revealed that proteins produced using the GRP system were structurally and functionally active. Thus, the GRP system is suitable for both the large- and small-scale preparation of recombinant proteins for functional and structural analyses.

Keywords: β -1,3-glucan recognition protein/affinity tag/
curdlan/glutathione S-transferase/recombinant protein

Introduction

The attachment of affinity-tags is a useful method for the preparation of recombinant proteins. In general, affinity-tags are paired with specific ligands immobilized on a solid matrix, with the following affinity-tag/ligand pairings in

widespread use: (i) glutathione S-transferase (GST) and glutathione (Smith and Johnson, 1988); (ii) polyhistidine peptide (His-tag) and metal chelate (Porath *et al.*, 1975); (iii) DYKDDDDK peptide (FLAG-tag) and anti-FLAG monoclonal antibody (Hopp *et al.*, 1988); (iv) WSHYPQFEK peptide (Strep-tag II) and modified streptavidin (Strep-Tactin) (Schmidt *et al.*, 1996); (v) maltose-binding protein (MBP) and amylose (di Guan *et al.*, 1988); and (vi) chitin-binding domain and chitin (Chong *et al.*, 1997). Each of these affinity systems affords both advantages and disadvantages. The size of the tag is small in the FLAG-tag, Strep-tag II and His-tag systems, whereas GST and MBP are both large protein tags composed of 211 and 396 amino acid residues, respectively, and the solubility of the fusion proteins of interest is increased in comparison with those when short peptide affinity tags are used. FLAG-tag is a popular affinity-tag for protein expression and purification in molecular biology. The immobilized anti-FLAG monoclonal antibodies, however, are expensive, which prevents large-scale protein preparation for drug screening and structural studies. Recombinant proteins are often expressed in an insoluble fraction in host cells, which must then be solubilized and purified under denaturing conditions, such as a 6-M guanidine-HCl solution, and refolded to obtain the native protein. Metal-chelating affinity systems can be used even under denaturing conditions, and are suitable for this purpose. Thus, a variety of affinity tag systems is still required for the production of recombinant proteins.

β -1,3-Glucan recognition protein (β GRP), first isolated from the hemolymph of the silkworm, *Bombyx mori*, is known as a sensor protein for β -1,3-glucan, a fungus cell wall component. β GRP recognizes the invasion of fungi and immediately evokes the innate immune responses, including the prophenol oxidase cascade and the Toll pathways. The β -1,3-glucan recognition domain of β GRP is composed of the residues from 1 to 102 (Ochiai and Ashida, 1988, 2000). Nuclear magnetic resonance (NMR) analysis of the interaction between the β -1,3-glucan recognition domain of β GRP and laminarin, a water soluble β -1,6 branched β -1,3-glucan polymer (Takahasi *et al.*, 2009), as well as the crystal structure in complex with the β -1,3-glucan hexamer (Kanagawa *et al.*, 2011), indicated that β GRP specifically bound to the triple-helical structure of β -1,3-glucan. The affinity of β GRP for β -1,3-glucan was high and β GRP could not be released under standard conditions, such as high salinity, to wash through the non-specifically bound proteins. This evoked the idea for an affinity purification system for recombinant proteins using β GRP and β -1,3-glucan.

β GRP associates with two natural products containing β -1,3-glucan, curdlan and laminarin. Curdlan, a linear β -1,3-glucan polymer, is insoluble in aqueous solution and, hence, is suitable for use in the affinity matrices. Furthermore, curdlan can be solubilized under highly

alkaline conditions, and reconstituted by either neutralization or by heating to over 60°C, which allows curdlan to be formed into affinity beads (Tada et al., 1998).

Here, we report an affinity purification system using the specific interaction between β -1,3-glucan-binding domain (GRP-tag) of β GRP and curdlan beads, designated the 'GRP system'. Moreover, we could readily prepare curdlan beads by the solubilization of curdlan in an alkaline solution, followed by neutralization, sonication, centrifugation and decantation. We prepared several proteins using the GRP system, and demonstrated that the purity, yield and activity of the proteins were suitable for biochemical and biophysical studies. It can be concluded that the GRP system is useful for both the large- and small-scale preparation of recombinant proteins for functional and structural studies at low cost.

Materials and methods

Construction of the expression vectors pET-GRP-3C-His and pET-GST-3C-His

The DNA fragment coding silkworm β GRP (1–111), named the *GRP-tag*, was amplified by polymerase chain reaction (PCR) with the primers GGAATTCCATATGTACGAGGC ACCACCGGC and CATGCCATGGAGCCCTCTGTGTTTA CTGGATT from silkworm β GRP cDNA (NM_001043375.1) (Ochiai and Ashida, 2000) as a template, and was digested using *NdeI* and *NcoI*. The digested DNA fragment was cloned into pET-22b (+) (Novagen) using the same sites. The resultant plasmid was named pET-GRP-His. The DNA fragment coding the HRV 3C protease recognition sequence was amplified by PCR from the pGEX-6P-1 plasmid (U78872.1) (GE healthcare) using the primers CATGCCATGGCACTGGAA GTTCTGTTCCAGGGG and CGTCAGTCAGTCACGAT GCG, and was digested using *NcoI* and *XhoI*. The digested DNA fragment was cloned into pET-GRP-His using the same sites. The resultant plasmid was named pET-GRP-3C-His.

The DNA fragment coding *glutathione S-transferase*, named the *GST-tag*, was amplified by PCR using the primers GGAATTCCATATGTCCCCTATACTAGGTTATTGG and CATGCCATGGCATCCGATTTTGGAGGATGGT from the pGEX-6P-1 plasmid, digested using *NdeI* and *NcoI* and cloned into the same sites of pET-GRP-3C-His. The resultant plasmid was named pET-GST-3C-His.

Construction of the plasmids for the expression of GRP- and GST-tagged proteins

The DNA fragment coding human RIG-I with a stop codon was amplified by PCR with the forward primer CGGGATCCATGACCACCGAGCAGCGACGC, the reverse primer CCGCTCGAGTTATTTGGACATTTCTGCTGGATC, and human RIG-I cDNA (NM_014314.3) as a template, digested using *BamHI* and *XhoI*, and cloned into the same sites of both pET-GRP-3C-His and pET-GST-3C-His, respectively. The resultant plasmids were named pET-GRP-3C-RIG-I and pET-GST-3C-RIG-I, respectively, and they did not contain a hexahistidine tag at the C-terminus of the coding proteins. The genes for Caf1 (NM_013354.5), TobN, and the N-terminals of Tob (NM_005749.2) and IRF3 (NM_001571.5) were also cloned into the pET-GRP-3C-His and pET-GST-3C-His plasmids in a similar manner.

