Chitin- and Streptavidin-Mediated Affinity Purification Systems: A Screening Platform for Enzyme Discovery

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Abstract: Affinity purification of recombinant proteins is an essential technique in biotechnology. However, current affinity purification methods are very cost intensive, and this imposes limits on versatile use of affinity purification for obtaining purified proteins for a variety of applications. To overcome this problem, we developed a new affinity purification system which we call CSAP (chitin- and streptavidin-mediated affinity purification) for low-cost purification of *Strep*-tag II fusion proteins. The CSAP system is designed to utilize commercially available chitin powder as a chromatography matrix, thereby significantly improving the cost-efficiency of protein affinity purification. We investigated the CSAP system for protein screening in 96-well format as a demonstration. Through the screening of 96 types of purified hemoproteins, several proteins capable of the catalytic diastereo-divergent synthesis of cyclopropanes were identified as candidates for an abiotic carbene transfer reaction.

Introduction

Protein purification is an essential process in biotechnology and is required for protein research.[1] In particular, affinity chromatography, which recognizes a protein tag, is highly advantageous in terms of its selectivity and processes simplicity compared to other conventional chromatography methods.[2] However, current affinity chromatography systems are quite cost-intensive due to the use of expensive chromatography matrices. As a result, affinity chromatography is rarely employed for certain purposes, such as large-scale purification of recombinant proteins or high-throughput screening of diverse protein libraries.

Against this background, our group and others have previously developed a number of custom-designed affinity chromatography methods to reduce the cost of protein purification.[3] These methods have mainly utilized “self-made” chromatography matrices containing inexpensive polysaccharides such as starch[3a-d], agarose[3e], curdlan[3f] and chitosan[3g]. While these matrices are cost-effective, time-consuming and complicated procedures are required to prepare them. Moreover, these methods mostly employ large polysaccharide-binding proteins as fusion tags, which may affect the physiological properties of the protein of interest (POI). To overcome these limitations, low-cost affinity purification methods using “ready-prepared” chromatography matrices and small peptide tags are in high demand.

Accordingly, we here designed a new low-cost affinity purification system, which we call CSAP (chitin- and streptavidin-mediated affinity purification). This system is designed to purify *Strep*-tag II[4] fusion proteins. The CSAP system is based on ChSav, an engineered streptavidin variant[4] fused to a chitin-binding protein[5] (Figure 1a). Since ChSav has sufficient binding ability for both chitin and *Strep*-tag II peptide, a POI provided with the *Strep*-tag II sequence can be specifically immobilized on a chitin-based chromatography matrix via ChSav and purified by elution with a biotin-containing solution (Figure 1b and 1c). Given the low cost of commercially-available chitin powder and the size of the *Strep*-tag II sequence, which consists of only 8 amino acids, we recognized that the CSAP system would provide a powerful and versatile platform for the purification of recombinant proteins.

