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Improved recovery of active GST-fusion proteins from insoluble aggregates: solubilization and purification conditions using PKM2 and HtrA2 as model proteins

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The glutathione S-transferase (GST) system is useful for increasing protein solubility and purifying soluble GST fusion proteins. However, purifying half of the GST fusion proteins is still difficult, because they are virtually insoluble under non-denaturing conditions. To optimize a simple and rapid purification condition for GST-pyruvate kinase muscle 2 (GST-PKM2) protein, we used 1% sarkosyl for lysis and a 1 : 200 ratio of sarkosyl to Triton X-100 (S-T) for purification. We purified the GST-PKM2 protein with a high yield, approximately 5 mg/L culture, which was 33 times higher than that prepared using a conventional method. Notably, the GST-high-temperature requirement A2 (GST-HtrA2) protein, used as a model protein for functional activity, fully maintained its proteolytic activity, even when purified under our S-T condition. This method may be useful to apply to other biologically important proteins that become highly insoluble in the prokaryotic expression system. [BMB reports 2011; 44(4): 279-284]

INTRODUCTION

Proper systems are necessary for rapid and high-yield purification of functionally active proteins to investigate the structures and functions of proteins using biochemical and biophysical methods. A method frequently used for this purpose is the glutathione S-transferase (GST) fusion system introduced by Smith and Johnson (1). The GST-fusion system is an affinity protein purification system, which can inducibly express GST-fusion proteins in *E. coli* and purify the fusion proteins in their native forms by applying GST binding characteristics to glutathione (2, 3). Because this system can only be used to purify soluble proteins, the insolubility of many GST-fusion proteins still remains a major limitation with this approach (4-7).

To overcome this limitation, methods for improving protein solubility have been designed using various reagents that remove the interactions involved in the formation of protein aggregates during purification steps of proteins expressed in a prokaryotic system (8-10). An example of such a method is purification of the chicken pyruvate kinase muscle 2 (cPKM2) recombinant protein with amino acids 17-476 (7). This study has shown that the insoluble GST-fusion protein can be solubilized using the alkyl anionic detergent *N*-lauroylsarcosine sodium salt (sarkosyl), and the binding affinity of the sarkosyl-solubilized GST-fusion protein to glutathione beads was improved by subsequent treatment with nonionic detergent Triton X-100. Nevertheless, in practice, technical hurdles still exist during the purification of primarily insoluble proteins under non-denaturing conditions.

In the present study, the full-length mouse PKM2 and the serine protease high-temperature requirement A2 (HtrA2) were selected as model proteins for protein purification and functional activity tests, respectively. The purification conditions of these GST-fusion proteins were optimized by various combinations of sarkosyl and Triton X-100 at different ratios, and the proteolytic activity of the serine protease HtrA2 was analyzed to determine the effects of the ionic detergent sarkosyl on its activity. Our study shows that the S-T purification method not only solubilizes insoluble proteins expressed in *E. coli*, thereby facilitating the purification of a large amount of proteins with high yields, but also is an optimal method with no adverse effects on protein function.

RESULTS AND DISCUSSION

Selection of a challenging model protein for purifying proteins insoluble during prokaryotic expression

Protein insolubility observed during protein expression and purification is a limitation in the purification of a large quantity of soluble proteins (11-15). To overcome this limitation, the protein solubilization and purification conditions were optimized to increase the solubility and binding affinity of GST-fusion proteins to glutathione beads in their native forms. Based