The expression plasmid for GRP-3C-RIG-I-His was also constructed for the purpose of two-step affinity purification. The DNA fragment coding human RIG-I without a stop codon was amplified by PCR with the primers CGG GATCCATGACCACCGAGCAGCGACGC and CCGCTC GAGTTTGGACATTTCTGCTGGATCAAATG from human RIG-I cDNA, and digested with *BamHI* and *XhoI*. The digested DNA was cloned into the same sites of pET-GRP-3C-His. The resultant plasmid was named pET-GRP-3C-RIG-I-His. The genes for Uba1 (NM_003334.3) and UbcH5B (NM_003339.2) were also cloned into pET-GRP-3C-His for two-step purification in a similar manner.

Preparation of the curdlan beads by sonication method

Curdlan powder (0.1 g; Wako) was dissolved in 10 ml of 0.1 M NaOH at 25°C. The curdlan solution was centrifuged at 30 000 g at 25°C for 10 min and the supernatant was collected. The supernatant was mixed with 0.5 ml of 10% Tween 20 and 10 ml of 1-butanol, and sonicated at a power of 50 W using a Branson Model 250 sonicator at an output setting of 5 for 10 s on ice. The sonicate was neutralized by 50 μ l of glacial acetic acid and was further sonicated at an output setting of 5 for 10 s on ice. This neutralization reaction was repeated six times. The reaction mixture was centrifuged at 500 g at 4°C for 5 min and the supernatant was discarded. The precipitate was suspended with 40 ml of deionized water and centrifuged at 500 g at 4°C for 5 min. This step was repeated three times. The precipitate was then suspended with 20% ethanol to form a 20% slurry and stored at 4°C. The curdlan beads were equilibrated with a 10-bed volume of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 20 mM imidazole and 5 mM sodium azide prior to use for affinity purification.

Expression and purification of the GRP- and GST-tagged proteins

Escherichia coli BL21 (DE3) competent cells were transformed with expression plasmids pET-GRP-3C-TobN, pET-GST-3C-TobN, pET-GRP-3C-Caf1, pET-GST-3C-Caf1, pET-GRP-3C-IRF-3, pET-GST-3C-IRF-3, pET-GRP-3C-RIG-I and pET-GST-3C-RIG-I. A single colony was inoculated into 5 ml of luria-bertani (LB) medium containing 50 μ g ml⁻¹ carbenicillin, and incubated at 37°C for 18 h with constant shaking (~180 rpm). A 0.1 ml aliquot of the cell culture was then added to 10 ml of LB medium containing 50 μ g ml⁻¹ carbenicillin, and incubated at 37°C with constant shaking (~120 rpm) until the OD600 reached 0.6. Expression of the recombinant proteins was induced by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.01 mM. The culture was further incubated at 16°C for 24 h with gentle agitation (~90 rpm). The cells were harvested by centrifugation at 4000 g at 4°C for 15 min, and were resuspended in 1 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, 1% Triton X-100, 5 mM sodium azide, 7.5 kU/ml rLysozyme (Merck), 25 U/ml Benzonase (Sigma) and a protease inhibitor cocktail (Nakalai Tesque).

The cell suspension was lysed three times using an Ultra Sonic Homogenizer UH-50 (SMT Co., Ltd) at an output setting of 8 for 20 s on ice, and the lysate was centrifuged at 20 000 g at 4°C for 30 min. The supernatants containing the GRP- and GST-tagged proteins were mixed with 0.2 ml of

20% slurry of curdlan beads and 0.2 ml of 20% slurry of glutathione sepharose 4B (GE healthcare), respectively, and incubated at 16 h at 4°C with gentle agitation. The beads were collected by centrifugation at 500 g at 4°C for 5 min, and washed twice with 1 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 1 M NaCl, 5 mM sodium azide. The beads were then suspended in 0.25 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, 1% Triton X-100 and 5 mM sodium azide, and the GRP-tagged proteins were treated with 0.5 μ g of GST-HRV3C protease at 4°C for 16 h.

Two-step affinity purification for the GRP-tagged proteins

Escherichia coli BL21 (DE3) competent cells were transformed with the expression plasmid pET-GRP-3C-RIG-I-His. Twenty-five milliliters of the cell lysate containing the GRP-3C-RIG-I-His protein was applied to a 4-ml Ni-NTA Superflow affinity column (Qiagen) equilibrated with 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole and 5 mM sodium azide. The column was washed with 10 column volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 20 mM imidazole and 5 mM sodium azide and 10 column volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 1 M NaCl and 5 mM sodium azide. GRP-RIG-I-His was eluted with five column volumes of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 250 mM imidazole and 5 mM sodium azide. The eluate was mixed with 0.5 ml of 20% slurry of curdlan beads at 4°C for 16 h with gentle agitation. The beads were washed twice with 10 ml of 50 mM sodium phosphate buffer (pH 8.0) containing 1 M NaCl, and 5 mM sodium azide. The beads were suspended with 1 ml of 100 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and the GRP-3C-RIG-I-His was digested by adding 10 μ l of the GST-tagged HRV-3C protease at 4°C for 16 h. RIG-I-His released from the curdlan beads was recovered from the supernatant after centrifugation at 500 g at 4°C for 5 min. Both Uba1-His and UbcH5B-His were similarly purified using a two-step affinity chromatography method.

Circular dichroism spectroscopy

RIG-I-His, purified by two-step purification, was loaded onto a Hi-Load 26/60 Superdex 200pg gel-filtration column equilibrated with 20 mM sodium phosphate buffer (pH 6.8) containing 150 mM NaCl. The eluted fractions containing RIG-I-His were collected and analyzed using a circular dichroism spectrometer J-725 (Jasco). The Far-UV spectrum from 200 to 260 nm was recorded in a 1-mm path length quartz cell at 20°C. The protein concentration was adjusted to 50 μ M with 20 mM sodium phosphate (pH 6.8) containing 150 mM NaCl. An average of eight scans were recorded at 0.1-nm intervals at a rate of 2 s per point and at a scan speed of 20 nm s⁻¹.

