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# Application of volcanic ash particles for protein affinity purification with a minimized silica-binding tag

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**We recently reported that the spore coat protein, CotB1 (171 amino acids), from *Bacillus cereus* mediates silica biomineralization and that the polycationic C-terminal sequence of CotB1 (14 amino acids), designated CotB1p, serves as a silica-binding tag when fused to other proteins. Here, we reduced the length of this silica-binding tag to only seven amino acids (SB7 tag: RQSSRGR) while retaining its affinity for silica. Alanine scanning mutagenesis indicated that the three arginine residues in the SB7 tag play important roles in binding to a silica surface. Monomeric L-arginine, at concentrations of 0.3–0.5 M, was found to serve as a competitive eluent to release bound SB7-tagged proteins from silica surfaces. To develop a low-cost, silica-based affinity purification procedure, we used natural volcanic ash particles with a silica content of ~70%, rather than pure synthetic silica particles, as an adsorbent for SB7-tagged proteins. Using green fluorescent protein, mCherry, and mKate2 as model proteins, our purification method achieved 75–90% recovery with ~90% purity. These values are comparable to or even higher than that of the commonly used His-tag affinity purification. In addition to low cost, another advantage of our method is the use of L-arginine as the eluent because its protein-stabilizing effect would help minimize alteration of the intrinsic properties of the purified proteins. Our approach paves the way for the use of naturally occurring materials as adsorbents for simple, low-cost affinity purification.**



[**Key words:** Affinity purification; Arginine; Fusion tag; Silica-binding peptide; Volcanic ash; *Bacillus cereus* CotB1]

Affinity purification of recombinant protein is a common technique for facilitating the one-step purification of proteins of interest from a crude extract. An affinity tag is genetically fused to a target protein, and the resultant fusion protein is adsorbed onto a solid support via the interaction between the tag and the appropriate ligand immobilized on the support. The fusion protein is released and recovered from the solid support by the addition of an eluent that disrupts the interaction between the tag and ligand. Various proteins, domains, and peptides have been used as affinity tags (1–3), and the corresponding affinity resins have been made commercially available (1). However, affinity resins that are used commonly are rather expensive, making purification expensive (4).

Several attempts have recently been made to use silica-binding proteins/peptides as affinity tags, because inexpensive, unmodified silica ($SiO_2$) particles can be used as both the support as well as and ligand for the tag (5–7). Examples of such elutable, silica-binding tags include bacterial ribosomal protein L2 (also known as Si-tag) (8,9) and a Car9 peptide (5). The Si-tag shows high affinity for silica and is suitable for stable immobilization of Si-tagged fusion proteins on silica materials. However, with regard to affinity purification, a high affinity may be disadvantageous because it requires rather harsh conditions (e.g., high concentration of divalent cations such as 2 M $MgCl_2$) for the elution of Si-tagged fusion proteins from silica surfaces (6,7). Moreover, the large size of the Si-tag (273 amino acids) may negatively affect the intrinsic properties and function of the fusion protein. In contrast, the Car9 tag is a much shorter silica-binding tag (12 amino acids) and can be eluted under milder conditions (e.g., 1 M L-lysine) (5). However, it is still larger than commonly used affinity tags such as His-tag and FLAG tag, which are 6–10 and 8 amino acids, respectively. These drawbacks motivated us to develop an improved silica-based affinity purification method using a shorter silica-binding tag and milder elution conditions.

We previously reported that silica is deposited on the coat of *Bacillus cereus* spores as a protective coating against acid (10). Furthermore, gene disruption analysis previously revealed that the spore coat protein, CotB1 (171 amino acids), mediates silica biomineralization (11). The polycationic C-terminal sequence of CotB1 (14 amino acids corresponding to residues 158–171), designated CotB1p, was also found to serve as a short silica-binding tag (12). Here, we further shortened the length of this peptide to only seven amino acids while retaining the affinity for silica. We then developed a silica-based affinity purification method using the resultant short silica-binding peptide as an affinity tag, in which the protein of interest is eluted from silica under very mild conditions with 0.3–0.5 M L-arginine. Finally, we also demonstrated that naturally occurring silica-containing volcanic ash (designated *Shirasu*),

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instead of synthetic, pure silica particles, can be used as an affinity support in our method. Shirasu is mainly composed of silica (71–73%) and alumina ($Al_2O_3$) (13–15%) (13) and is widely distributed in large quantities in the southern Kyushu region of Japan (14). Size-separated Shirasu particles, depending on their size, are used for various applications, such as construction materials, thermal insulation materials, facial scrub, and so on. The availability of particles with a well-defined size makes this material suitable as an affinity support. The use of the low-cost natural particles as an affinity support enables further reduction in purification cost.