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on a previous study for purifying the cPKM2 (aa 17-476) protein purification study, we selected the mouse full-length PKM2 (aa 1-531) as a model protein, which is supposed to be present mostly in the insoluble fraction during conventional purification (7, 16). First, we compared the amino acid sequence homology of PKM2, cPKM2, and human PKM2 (hPKM2) (Supplementary Table 1, Supplementary Fig. 1A). The sequence identity of cPKM2 was almost 85% to those of both hPKM2 and PKM2, whereas PKM2 revealed 96% sequence identity to hPKM2. This alignment result suggests that the structure and function of hPKM2 may be much closer to those of PKM2 than to those of cPKM2; thus, a purification system for PKM2 must be developed to provide a useful reagent for characterizing the common biochemical properties of the PKM2 proteins derived from different species. Subsequently, to examine the correlation between amino acid composition of the proteins and protein solubility, we analyzed the distribution of polar and nonpolar amino acid residues on both the PKM2 and cPKM2 proteins (Supplementary Fig. 1B). The protein hydrophobicity was presented as the Kyte-Doolittle scale by the CLC bio program, which is a widely applied scale for delineating hydrophobic characteristics of proteins. Positive and negative values represent the hydrophobic and hydrophilic properties of proteins, respectively (17). The N-termini of both the PKM2 and cPKM2 proteins (from the N to A2 domains) showed almost similar hydrophobicity, whereas amino acid clusters (blue box, aa 388-414, aa 468-480) with higher hydrophobicity existed in the C domain of PKM2. This finding as well as that of the previous study suggests that PKM2 can be used as a model protein to solve protein insolubility problems in the prokaryotic expression system and to establish optimal conditions for protein purification.

PKM2, as the model protein, shows high insolubility in the prokaryotic expression system

To investigate whether the PKM2 protein is highly insoluble under non-denaturing conditions, we expressed PKM2 as a GST-fusion protein in *E. coli* and lysed cells with a typical EBC lysis buffer containing the nonionic detergent 0.5% NP40 (Supplementary Fig. 2). The GST-PKM2 protein with a molecular weight of approximately 85 kDa was expressed at high levels (40 mg/L *E. coli* culture, T lane). Total cell lysates (T) were separated by centrifugation as precipitates (P, NP40-insoluble precipitate plus some cell debris) and supernatants (S, NP40-soluble fraction), and the protein samples were analyzed on a 13% SDS-polyacrylamide gel to assess protein solubility. Over 95% of the total GST-PKM2 (38 mg) was present in the NP40-insoluble fraction (P), indicating that high levels of GST-PKM2 are virtually insoluble under non-denaturing conditions. Additionally, a decrease in protein solubility may be due to the amino acid clusters within the C domain of PKM2. Thus, GST-PKM2 can be used as an insoluble model protein to establish the optimal conditions for purifying proteins that become highly insoluble under non-denaturing conditions.

A dramatic increase in the solubility of the GST-PKM2 protein using the alkyl anionic detergent sarkosyl

The GST portion of the fusion protein improves the solubility of target proteins. Nevertheless, many GST-fusion proteins tend to aggregate rapidly and often become insoluble when overexpressed in *E. coli* and purified under non-denaturing conditions (Supplementary Fig. 2) (10, 18, 19). Generally, it is necessary to break interactions, such as hydrophobic interactions, hydrogen bonds, van der Waals forces, and ionic interactions, to improve protein solubility involved in protein aggregation (20-22). Compounds used for protein solubilization include chaotropic agents, detergents, reducing agents, and salts (20, 23). Notably, detergents are well-established and useful reagents to disrupt the hydrophobic interactions present in protein aggregates and thus to increase protein solubility. NP40 and Triton X-100 are nonionic detergents with a phenyl ring between an uncharged alkyl chain (hydrocarbon straight chain) and a hydrophilic ether group (23, 24) (Supplementary Fig. 3). These nonionic detergents have been used widely to purify proteins into their biologically active forms without denaturation. Several ionic detergents have detrimental properties on proteins, such as denaturation, and substantially destroy protein function. Nevertheless, they could be widely used to solubilize insoluble protein aggregates (7, 10, 25-27).