ATPase assay

RIG-I-His was incubated for 15 min at room temperature in the buffer (20 mM Tris (pH 8.0), containing 1.5 mM MgCl₂, 1.5 mM DTT) with or without 1 μ g polyI:C. ATP was then added at a final concentration of 1 mM, and the mixture was incubated at 37°C for 15 min followed by phosphate determination using BIOMOL GREEN Reagent (BIOMOL Research Laboratories) (Takahasi *et al.*, 2008).

Expression and purification of UbcH5B using the GB1-fusion system

The fragment coding UbcH5B was cloned into pGB1HPS (Kobashigawa *et al.*, 2009). UbcH5B was expressed as a fusion protein with an N-terminus GB1, hexahistidine tag and HRV3C protease recognition site using *E. coli* strain Rossetta (DE3) at 25°C. Uniformly ¹⁵N-labeled protein was prepared by culturing cells in M9 minimum medium using ¹⁵NH₄Cl as a sole nitrogen source. UbcH5B was purified using Ni²⁺-affinity column chromatography, followed by HRV3C protease digestion to remove the tag, and then further purified by gel filtration chromatography on Superdex 75 (GE Healthcare).

In vitro ubiquitination assay

In vitro ubiquitination assay was performed as described previously (Kobashigawa *et al.*, 2011). Briefly, the ubiquitination reaction was carried out at 30°C in 25 ml of reaction solution containing 20 mM HEPES-KOH (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 0.25 μ g Uba1-His derived from the GRP-tagged proteins or commercial Uba1 E1 (Sigma), 0.5 μ g UbcH5B, 5.0 μ g Cbl-b (39–426), 0.5 μ g N-terminus hexahistidine tag-attached ubiquitin and 10 μ g creatine kinase. The reaction was terminated at 0, 30, 60 and 120 min for western blotting analysis. Ubiquitination was monitored by immunoblotting using peroxidase-conjugated anti-polyhistidine antibody (Sigma).

NMR spectroscopy

All NMR experiments were carried out at 25°C on a Varian Inova 500 MHz NMR spectrometer equipped with four radio frequency channels and pulse-field gradients. The protein sample concentration was 300 μ M for both proteins prepared using the GB1 and GRP fusion systems. For all measurements, the sample solution contained 20 mM MES (pH 6.3), 1 mM CaCl₂, 2 mM DTT and 150 mM NaCl in 90% H₂O/10% ²H₂O.

Results

The expression plasmids for the GRP system

Our previous structural study revealed that the N-terminal 102 residues of β GRP forms a structural domain, which tightly binds to β -1,3-glucan, including both curdlan and laminarin (Takahasi *et al.*, 2009). This finding evoked the idea for an affinity chromatography system using the specific interaction between the N-terminal domain of β GRP and curdlan. We constructed the expression plasmid for the N-terminal domain, from 1 to 111 residues, of β GRP, referred to as a GRP-tag (Fig. 1A), which can be fused with the proteins of interest. As a basic construct, we used the plasmid pET-22b(+), which consists of a T7 promoter/operator, Shine-Dalgarno sequence, T7 terminator, β -lactamase gene and pBR322 replication origin. The region between *Nde*I and *Nco*I, which contains an initiating methionine codon and the *pelB* coding sequence of pET-22b (+), was replaced by the GRP-tag sequence amplified by PCR. The DNA fragment coding the cleavage sequence of the HRV 3C protease was inserted between the GRP-tag and the multiple-cloning site. The resultant plasmid, designated pET-GRP-

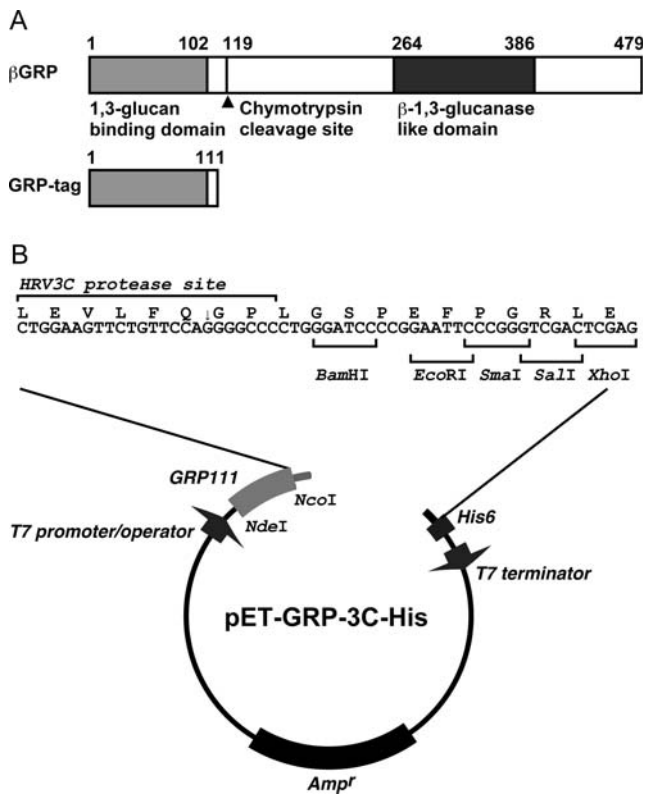


Fig. 1. Schematic representation of the GRP system. (A) Silk worm βGRP and the GRP-tag. (B) The sequence of the multicloning site of the pET-GRP-3C-His expression plasmid.

3C-His, can be used for the expression of the recombinant proteins fused to the GRP-tag at the N-terminus and to the hexahistidine-tag (His-tag) at the C-terminus (Fig. 1B). As a control, the expression plasmid of the GST-tagged protein was constructed. The resultant plasmid was designated pET-GST-3C-His.

Expression of the GRP-tagged proteins in *Escherichia coli*

As the target proteins of interest to be fused with the GRP-tag, we selected the N-terminal domain of Tob (TobN), CCR4-associated factor 1 (Caf1), interferon regulatory factor 3 (IRF-3) and retinoic acid-inducible gene I protein (RIG-I). TobN has 138-amino acid residues (16 kDa) and is composed of a domain characteristic of the BTG/Tob antiproliferative protein family proteins (Matsuda et al., 1996). Caf1 contains 285-amino acid residues (33 kDa) and is known to be a major component of poly(A)-deadenylase in mammalian cells (Bogdan et al., 1998). IRF-3 is a 427-amino acid residue protein (47 kDa) and is known to be a transcription factor activated by microbial invasion to produce a variety of cytokines including type-I interferons (Au et al., 1995). RIG-I is a DExD/H-box ATPase consisting of 925-amino acid residues (106 kDa) and is known to be a cytosolic viral RNA sensor responding to invasion by RNA viruses including paramyxoviruses, influenza viruses, Japanese encephalitis viruses and hepatitis C viruses (Yoneyama et al., 2004).