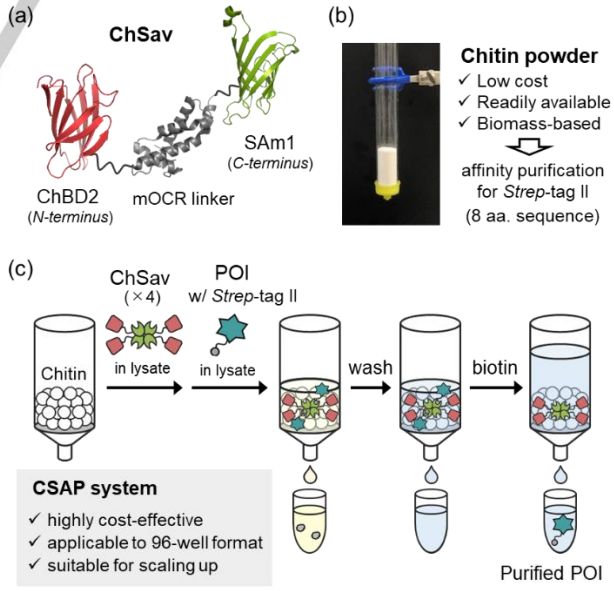

Figure 1. (a) Illustration of fusion protein ChSav (monomeric form), an engineered streptavidin variant (SAm1)[4] fused to a chitin binding domain of chitinase from *T. kodakarensis* KOD1 (ChBD2)[5] via monomeric overcome classical restriction protein (mOCR) linker[6]. (b) Chitin powder packed in a chromatography column. (c) Schematic illustration of the CSAP system to obtain the purified POI. ChSav forms tetrameric structure due to the streptavidin domain.

Results and Discussion

To demonstrate the concept of the CSAP system, we first constructed the fusion protein ChSav. As a component of ChSav, a chitin binding domain of chitinase (ChBD2) from hyperthermophilic archaeon *T. kodakarensis* KOD1 was selected due to its strong binding affinity for chitin.[5] The gene of ChBD2 was cloned into the pET-21b vector as a fusion with an engineered version of streptavidin known as SAm1, a high affinity variant for *Strep*-tag II reported by Skerra *et al.* (Figure 1a and S1).[4] The resulting expression plasmid, pET-21b-ChSav, was transformed into the *E. coli* BL21-Gold(DE3) strain, and ChSav was then expressed according to the protocol of the conventional pET expression system. ChSav in the *E. coli* cell lysate was found to bind to chitin powder with high specificity and high binding capacity as confirmed by the SDS-PAGE analysis (Figure S2). Next, two-step affinity chromatography for the *Strep*-tag II fusion protein was performed using ChSav and the chitin powder (Figure S3). We selected GFPuv, a green fluorescent protein variant,[7] as a target for the affinity purification. By sequentially loading two different kinds of cell lysates containing ChSav and GFPuv onto the chitin powder, GFPuv with *Strep*-tag II was successfully purified with high selectivity. It should be noted that throughout this process, commercially-available chitin powder was used as received without any purification or modification. This accessibility will be helpful in using chitin powder as a low-cost chromatography matrix (Table S1). Furthermore, the *Strep*-tag II fusion protein was able to be eluted under mild condition by using low-concentration biotin solution (2.5 mM). This feature provides practical advantages in the downstream process of protein research and reduces the cost of elution buffer (Table S2).

Motivated by this promising result, we then set out to improve the experimental procedure for the CSAP system. The above-mentioned purification procedure requires additional cell-lysis and protein-extraction processes to prepare the *E. coli* cell lysates containing ChSav (see Supplementary). To minimize the efforts associated with these time-consuming and labor-intensive processes, we developed following two affinity purification systems (CSAP-1 and CSAP-2): Each of these utilizes two different expression plasmids designated pNCMO2-ChSav and pAR3b-ChSav.

Development of the CSAP-1 system with pNCMO2-ChSav

The CSAP-1 system was designed to utilize the secretory expression system of *B. choshinensis* HPD31-SP3 to prepare a ChSav-containing solution.[8] Compared to the conventional intracellular expression system, the secretory expression is beneficial because the secreted recombinant proteins can be collected via a simple centrifugation process without cell lysis. A secretory expression plasmid pNCMO2-ChSav was prepared, which encodes the gene of ChSav under the *sec* signal sequence (Figure 2a). The *B. choshinensis* HPD31-SP3 cell harboring pNCMO2-ChSav was confirmed to express ChSav in a culture supernatant with a high expression level. The secreted ChSav binds specifically to chitin powder (Figure 2b).