We lysed *E. coli* cells with various concentrations of alkyl anionic sarkosyl consisting of an anionic head group and a hydrocarbon straight chain to improve protein solubility by disrupting hydrophobic interactions present in the NP40-insoluble GST-PKM2 protein aggregates (Fig. 1, Supplementary Fig. 3). In contrast to the highly insoluble properties of GST-PKM2 under the sarkosyl-free condition, an increase in the sarkosyl concentration dramatically increased the GST-PKM2 solubility. In 0.5% sarkosyl, 75% of all of the GST-PKM2 was present in the insoluble fraction, and 24% was present in the soluble fraction (Fig. 1A). Notably, the levels of 1%- and 2%-sarkosyl soluble GST-PKM2 were 74% (29.6 mg/L culture) and 92% (36.8 mg/L culture) of the total GST-PKM2, respectively (Fig. 2B). The results demonstrate that the solubility of GST-PKM2 increased up to 23 times in the presence of sarkosyl compared to that under sarkosyl-free conditions. This sarkosyl-solubilization method is rapid, simple, and efficient for recovering GST-PKM2 at high levels.

Purification of the GST-PKM2 protein using Triton X-100

The GST-fusion protein can be readily purified by affinity chromatography using glutathione Sepharose 4B under non-denaturing conditions (28-30). After the cell lysates were solubilized with 2% sarkosyl, the protein samples were further diluted in STE buffer to a final concentration of 0.008% sarkosyl to reduce the detrimental effects of ionic detergents on the proteins. Most of the GST-PKM2 was still in the soluble fraction; however, only 2.3% of the total solubilized GST-PKM2 protein was bound to the glutathione beads (Fig. 2, 0% Triton X-100). The final concentration of sarkosyl was further diluted

by more than 200 times; however, most of GST-PKM2 may have been already denatured under the 2% sarkosyl condition, or the residual amounts of ionic detergent sarkosyl may have substantially reduced the binding affinity of GST-PKM2 to glutathione. Several studies have indicated that nonionic detergents can sequester the ionic detergent sarkosyl by forming mixed micelles with sarkosyl; thus, facilitating the binding of the GST-fusion protein to glutathione beads (1, 31). Thus, we simply tested the binding affinity of GST-PKM2 to glutathione beads by adding different concentrations of Triton X-100 (Fig. 2A). The binding affinity of GST-PKM2 to glutathione beads increased to 7.5% in the presence of 2% Triton X-100, which is approximately 3 times higher than that with no Triton X-100 (Fig. 2B). This result indicates that Triton X-100 has a sequestering effect on ionic detergent sarkosyl for the interaction between GST and glutathione under ionic detergent conditions.

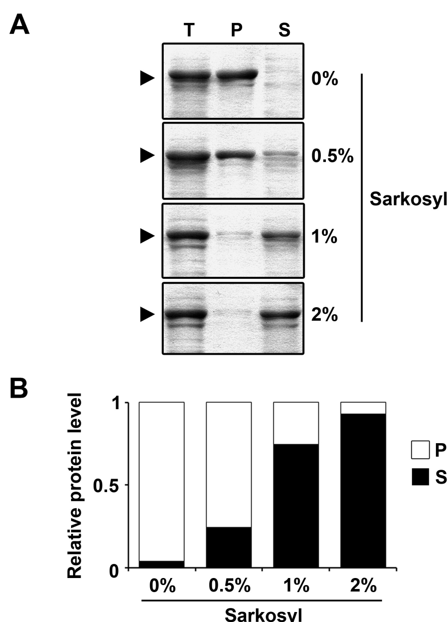

Fig. 1. Solubilization of the GST-PKM2 protein using the alkyl anionic detergent *N*-lauroylsarcosine sodium salt (sarkosyl). (A) The increase in solubility of the GST-PKM2 protein after using ionic detergent sarkosyl. GST-PKM2-expressing *E. coli* cells were lysed with STE buffer containing the indicated concentrations of sarkosyl. The sarkosyl-insoluble (precipitate, P) and -soluble (supernatant, S) fractions were separated by centrifugation at 13,400 × g for 15 min at 4°C. The arrowhead indicates 85 kDa GST-PKM2. (B) Quantification of the solubility of GST-PKM2. The GST-PKM2 band intensity in each fraction was determined by densitometric quantification using Multi gauge V3.0 software. The relative values of the amount of GST-PKM2 in the P and S fractions were expressed as the ratio of the band intensity in each fraction to the sum of those in the P and S fractions (the sum = 1).