The DNA fragments coding TobN, Caf1, IRF-3 and RIG-I were cloned into the multiple-cloning site of pET-GRP-3C-His and pET-GST-3C-His, respectively. *Escherichia coli* BL21(DE3) strains was transformed by the expression

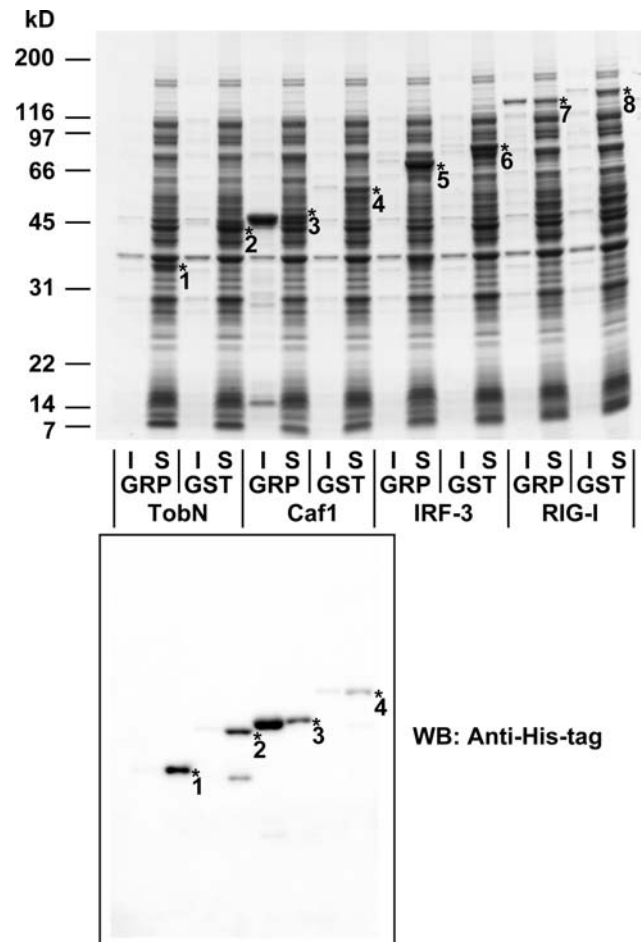


Fig. 2. SDS-PAGE profiles of protein expression (upper panel). The insoluble (I) and soluble (S) fractions of *E. coli* BL21 (DE3) cells expressing the GRP- and GST-tagged proteins at 16°C for 16 h after induction are displayed. *1, GRP-TobN; *2, GST-TobN; *3, GRP-Caf1; *4, GST-Caf1; *5, GRP-IRF-3; *6, GST-IRF-3; *7, GRP-RIG-I; *8, GST-RIG-I. Western blot analysis of the hexahistidine-tagged proteins detected by His-Probe HRP reagent (lower panel). As we have not constructed the hexahistidine-tag attached GST-IRF-3 and GST-RIG-I, we did not perform western blot analysis for both IRF-3 and RIG-I.

plasmids pET-GRP-3C-TobN, pET-GRP-3C-Caf1, pET-GRP-3C-IRF-3 and pET-GRP-3C-RIG-I, respectively. Expression of the GRP-fusion proteins was induced by the addition of 0.01 mM IPTG and further incubation at 16°C for 24 h. After harvest, the cells were disrupted by sonication, and the proteins in the insoluble and soluble fractions were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (lanes marked GRP in Fig. 2). GRP-TobN (30 kDa) and GRP-IRF-3 (62 kDa) were observed mainly in the soluble fraction. On the other hand, GRP-Caf1 (47 kDa) and GRP-RIG-I (120 kDa) were observed in both the soluble and insoluble fractions, with GRP-Caf1 observed mainly in the insoluble fraction.

In order to compare both the expression level and the solubility of the GRP-tagged proteins with those of the GST-tagged proteins, the expression of GST-TobN (43 kDa), GST-Caf1 (60 kDa), GST-IRF-3 (74 kDa) and GST-RIG-I (134 kDa) was performed under the same conditions as those used for the GRP-tagged proteins (lanes marked GST in Fig. 2). All of the GST-tagged proteins were expressed mainly in the soluble fractions. The expression levels of GRP-tagged proteins in the soluble fractions were similar, or

two or three times higher than those of the GST-tagged proteins, except for RIG-I. Interestingly, the total expression levels of GRP-tagged proteins were higher than those of GST-tagged proteins (upper panel in Fig. 2).

We further analyzed the expression levels of the GRP-tagged and GST-tagged TobN and Caf1 proteins with the hexahistidine tag at the C-terminus by the western blot analysis using a hexahistidine tag detection reagent, His-probe HRP (Pierce). As we have not constructed the hexahistidine-tag attached GST-IRF-3 and GST-RIG-I, we did not perform western blot analysis for both IRF-3 and RIG-I. The result indicated that the expression level of GRP-TobN protein was 1.6-fold higher than that of GST-TobN in the supernatant of bacterial lysate. The expression level of GRP-Caf1 was 3.0-fold higher than that of GST-Caf1 in the supernatant of bacterial lysate. The relative amounts of soluble vs. insoluble expression of GRP-TobN, GST-TobN, GRP-Caf1 and GST-Caf1 were 69.8, 27.8, 0.3 and 4.5-fold, respectively (lower panel in Fig. 2).