## MATERIALS AND METHODS

**Materials** Silica particles (α-quartz), ~0.8 μm in diameter, were purchased from Soekawa Chemical Co., Ltd. (Tokyo, Japan) and used without any pretreatment. The Shirasu particles, prepared as described below, were provided by the Kagoshima Prefectural Institute of Industrial Technology. The Shirasu deposit was harvested in Kushira-cho, Kanoya City, Kagoshima, Japan, and was screened through 5 mm and 38 μm mesh sieves and the resultant fine particles were used in this study.

**Construction of expression plasmids** To construct expression plasmids for green fluorescent protein (GFP) fusions with truncated sequences of CotB1p, inverse PCR was employed using pET-GFP-CotB1p (12) as a template with the following primer pairs: CB1M-F1/CB1M-R1, CB1M-F2/CB1M-R2, and CB1M-F3/CB1M-R3 (all primers are listed in Supplementary Table S1). The PCR products were self-ligated to construct pET-GFP-SB8, pET-GFP-SB10, and pET-GFP-SB7, respectively. Subsequently, plasmid pET-GFP-SB7 was used as a template for an additional round of inverse PCR with primer pairs of CB1M-F4/CB1M-R4 and CB1M-F5/CB1M-R5 to generate two additional plasmids pET-GFP-SB7v1 and pET-GFP-SB7v2, respectively.

To construct seven alanine-scanning mutants of the SB7 peptide (see Results), inverse PCR was employed using pET-GFP-SB7 as a template; SB7M-R and one of SB7M-F (1–7) were used as primers and the PCR products were self-ligated.

An expression plasmid for the hepta-arginine-tagged GFP was constructed as follows: A DNA fragment encoding the seven arginine residues was prepared by annealing synthetic oligonucleotides R7-S and R7-AS (Supplementary Table S1). The resultant double-stranded DNA carried a 5′-AGCT overhang at the upstream end and a 5′-CTAG overhang at the downstream end, identical to the cohesive ends of *Hin*dIII- and *Avr*II-digested fragments, respectively. The DNA fragment was ligated with *Hin*dIII- and *Avr*II-digested pET-GFP-CotB1 (12) to construct pET-GFP-R7.

To construct an expression plasmid for mCherry (15) with an N-terminal SB7 tag (SB7-mCherry), a two-step inverse PCR was employed using pET-CotB1p-SC (12) as a template with the primers SB7-SC-F and SB7-SC-R in the first-round PCR. The amplified DNA were self-ligated and then used as a template in the second-round PCR with the primers SB7-mC-F and SB7-mC-R. The resultant DNA was self-ligated to construct pET-SB7-mCherry.

To construct an expression plasmid for mKate2 (16) with an N-terminal SB7 tag (SB7-mKate2), a DNA fragment encoding SB7-mKate2 (nucleotide and amino acid sequences are shown in Supplementary Fig. S1) was synthesized by Invitrogen/Thermo Fisher Scientific Inc. (Waltham, MA, USA) and cloned into *Nde*I- and *Bam*HI-digested pET-21b (Novagen/Merck KGaA, Darmstadt, Germany) by using an In-Fusion HD cloning kit (Clontech/Takara Bio Inc., Shiga, Japan). The resultant plasmid was designated pET-SB7-mKate2.

**Expression of recombinant proteins** The expression plasmids were introduced into *Escherichia coli* Rosetta 2(DE3) (Novagen/Merck KGaA). The transformants were grown at 37°C in 2× YT medium (17) supplemented with 50 μg/mL kanamycin (or 50 μg/mL carbenicillin in the case of SB7-mKate2 expression), 30 μg/mL chloramphenicol, and 1% (w/v) glucose. When the culture reached an optical density of 0.5 at 600 nm, 0.2 mM isopropyl-β-D-thiogalactopyranoside was added to the medium to induce expression of recombinant proteins. After an additional 3 h of cultivation at 37°C, the cells were harvested by centrifugation and the resulting pellets were stored at −80°C until use.

**Silica-binding assay of recombinant proteins** Pellets of recombinant cells harvested from 1 mL cultures (typically 10 mg wet weight of the cells) were suspended in 0.5 mL of 25 mM Tris–HCl buffer (pH 8.0) containing 0.5% (v/v) Tween 20, and disrupted by sonication. After centrifugation at 20,000 ×*g* for 30 min, each supernatant (cleared cell lysate) containing 0.5 mg of total protein was mixed with 0.5 mL of silica-particle suspension in the same buffer (final silica concentration of 40 mg dry weight/mL) for 5 min at room temperature. Silica particles with the bound protein were collected by centrifugation at 5000 ×*g* for 2 min and then washed three times with 1 mL of the same buffer. After the supernatant was carefully removed, proteins that still bound to the particles were released by boiling in sodium dodecyl sulfate (SDS) gel-loading buffer (17) for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12.5%). Gels were stained with Coomassie brilliant blue R-250, and the target protein was quantified by densitometric analysis using ImageJ software version 1.41 (18).