Determining the optimal ratio of sarkosyl and Triton X-100 to purify the GST-PKM2 protein

The yield of GST-fusion proteins during purification depends substantially on the properties of the fusion proteins, the host *E. coli* strain, culture, lysis, and purification conditions (32). As much as 10 mg GST-fusion protein per liter culture could be recovered; however, the average yield is approximately 2.5 mg/L culture (32, 33). Based on these criteria and to establish the optimal conditions for purifying the GST-fusion proteins, protein samples were solubilized with various sarkosyl concentrations, and GST-PKM2 was purified by selectively binding to glutathione beads in the presence of 2% Triton X-100 (Supplementary Fig. 4). The binding affinity of GST-PKM2 to glutathione increased up to 19% under the 1% sarkosyl lysis condition, which was 2.5 times higher than that from protein samples solubilized with 2% sarkosyl (Supplementary Fig. 4A). Therefore, the protein yield was approximately 5.5 mg/L culture, which was 33 times higher than that obtained from samples processed under conventional non-denaturing conditions (0% sarkosyl, Supplementary Fig. 4B). Although the solubility of GST-PKM2 lysed with 2% sarkosyl was 1.2 times higher than that lysed with 1% sarkosyl (Fig. 1), the binding affinity to the glutathione beads was 3 times lower than that of the 1%

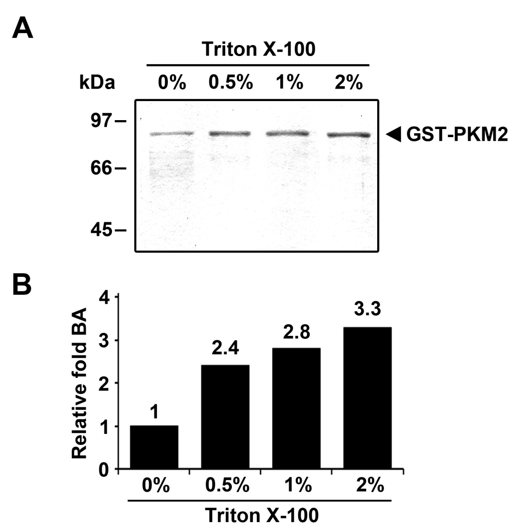

Fig. 2. Increase in the binding affinity of GST-PKM2 to glutathione Sepharose 4B by treatment with the nonionic detergent Triton X-100. (A) Purification of the soluble GST-PKM2 protein. The GST-PKM2 protein was solubilized with 2% sarkosyl, diluted further in STE buffer to a 0.008% final concentration, and purified by selective binding to glutathione Sepharose 4B beads in STE buffer containing the indicated concentrations of Triton X-100. (B) Quantification of relative-fold binding affinity of GST-PKM2 to glutathione beads at different Triton X-100 concentrations. Values are expressed as relative-fold binding affinity (BA) of GST-PKM2 to glutathione beads relative to control (BA at 0% Triton X-100 refers to 1).