Affinity purification of the GRP-tagged proteins using curdlan beads

In order to examine whether the GRP-tagged proteins could be purified using the curdlan beads, the soluble fractions containing GRP-tagged TobN, Caf1, IRF-3 and RIG-I were mixed with the curdlan beads, washed with phosphate buffer containing 1 M NaCl, and then the proteins bound on the beads were analyzed by SDS-PAGE (lanes marked GRP in Fig. 3). All of the GRP-tagged proteins were purified on the beads and their molecular weights were in agreement with the calculated weights. We quantified the amount of GRP-TobN, GRP-Caf1, GRP-IRF3 and GRP-RIG-I expressed and bound to the curdlan beads with the purified GRP-UbcH5B as an internal concentration standard by western blot analysis using anti-GRP antibody (Ochiai *et al.*, 1992). The purity of each GRP-tagged protein on the curdlan beads was estimated by imageJ software. The yields and

purities of GRP-tagged proteins were summarized in Table II. In terms of non-specific binding to the curdlan beads, the purity of the GRP-tagged proteins was comparable to that of the GST-tagged proteins bound on the glutathione beads (lanes marked GST in Fig. 3). Although the expression level of GRP-RIG-I in the soluble fraction was similar to that of GST-RIG-I (Fig. 2), the amount of GRP-RIG-I on the curdlan beads was approximately two times higher than that of GST-RIG-I on the glutathione beads. This situation was not changed by increasing the volume of the GST-beads. Half of the GST-RIG-I in the soluble fraction passed through the glutathione beads at the washing step. Although further analysis is required, half of the GST-RIG-I in the soluble fraction is thought to form a micro aggregate due to incomplete folding. These results indicated that the GRP-tag could induce better conformation of the recombinant proteins than did the GST-tag in *E. coli*.

Chemical stability of the GRP-tagged protein–curdlan bead complex

The purification conditions for the affinity chromatography systems are restricted by the physical and chemical stabilities between the affinity tag and its ligand. Hence, we studied the stabilities of the association of the GRP-fusion protein to the curdlan beads in pH range between 4 and 9. First, GRP-RIG-I was solubilized in a buffer with a pH between 4 and 9. After centrifugation, each supernatant was mixed with the curdlan beads and the unbound proteins were washed out with buffer at each pH. The complexes of GRP-RIG-I with the curdlan beads were digested with GST-HRV 3C proteases and both supernatants and precipitates were analyzed by SDS-PAGE (Fig. 4A). GRP-RIG-I was stably bound to the curdlan beads between pH 4 and 9, and was completely digested with GST-HRV between pH 7 and 9 (Fig. 4A). GRP-RIG-I was not digested under pH 5 and only partially digested at pH 6, as these conditions were outside the optimal pH range of HRV 3C protease (between pH 7 and 8.5). Moreover, the digested RIG-I was present in the precipitate at pH 6, as this condition was close to the isoelectric point (calculated pH of 6.03). Thus, the GRP-system is robust across a wide pH range of between 4 and 9, whereas conventional affinity purification systems including GST-tag, MBP-tag and His-tag cannot be used at a pH below 6.

Next, we studied the chemical stability of the affinity purification systems. Almost all of the affinity-tag systems exhibit instability in the presence of high concentrations of urea (GST-tag) or imidazole (His-tag). In order to determine the chemical stability of the complexes of the GRP-tagged proteins with the curdlan beads, we performed binding experiments between the curdlan beads and the GRP-tagged proteins dissolved in the aqueous solutions containing high concentration of the reagents listed in Table I. The GRP-tagged proteins stably bound to the curdlan beads, except in the presence of 8 M urea (Table I and Fig. 4B). Hence, the eluate of the conventional affinity-tag systems involving GST-tag, His-tag, MBP-tag and Streptag-II-tag can be directly loaded onto the GRP-system for secondary affinity purification.

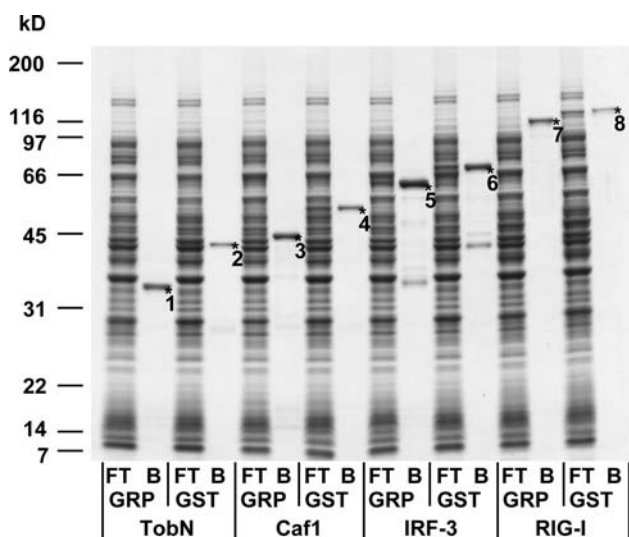


Fig. 3. Affinity purification of the GRP- and GST-tagged proteins using the curdlan beads and the glutathione beads. SDS-PAGE analysis of the proteins in flow-through and on the curdlan beads after washing. FT, flow-through proteins; B, proteins bound on beads; *1, GRP-TobN; *2, GST-TobN; *3, GRP-Caf1; *4, GST-Caf1; *5, GRP-IRF-3; *6, GST-IRF-3; *7, GRP-RIG-I; *8, GST-RIG-I.

Table I. Summary of curdlan-binding ability of GRP-tag under chemicals

| Reagents ^a | Concentration | Curdlan-binding ability of GRP-tag | Reagents ^a | Concentration (M) | Curdlan-binding ability of GRP-tag |
|-----------------------|---------------|------------------------------------|-------------------------|-------------------|------------------------------------|
| Triton X-100 | 10% | + | DTT | 0.1 | + |
| | 20% | + | | 0.2 | + |
| NaCl | 2 M | + | Reduced glutathione | 0.1 | + |
| | 4 M | + | | 0.2 | + |
| Urea | 4 M | + | Imidazole | 0.5 | + |
| | 8 M | - | | 1 | + |
| L-Arg | 0.5 M | + | Maltose | 0.1 | + |
| | 1 M | + | | 0.2 | + |
| EDTA | 0.1 M | + | <i>d</i> -Desthiobiotin | 0.01 | + |
| | 0.2 M | + | | 0.02 | + |

^aAll reagents were dissolved in 100 mM Tris and adjusted to pH 8.0 with HCl or NaOH.

Table II. Yield and purity of GRP-tagged proteins

| | TobN ^a | Caf1 ^a | IRF3 ^a | RIG-I ^a | UbcH5B-His ^b |
|-------------------------------------|-------------------|-------------------|-------------------|--------------------|-------------------------|
| Yield (mg per 1 l culture) | 8 | 23 | 26 | 13 | 10 |
| Purity of one-step purification (%) | 88 | 87 | 61 | 87 | 82 |
| Purity of 2 step purification (%) | - | - | - | - | 93 |

^aThe yield was determined by either western blotting using anti-GRP antibody

^bThe yield was determined by absorbance at 280 nm.