**Affinity purification of SB7-tagged proteins using silica or Shirasu particles as an adsorbent** Pellets of recombinant cells expressing GFP with a C-terminal SB7 tag (GFP-SB7), SB7-mCherry, or SB7-mKate2 harvested from 5–10 mL cultures (~0.05–0.1 g wet weight of cells) were suspended in 2 mL of 25 mM Tris–HCl buffer (pH 8.0) containing 0.5% (v/v) Tween 20 and disrupted by sonication. After centrifugation at 20,000 ×*g* for 30 min, cleared cell lysates was mixed with 0.4 g dry weight of silica particles or with 0.35 g dry weight of Shirasu particles by gentle rotation for 5 min at room temperature. After centrifugation at 5000 ×*g* for 2 min, the particles were washed three times with 2 mL of the same buffer. Proteins were eluted by resuspending the particles with 2 mL of 25 mM Tris–HCl buffer (pH 8.0) containing 0.5 or 0.3 M L-arginine for silica and Shirasu particles, respectively. After 5-min incubation at room temperature, the suspension was centrifuged at 5000 ×*g* for 2 min. The resultant supernatant containing the eluted proteins was collected. The elution step was repeated (total volume of eluted fractions was 4 mL).

For comparison, GFP-SB7, which also contains an N-terminal His-tag, was purified from the same amount of cells by His-tag affinity purification using Ni-NTA agarose (Qiagen GmbH, Hilden, Germany). Purification was performed per the batch purification protocol provided by the manufacturer.

**Protein assay** The protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with bovine serum albumin as the standard.

**Nucleotide sequence data** The nucleotide sequence of SB7-mKate2 is available in the DDBJ/EMBL/GenBank databases under the accession number LC147380.

## RESULTS

**Minimization of the silica-binding CotB1p tag** Shorter affinity tags are more preferable than longer ones because the former are less likely to affect the intrinsic properties and function of the fusion partner. To minimize the length of the 14-amino-acid silica-binding CotB1p tag (SGRARAQRQSSRGR), we fused the following three truncated mutants of CotB1p to the C-terminus of GFP: T1 (SGRARAQR, corresponding to residues 1–8 of the CotB1p peptide), T2 (RAQRQSSRGR, corresponding to residues 5–14 of the CotB1p peptide), and T3 (RQSSRGR, corresponding to residues 8–14 of the CotB1p peptide). The affinities of these peptide for silica in 25 mM Tris–HCl buffer (pH 8.0) containing 0.5% (v/v) Tween 20 were compared to that of the original CotB1p; the surfactant Tween 20 was added to the buffer to prevent weak, non-specific interactions with the surface of silica particles. Consistent with our previous observation (12), densitometric analysis of the protein bands obtained on SDS-polyacrylamide gels showed that more than 80% of GFP-CotB1p bound to silica particles, whereas GFP alone hardly bound to silica under the conditions used (Fig. 1). Although the truncated mutant T1 showed relatively weak binding (about 70% of the protein bound to silica particles), affinities of T2 and T3 were comparable to that of the original CotB1p (Fig. 1). Because further truncation of the smallest peptide T3 (i.e., removal of the first or seventh arginine residue of the RQSSRGR sequence) caused significant reduction in affinity for silica (Fig. 1, T3-v1 and T3-v2), we chose T3 (hereafter renamed as SB7 tag) as the optimal peptide for further investigation, as its short length did not compromise its high affinity.

**Alanine scanning mutagenesis of the SB7 peptide** High abundance of positively charged arginine residues in the SB7 tag (and original CotB1p) suggested that these residues play important roles in binding to silica. Electrostatic attraction between positively charged amino acid residues and the negatively charged silica surface has been reported to be a major driving force of cationic silica-binding proteins/peptides (5,7,8,19–23). Consistent with these previous findings, the binding of the SB7 tag was pH-dependent; GFP-SB7 bound efficiently to silica at a pH range of 7.5–8.5 (data not shown), at which the SB7 tag and silica surface have high net positive and negative charges, respectively. To gain a better understanding of the binding mechanism of SB7, we performed alanine scanning mutagenesis for all seven residues of

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