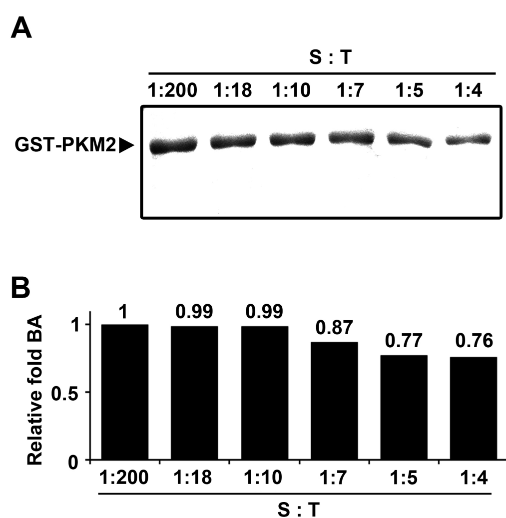

Fig. 3. Optimal ratio of a 1 : 200 ratio of sarkosyl to Triton X-100 (S-to-T) for purifying GST-PKM2. (A) *E. coli* cells were solubilized with STE buffer containing 1% sarkosyl. The sarkosyl-soluble fraction (10 μl) was diluted in 1 ml STE buffer to a final concentration of 2% Triton X-100 and further adjusted with sarkosyl to the indicated ratio of S : T, followed by incubating with glutathione Sepharose 4B. (B) Quantification of relative-fold BA of GST-PKM2 to glutathione beads at different combinations of S and T (refer to 1 at the S : T ratio of 1 : 200). All samples in Fig. 1-3 were lysed in 1× SDS sample buffer, incubated for 5 min at 95°C, and resolved on 13% SDS-PAGE. Protein bands were visualized by staining with Coomassie Brilliant Blue dye.

sarkosyl lysis, leading to yields of approximately 2.3 mg/L culture of GST-PKM2.

To examine the effects of GST-PKM2 binding affinity to glutathione at various ratios of sarkosyl (S) to Triton X-100 (T) (S : T), the protein samples were solubilized with 1% sarkosyl and diluted with STE buffer to give different S to T ratios at a fixed concentration of 2% Triton X-100, based on the conditions shown in Supplementary Fig. 4A (Fig. 3). The binding affinity of GST-PKM2 to glutathione was maintained at up to the S to T ratio of 1 : 10 and gradually decreased according to the decrease in the S to T ratio (Fig. 3B). In summary, we have described the optimal conditions for solubilizing and purifying protein aggregates with high yields of GST-fusion proteins in their soluble native state. *E. coli* cells were lysed with 1% sarkosyl, followed by a 100-fold dilution with STE buffer and subsequent addition of Triton X-100 at a 2% final concentration, and the GST-fusion proteins were purified by selective binding to glutathione beads.

Assessing the catalytic function of the high temperature requirement A2 (HtrA2) that was purified under the sarkosyl-Triton X-100 (S-T) purification condition

We used the serine protease HtrA2 as a model for assessing protein function to examine the effect of the S-T purification conditions on protein function (Fig. 4). The advantage of using HtrA2 is that it could be used readily to analyze the effect of sarkosyl on enzymatic activity with the *in vitro* β-casein cleavage assay (34). Notably, β-casein was cleaved approximately 50% by HtrA2 purified under the S-T condition at 37°C for 60 min (lane 5), which was comparable to HtrA2 purified under NP40-EBC conditions (lane 4). This result indicates that the GST-fusion proteins may maintain their structures in the soluble native form during processing under the S-T condition.

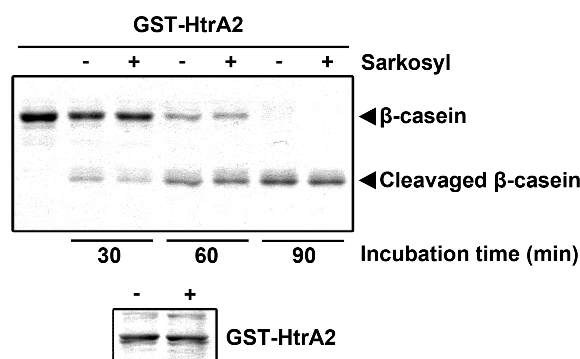

Fig. 4. Preparation of enzymatically active HtrA2 under sarkosyl-Triton X-100 purification conditions. Wild-type GST-HtrA2 protein was purified by a conventional (-, lysed with EBC buffer) or S-T purification (+, lysed with 1% sarkosyl-STE buffer) method described in Supplementary Fig. 4. The purified GST-HtrA2 protein was incubated at 37°C with 15 μg β-casein as an exogenous substrate for serine protease. The reaction mixture were resolved by 13% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue dye.

We have established the optimal conditions to effectively purify proteins with high yields, using PKM2 as an insoluble protein and HtrA2 as an enzyme for assessing protein function. Therefore, our study has provided a method and information for generating useful reagents for biochemical and biophysical studies of proteins essential for cellular processes. However, the easily formed insoluble aggregates in the prokaryotic system greatly limit studies of their structure and function.

MATERIALS AND METHODS

Detailed information is described in Supplementary data.

Acknowledgements

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