The purity was estimated from the intensity of the band stained with Coomassie Brilliant Blue of SDS-PAGE using imageJ software.

Analysis of the catalytic activity and structure of the recombinant proteins produced by the GRP system

The association of GRP to the curdlan beads was stable even in the presence of various reagents, including high concentration of imidazole, so that we could apply a two-step affinity purification to obtain intact proteins using both GRP and hexahistidine tags. RIG-I was used for this purpose, as it exceeds 100 kDa and is partially degraded by endogenous *E. coli* protease. Immediately after the first-step chromatography, the elution fraction of the Ni-chelating resin contained several degraded GRP-RIG-I-His proteins (lanes marked Ni in Fig. 5A). After the second chromatography using the curdlan beads, the full length GRP-RIG-I-His was concentrated on the beads as the major band. GRP-RIG-I-His was digested with GST-HRV3C protease, and RIG-I-His was released into the flow-through fraction (lanes marked SC in Fig. 5A).

In order to evaluate whether the proteins prepared by the GRP system were structurally and functionally active, we measured the secondary structure and RNA-dependent ATPase activity of RIG-I. The fractional composition of the secondary structure of RIG-I was estimated from the circular dichroism (CD) spectrum using the manufacturer's program. CD measurement revealed that RIG-I consisted of 61% α -helix, 13% β -strand and 26% coil or random structure. RIG-I obtained using the GRP system was considered to possess a properly folded structure (Fig. 5B). Finally, the ATPase activity of RIG-I was analyzed in the presence or absence of Poly I:C. RIG-I derived from the GRP-tagged protein exhibited ATPase activity only in the presence of

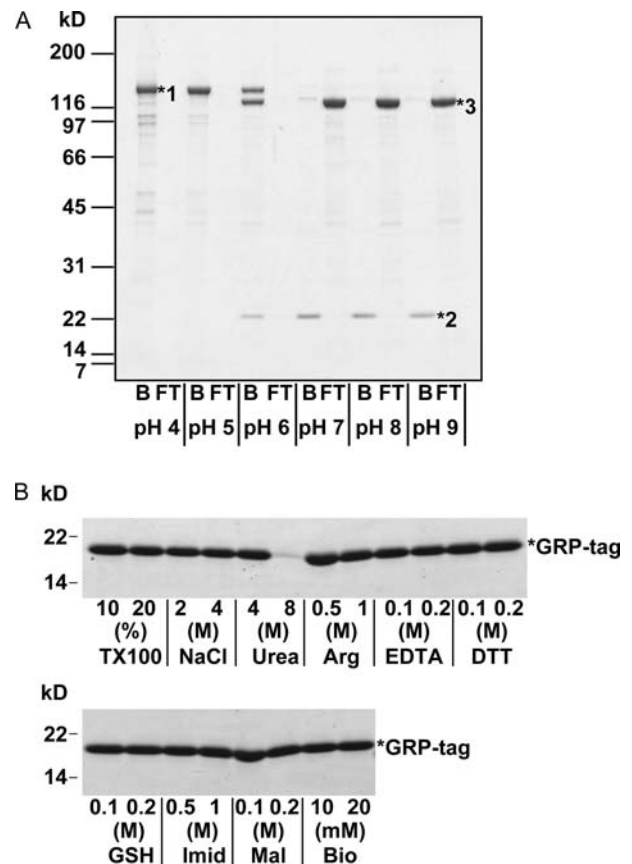


Fig. 4. Chemical stability of the complexes of the GRP-tag and the curdlan beads. (A) The stability of the GRP-tag–curdlan complexes between pH 4 and 9. GRP-RIG-I solubilized in a buffer at a pH of 4, 5, 6, 7, 8 and 9 was mixed with the curdlan beads, respectively. The beads of the each sample were washed with the same buffer and the remaining GRP-RIG-I on the beads was digested with GST-HRV 3C protease. After digestion, the proteins on the beads (Lane B) and in the flow-through of the beads (FT) were analyzed by SDS-PAGE. *1, GRP-RIG-I; *2, GRP-tag; *3, RIG-I. (B) The stability of the GRP-tag–curdlan complexes in the presence of chemical compounds generally used in protein purification. The complexes of the GRP-tag with the curdlan beads were mixed with the solutions as indicated in Table I and further washed with the same solutions, respectively. The residual complexes on the beads after washing were analyzed by SDS-PAGE.

Poly I:C, whereas no ATPase activity was detected in its absence (Fig. 5C). Thus, the structural analysis as well as enzymatic activity of RIG-I supported the notion that RIG-I produced using the GRP system was correctly folded, and