Based on this result, we next demonstrated use of the CSAP-1 system for purification of hemoproteins with a *Strep*-tag II sequence (Figure 2c). First, the chitin powder was incubated with the culture supernatant of *B. choshinensis* HPD31-SP3 cell to immobilize ChSav on the surface. The resulting chitin powder chromatography matrix was then packed into a 96-well filter plate (Figure S4). Next, *E. coli* cell lysates expressing each hemoprotein were loaded onto the filter plate for the separation of cellular impurities. Finally, the bound proteins were eluted with a biotin solution to obtain the purified protein solution. As confirmed by SDS-PAGE, all hemoproteins were successfully purified by the CSAP-1 system with high efficiency (Figure 2d). It is noteworthy that this CASP-1 system has functioned well regardless of the size and the structure of the POI, although some proteins with low expression levels were obtained at relatively low concentrations. Furthermore, the efficiency of the purification is not affected by placement of the *Strep*-tag II sequence at the N-terminus or the C-terminus (Table S3).

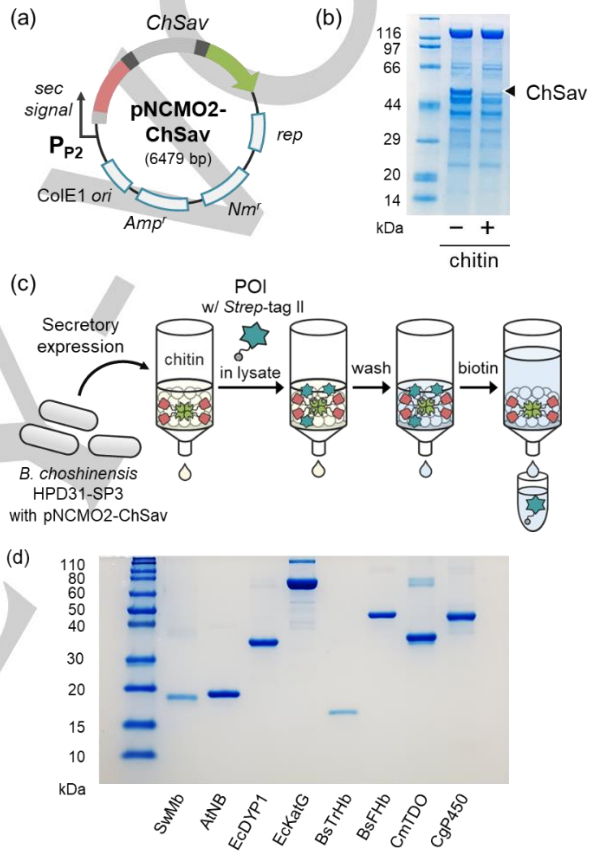

Figure 2. Overview of the CSAP-1 system. (a) Secretory expression plasmid pNCMO2-ChSav. (b) SDS-PAGE analysis of cell culture supernatants of the *B. choshinensis* HPD31-SP3 strain harboring pNCMO2-ChSav. The supernatants were incubated with (+) or without (–) chitin powder to determine the binding ability of ChSav (46.2 kDa). (c) Schematic diagram of the CSAP-1 system. ChSav secreted into the media was directly utilized for the affinity chromatography. (d) Representative results of SDS-PAGE analysis on protein solutions purified using the CSAP-1 system. SwMb: myoglobin from *P. catodon* (19.4 kDa), AtNB: nitrobindin from *A. thaliana* (20.5 kDa), EcDYP1: dye-decolorizing peroxidase YfeX from *E. coli* (34.7 kDa), EcKatG: catalase-peroxidase KatG from *E. coli* (81.7 kDa), BsTrHb: truncated hemoglobin from *B. subtilis* (17.3 kDa), BsFHb: flavohemoglobin from *B. subtilis* (46.3 kDa), CmTDO: tryptophan 2,3-dioxygenase from *C. metallidurans* (36.5 kDa), CgP450: cytochrome P450 CreJ from *C. glutamicum* (47.9 kDa).