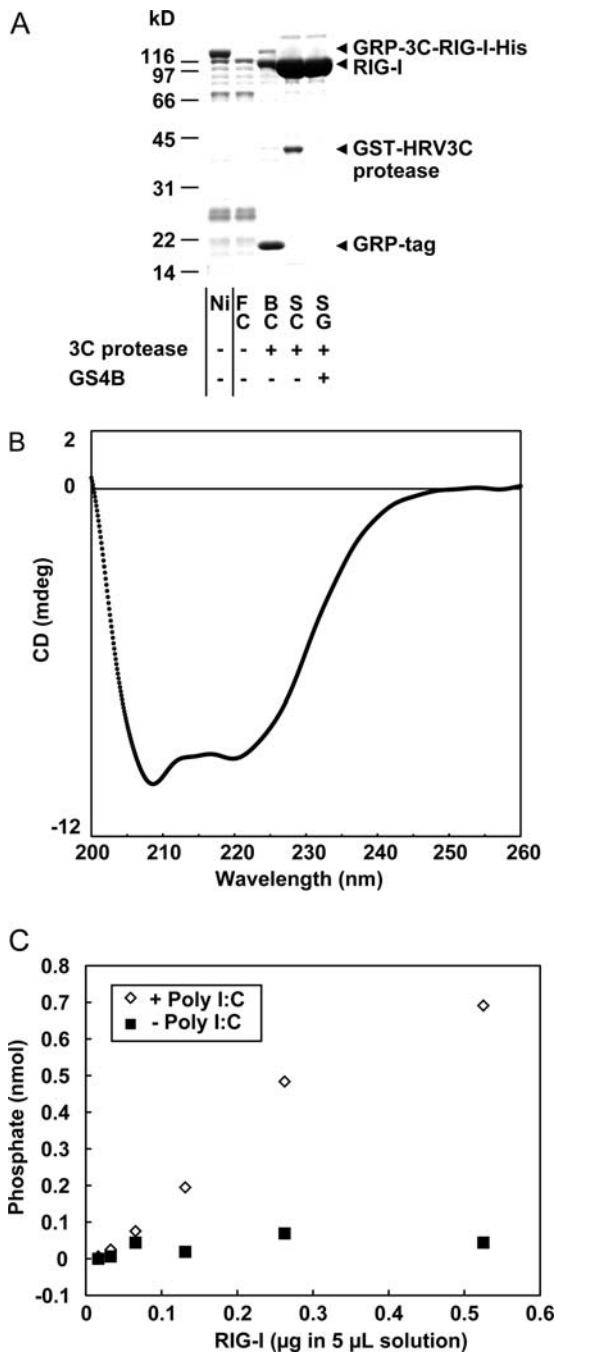


Fig. 5. Two-step affinity purification of the RIG-His protein. (A) SDS-PAGE analysis of each step in purification. Ni, eluate from Ni-NTA Superflow; FC, flow-through of eluate from Ni-NTA Superflow of the curdlan beads; BC, proteins bound on the curdlan beads after digestion with GST-HRV3C-His protease; SC, proteins released from the curdlan beads after digestion with GST-HRV3C protease; SG, proteins after removal of GST-HRV3C protease by glutathione sepharose 4B (GS4B). (B) Far-UV circular dichroism spectrum of the RIG-His obtained by the two-step affinity purification. (C) Assay of the ATPase activity of RIG-His.

could be used for further crystallographic analysis, NMR spectroscopy and drug screening.

Next, we examined the catalytic activity of ubiquitin-like modifier-activating enzyme 1 (Uba1), a large protein with the molecular weight of >110 kDa, and the E2 ubiquitin-conjugating enzyme UbcH5B prepared by the GRP system using a two-step affinity chromatography. To express

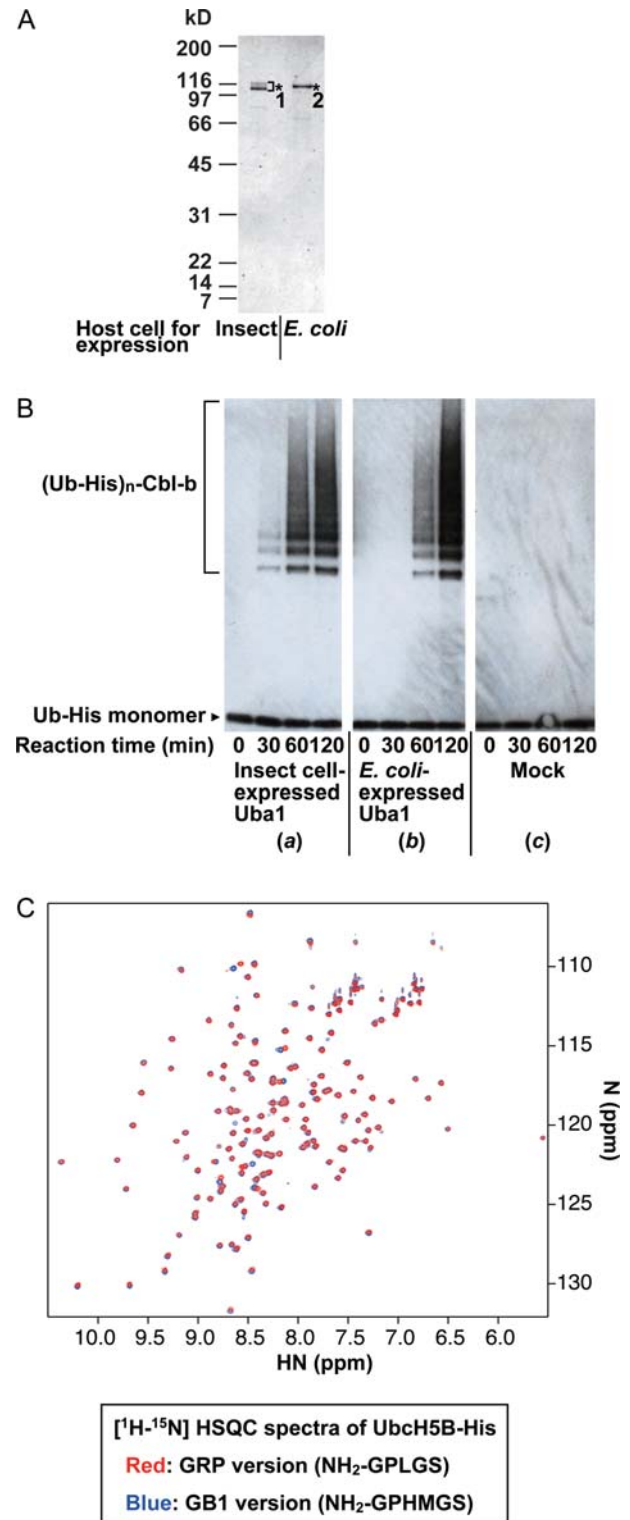


Fig. 6. Functional analyses and structural properties of the GRP-tagged ubiquitination-related proteins purified by two-step affinity purification. (A) SDS-PAGE analysis of Uba1 for *in vitro* ubiquitination assay. Lane 1: Insect cell-expressed commercial Uba1 without an affinity-tag and lane 2: *E. coli*-expressed Uba1 with a C-terminal His-tag after removal of the N-terminal GRP-tag. (B) Immunoblot with anti-histidine-tag antibody against the His-tagged ubiquitin after the ubiquitination reaction. A mixture of Cbl-b and ubiquitin was incubated with Uba1 for 0, 30, 60 and 120 min at 37°C. Lane a: commercial Uba1 expressed in insect cells. Lane b: monomer fraction of Uba1 derived from the GRP-tagged fusion protein expressed in *E. coli* as E1. Lane c: mock without Uba1. (C) ¹H-¹⁵N HSQC spectra of UbcH5B prepared using the GRP-fusion (red) and GB1-fusion systems (blue).

GRP-tagged Uba1-His, we used the mutant Uba1 with a 44-amino acid residue deletion at the amino terminus, because the deleted region had low homology to other species. The theoretical molecular weight of purified Uba1-His is 115 kDa, which was in agreement with the molecular weight obtained from SDS-PAGE (marked *2 in Fig. 6A). Uba1-His derived from the GRP-tagged protein was mixed with three proteins: the E2 ubiquitin-conjugating enzyme UbcH5B-His, the E3 ubiquitin ligase Cbl-b and His-tagged ubiquitin. After incubation, ubiquitinated Cbl-b was detected by immunoblotting using an anti-His-tag antibody (Fig. 6B). As a positive control, the ubiquitination reaction using a commercially available recombinant Uba1 expressed in insect cells (Sigma; marked *1 in Fig. 6A) was performed (Fig. 6B). It was revealed that the activity of Uba1 expressed in *E. coli* using the GRP system was similar to that expressed in insect cells.

In order to evaluate whether the GRP system was applicable to the production of proteins for use in structural biology, we prepared ¹⁵N-labeled UbcH5B-His from GRP-tagged protein by two-step affinity chromatography using the GRP system as the first step and the nickel-chelating affinity system as the second step. The purity of UbcH5B released from the curdlan beads at the first step and eluted from nickel-chelating beads was 82% (lane first in Supplementary Fig. S1 and Table II) and 93% (lane second in Supplementary Fig. S1 and Table II), respectively. After buffer exchange by gel filtration, the NMR spectrum of the labeled ¹⁵N-labeled UbcH5B was measured. The ¹H-¹⁵N HSQC spectrum of UbcH5B was well-dispersed and was almost consistent with the reference spectrum of UbcH5B prepared using the GB1-fusion system (Kobashigawa *et al.*, 2011) (Fig. 6C). The yield of purified ¹⁵N-labeled UbcH5B using the GRP system was ~10 mg, which is sufficient for structural analysis using NMR and X-ray crystallography.

Finally, we examined the binding capacity of the curdlan beads against GRP-UbcH5B protein. 10, 20, 50, 100, 200 and 500 µg of the purified GRP-UbcH5B (32 kDa) were mixed with 20 µl bed volume of the curdlan beads. Both the GRP-tagged proteins binding on the beads and the excess of those in the supernatant were detected by Coomassie Brilliant Blue staining on SDS-PAGE (Supplementary Fig. S2). The binding capacity of the curdlan beads was determined to be 5 mg of the GRP-UbcH5b-His per 1 ml of the beads. This means that 1 ml of the curdlan beads can bind to 0.2 µmol of GRP-fusion protein. Total binding capacity of the curdlan beads is similar to that of the glutathione beads shown in the manufacturer's manual (GE Healthcare). Thus, the GRP system is expected to be useful for the production of protein samples for structural studies at high yield and low cost.

Discussion

In this paper, we described the establishment of an affinity purification system using a GRP-tag and curdlan beads. Six GRP-tagged proteins expressed in *E. coli* were purified on the curdlan beads. The GRP-tag could be removed by GST-HRV 3C protease, and the target protein of interest could be released from the curdlan beads. Functional and structural analyses revealed that biologically active RIG-I, UbcH5B and Uba1 could be prepared using the GRP system.

Although the total amount of GRP-RIG-I in the soluble fraction after sonication was less than that of GST-RIG-I, the amount of GST-RIG-I bound to the affinity beads was less than the GRP-RIG-I bound to the curdlan beads. This suggests that GST-RIG-I could not be cleaved by HRV 3C protease because it partially forms a micro aggregate, unlike GRP-RIG-I.

The GRP-tag was stably bound to the curdlan beads at a pH of between 4 and 9. The binding stability at a pH <6 has an advantage in that the functional assay of recombinant proteins is possible on curdlan beads even under weakly acidic conditions. The chemical stability test exhibited that the complex formation between the GRP-tag and curdlan is stable in the presence of the elution buffers used in other affinity chromatography systems, including 10 mM reduced glutathione for GST-tag, 250 mM imidazole for His-tag, 10 mM maltose for MBP-tag and 2.5 mM *d*-desthiobiotin for Streptag-II. Large-sized proteins, such as RIG-I and UBA1, possess labile regions from which they can be easily degraded by proteases in *E. coli*. In such cases, the tandem affinity purification tag (TAP-tag) is useful for obtaining intact proteins (Rigaut *et al.*, 1999). To date, the combinations of affinity purification systems available are (i) GST-tag with His-tag and (ii) His-tag with Streptag-II. In this paper, we tested whether the GRP system could be used for the preparation of intact proteins directly from the eluate of metal-chelating affinity chromatography. From the chemical stability test, it is expected that GRP-tag could be used for the direct preparation of proteins from the eluate of GST-tag, MBP-tag, Streptag-II and so on. Hence, the GRP-system is thought to be suitable as a secondary step for TAP-tag.

At present, it is difficult to elute GRP-tagged proteins from curdlan beads under native conditions. This property presents a disadvantage in comparison with affinity-tags eluted with the ligands. However, in the case of on-gel digestion using the sequence-specific proteases, such as HRV 3C protease, the GRP-system is comparable with other affinity purification systems, such as the GST-system, in terms of protein purification. Of course, GRP-tagged proteins might be useful as the bait for pull-down binding assays and proteomics studies as they were eluted in the presence of 8 M urea (Fig. 4B) or 2% SDS in the SDS-PAGE sample buffer.

In many cases, affinity beads are packed and used in a column. However, the size of the curdlan beads used in this paper is so small that these pass through an end-fitting of a column. In order to solve this problem, we have tried to prepare a larger size of the curdlan beads that can be used for the gravity-flow affinity chromatography. The details of the new curdlan beads will be presented in a future paper.

In general, since affinity beads are expensive, they are re-generated and used repeatedly. However, impurities are accumulated on the beads even if they are washed adequately and the risk of contamination of the purified protein fraction is increased. In this respect, the curdlan beads could be used as disposable affinity beads due to their low cost. Although further analysis is required, the GRP-tagged proteins were found to be immobilized on curdlan beads in a one-step process without specific reagents, and thus the GRP system could be applicable to the large-scale preparation of immobilized enzymes in industrial bioreactors. The application of the GRP system for the preparation of proteins using other

expression hosts including insect and mammalian cells is now in progress.

Supplementary data

Supplementary data are available at *PEDS* online.

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