Development of the CSAP-2 system with pAR3b-ChSav

We also developed another affinity purification system, designated CSAP-2, in an attempt to further simplify the experimental procedure of our purification system. In the above-

mentioned CSAP-1 system, the ChSav-containing solution was prepared separately from the POI-containing cell lysates. Therefore, if both ChSav and POI could be expressed simultaneously in single cell, the subsequent affinity purification could be directly performed in one-step method using the simple chitin powder (Figure 3a). Based on this concept, a co-expression plasmid, pAR3b-ChSav, was designed for the CSAP-2 system. Since the pAR3b vector contains pACYC *ori* and the chloramphenicol resistance gene,[9] pAR3b-ChSav exhibits good compatibility with ColE1-type plasmids such as pET-21b which we used for the expression of various proteins of interest (Figures 3b and 3c). Moreover, pAR3b-ChSav encodes the ChSav gene under the control of the arabinose pBAD promoter. Therefore, the expression level of ChSav can be controlled independently from the pET expression system.

Using this pAR3b-ChSav, we first demonstrated the CSAP-2 system for the purification of GFPuv containing *Strep*-tag II sequence. The pET-21b plasmid encoding the GFPuv gene (pET-21b-GFPuv) was transformed into the chemically-competent *E. coli* BL21-Gold(DE3) cell harboring pAR3b-ChSav. The cells were then cultivated in LB media containing ampicillin and chloramphenicol, and expression of ChSav and GFPuv was independently induced upon the addition of arabinose and IPTG. As confirmed by the SDS-PAGE analysis, the expression systems for these two proteins were found to be orthogonal (Figure 3d). The co-expression of ChSav and GFPuv was only observed in the presence of both arabinose and IPTG. Furthermore, after the chemical lysis of the *E. coli* cell, GFPuv in the lysate was purified with high efficiency (approximately 90% recovery) with the affinity chromatography using the chitin powder (Figures 3e and S5). Control experiments without IPTG or arabinose induction reveal that the co-expression of ChSav plays an important role in the purification of the *Strep*-tag II fusions (Figure S6).

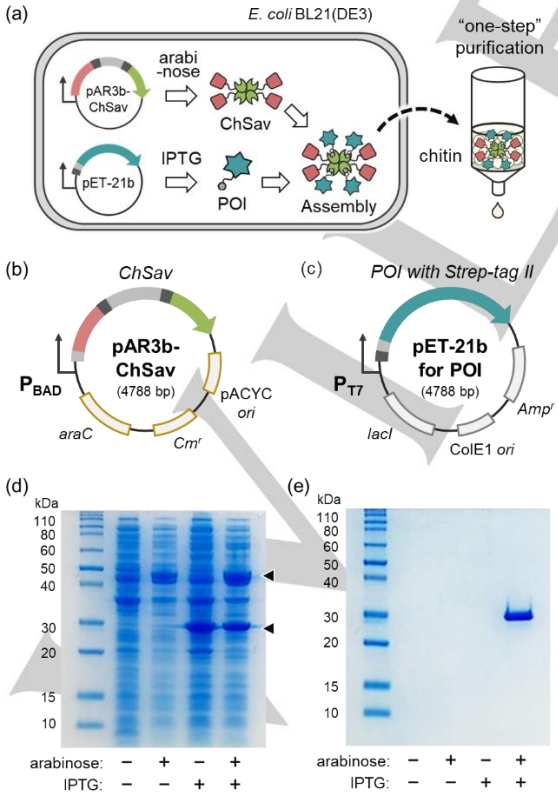

Figure 3. Overview of the CSAP-2 system, a "one-step" purification approach based on a co-expression system. (a) Schematic diagram of the CSAP-2 system. The POI containing *Strep*-tag II was co-expressed with ChSav and directly subjected to the affinity purification with chitin powder. (b) Co-expression plasmid pAR3-ChSav. (c) pET-21b expression plasmid for the POI containing *Strep*-tag II. (d) SDS-PAGE analysis of *E. coli* cells expressing ChSav (46.7 kDa) and GFPuv (28.9 kDa). The *E. coli* cells harboring pAR3-ChSav and pET21b-GFPuv were cultivated in the presence (+) or absence (−) of arabinose and IPTG. (e) SDS-PAGE analysis of GFPuv solutions purified by the CSAP-2 system with (+) or without (−) addition of arabinose and IPTG.

The CSAP-2 system also worked efficiently for the affinity purification of other proteins. For example, the one-step affinity chromatography of the CSAP-2 system allowed purification of various hemoproteins with diverse structure (Figure S7). As an exceptional case, some proteins, such as dye-decolorizing peroxidase YfeX (EcDYP1) and cytochrome P450 CreJ (CgP450), were obtained at relatively lower concentrations compared to the yields obtained from the CSAP-1 system. It is assumed that these proteins may interact unfavorably with the chitin binding domain of ChSav in solution and thus inhibit binding to the chitin powder. However, the CSAP-2 system still offers significant advantages in terms of its cost and process simplicity. As a demonstration, the CSAP-2 system was applied to large-scale purification of the *Strep*-tag II fusions. The *E. coli* cells harboring pET-21b-GFPuv and pAR3b-ChSav were cultivated on a litter scale (7.5 L total), and the expressed GFPuv was purified by suction filtration with 200 g of chitin powder (Figure 4a). Consequently, ca. 500 mg of GFPuv was successfully obtained from the 7.5 L of *E. coli* cell culture medium at high purity (Figures 4b and 4c). The binding capacity of chitin powder was calculated to be approximately 2.5 mg/g. Considering its simplicity and cost-efficiency, the CSAP-2 system has significant potential to be applied to industrial processes for production of recombinant proteins.

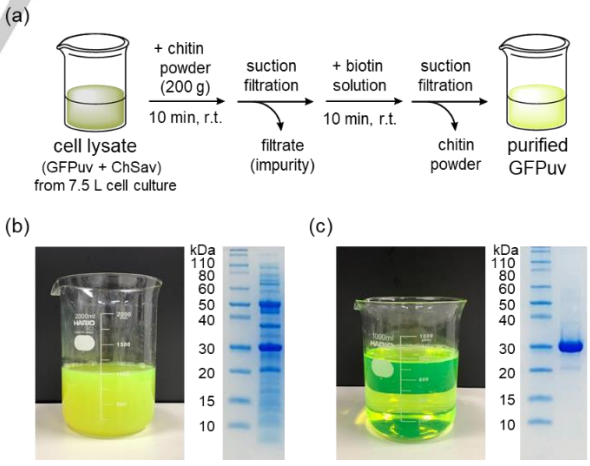

Figure 4. (a) Schematic illustration of the large-scale purification process using the CSAP-2 system. GFPuv containing the *Strep*-tag II sequence (28.9 kDa) was co-expressed with ChSav (46.7 kDa) at liter scale and purified by suction filtration with the chitin powder. (b) Photograph of the *E. coli* cell lysate before the purification and the result of SDS-PAGE analysis. (c) Photograph of the purified solution containing GFPuv and the result of SDS-PAGE analysis.

Demonstration of enzyme screening with CSAP system

Next, we demonstrated an application of the CSAP system for high-throughput screening of protein libraries to discover new

enzymes with high catalytic activity and stereoselectivity. Since the CSAP system allows enzyme activity assays to be conducted using purified proteins, development of a high-throughput screening platform based on the CSAP system was expected to offer substantial advantages in terms of accuracy and versatility compared to conventional screening platforms operating in a crowded cellular environment. Based on this concept, we screened a hemoprotein library to identify optimal enzymes for catalysis of cyclopropanation of styrene (**1**) with ethyl diazoacetate (**2**) (Figure 5). Several hemoproteins, such as cytochrome P450s[10], myoglobins[11], and their engineered variants[12], have been widely used to promote this cyclopropanation reaction. However, it remains to be clarified which types of hemoproteins present in nature are best suited for this abiotic reaction.

A library of pET expression plasmids was generated to encode diverse types of hemoproteins (e.g., globins, peroxidases, catalases, cytochrome P450s, tryptophan 2,3-dioxygenases, peroxynitrite isomerases, and nitric oxide synthases) as fusion proteins including the *Strep*-tag II sequence for the protein screening process (Tables S4–S9). The library was then expressed using the *E. coli* BL21-Gold(DE3) strain, and the expressed hemoproteins were purified using the CSAP-1 system in a 96-well format (Figures 5a, S8, and S9). The present screening platform with the CSAP-1 system was designed to include a heme cofactor supplementation step[13] during the purification to ensure that the catalytic activities of hemoproteins are equally evaluated in the holo-form and to increase the fidelity of the screening (Figure S10). Then, the purified hemoproteins were subjected to an activity assay to assess the cyclopropanation of **1** with **2** (Figure 5b). Yields, diastereomeric excess (de), and enantiomeric excess (ee) of the products **3** and **4** were determined by GC-FID (Figures 5c, S11, and S12).

As summarized in Figure 5c and Tables S4–S9, the screening revealed a clear tendency in the cyclopropanation activity of each hemoprotein. Most hemoproteins with globin folds (e.g., truncated hemoglobins, flavohemoglobins, and latex clearing proteins) exhibited high catalytic activity, whereas a family of heme-dependent peroxidases (e.g., dye-decolorizing peroxidases and catalase-peroxidases) was found to have little or no activity. Furthermore, two hemoproteins capable of catalyzing diastereo-divergent synthesis of cyclopropanes were successfully identified by the screening (Figure S13–15): Bacterial hemoglobin from *S. novella* (SnVHb) was found to afford (*S*,*S*)-**3** with high yield and high stereoselectivity (99% yield, >96% de, >98% ee) (Table S8, entry 70). In contrast, truncated hemoglobin from *S. roseum* (SrTrHb) gave a thermodynamically-unfavored diastereomer (*S*,*R*)-**4** with high stereo-selectivity (85% yield, >96% de, >98% ee) (Table S9, entry 88). We also determined Michaelis-Menten parameters and TONs for these two enzymes independently (Figures S16–S17, and Table S10). Particularly, SnVHb was found to exhibit a high k_{cat} value (4.9×10^4 min^{-1}), and both enzymes exhibited modest K_M values for styrene (**1**) (approximately 3–4 mM). This suggests that the high k_{cat} value mainly contributes to the cyclopropanation activity of the enzymes, and that the lack of structural similarity between styrene (**1**) and the native substrates may be responsible for the high K_M value. Overall, these results clearly indicate the utility of our screening platform with the CSAP system, and additional library screening is currently underway to discover new hemoproteins capable of producing the stereoisomers (*R*,*R*)-**3** and (*R*,*S*)-**4**, respectively.

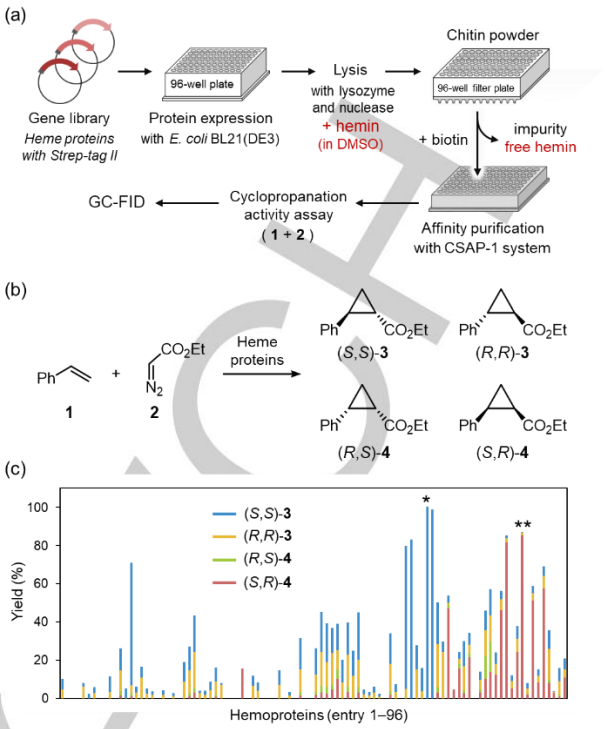

Figure 5. (a) Schematic illustration of enzyme screening using the CSAP-1 system. The POIs containing the *Strep*-tag II sequence were expressed in *E. coli* cell. The cells were then chemically lysed in the presence of excess hemin to supplement the heme cofactor. The resulting cell lysates were next purified using the chitin powder in a 96-well format. Finally, the purified POIs were subjected to the cyclopropanation activity assay. (b) Cyclopropanation of styrene (**1**) with ethyl diazoacetate (**2**) catalyzed by hemoproteins. Reaction conditions: **1** (6.0 mM), **2** (2.0 mM) and sodium dithionite (2.0 mM) in Tris-HCl buffer (100 mM Tris-HCl, 150 mM NaCl, 1.0 mM EDTA, 2.0 mM biotin, pH = 8.0) containing hemoproteins, 25 ºC for 17 h. (c) Screening results for the cyclopropanation activity of hemoproteins. Yields of (*S*,*S*)-**3**, (*R*,*R*)-**3**, (*R*,*S*)-**4**, and (*S*,*R*)-**4** are shown in blue, yellow, green, and red bars, respectively. *Bacterial hemoglobin from *S. novella* (entry 70). **Truncated hemoglobin from *S. roseum* (entry 88).

Conclusion

In conclusion, we have demonstrated the effectiveness of our new CSAP protein purification system which uses commercially-available chitin powder as a chromatography matrix for the purification of the *Strep*-tag II-containing proteins. The fusion protein ChSav enables various types of proteins to be purified with high efficiency. To further demonstrate the utility of our purification system, the CSAP system was applied to large-scale production of purified proteins and high-throughput screening of enzyme libraries. In particular, new hemoproteins capable of catalyzing the diastereo-divergent synthesis of cyclopropanes were succesfully identified through the screening. Considering (i) the cost efficiency of commercially-available chitin powders, (ii) the simplicity of the purification procedure, and (iii) the convenient use of the *Strep*-tag II fusion proteins, the present CSAP system has a significant potential to be widely used as a powerful purification platform for the recombinant proteins in a variety of applications.

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Keywords: protein purification • enzyme screening • chitin • hemoproteins • cyclopropanation

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[13] To supplement the heme cofactor for the apo-enzymes, *E. coli* cells expressing hemoproteins (from 750 µL of cell culture) were lysed in 150 µL of Tris-HCl buffer (100 mM Tris-HCl, 150 mM NaCl, 1.0 mM EDTA, pH = 8.0) which was supplemented with hemin (10 mM in DMSO, 0.3 µL), lysozyme (2.0 mg/mL in Tris-HCl buffer, 5.0 µL), and benzonase® nuclease (250 U/µL, 0.05 µL). The resulting lysates were loaded onto the chitin powder and free hemin not bound to the proteins was removed during affinity chromatography.

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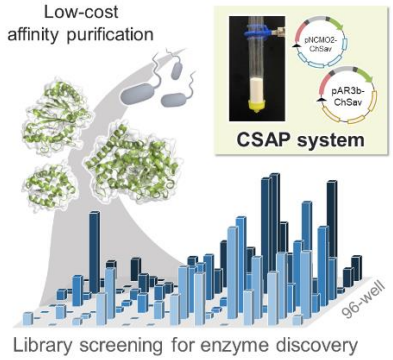
Insert graphic for Table of Contents here.



A new protein purification system, designated CSAP (chitin- and streptavidin-mediated affinity purification) system, has been developed for enzyme library screening. The system utilizes chitin powder as a chromatography matrix to purify *Strep*-tag II fusion proteins. Given the cost of chitin and the availability of *Strep*-tag II, the CSAP system offers a powerful platform for the various screening of purified proteins.