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Purification of Recombinant Proteins Based on the Interaction between a Phenothiazine-Derivatized Column and a Calmodulin Fusion Tail

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A method to purify proteins by fusing them to the Ca^{2+}-dependent protein calmodulin is described by using glutathione-S-transferase (GST) from *Schistosoma japonicum* as a model. Glutathione-S-transferase was genetically fused to calmodulin (CaM). The designed GST-CaM fusion protein has a selective factor Xa cleavage site located between the C-terminus of GST and the N-terminus of CaM. The recombinant fusion protein was expressed in *Escherichia coli*, and the crude cell extract was loaded onto a phenothiazine affinity column in the presence of Ca^{2+}. Calmodulin was used as an affinity tail to enable binding of the fusion protein to the phenothiazine column. Removal of Ca^{2+} with a calcium-complexing solution causes elution of the fusion protein. The GST-CaM fusion protein was then digested with factor Xa, and the target protein GST was isolated. The purity of the isolated GST was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Introduction

Protein engineering has been used for the design of proteins with desired characteristics. In addition, when the gene of the protein is known, recombinant DNA technology can be used to incorporate specific modifications into the sequence of amino acids of the desired protein that enables its isolation in a biologically active form. These changes can be introduced wither within the amino acid sequence of the protein or by adding polypeptide extensions (fusions) to the C- or N-terminus of the protein. Depending on the properties of the protein of interest and the purification scheme that would be appropriate for that protein, a variety of polypeptides of varying length and characteristics can be introduced by gene fusion as "tails" to the target protein (Ford et al., 1991). All fusion tails take advantage of a specific interaction between the fusion tail and the stationary phase, while the unmodified protein of interest is not capable of that interaction. To be able to remove the affinity tail from the fusion protein, a specific cleavage site can be genetically incorporated between a protein of interest and the affinity tail. Since not every available fusion tail and protease cleavage site can work with every protein, there is a need for the development of new fusion tail systems.

In this article, we used calmodulin (CaM) as the fusion tail and glutathione-S-transferase (GST) from *Schistosoma japonicum* as the model target protein. For the purification of the GST, we took advantage of the specific interaction between the calmodulin fusion tail and the drug phenothiazine in the presence of Ca^{2+} (Levin and Weiss, 1977). For that, a plasmid encoding a GST-CaM fusion protein was expressed in *Escherichia coli*. The fusion protein was loaded onto an affinity column prepared by immobilizing phenothiazine on silica particles (Hentz et al., 1996; Charbonneau et al., 1983; Jarret, 1986). Elution of the GST-CaM fusion protein was performed under mild conditions in the presence of a complexing agent such as EGTA. Removal of Ca^{2+} induces a conformational change in calmodulin that caused dissociation of the phenothiazine−calmodulin complex. Another goal of this study was to evaluate whether a factor Xa cleavage site can be used to cleave the protein of interest from the calmodulin affinity tail.

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Materials and Methods

Ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA) and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Aldrich (Milwaukee, WI). The 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES) and tris(hydroxymethyl)aminomethane (Tris) buffer salts were purchased from Research Organics (Cleveland, OH). Coagulation factor Xa and reduced glutathione (GSH) were purchased from Calbiochem (La Jolla, CA). The pGEX-5X-1 plasmid was obtained from Pharmacia Biotech (Uppsala, Sweden). Restriction enzymes *Xho*I, *Eco*RI, and the T4 ligase were purchased from Gibco (Gaithersburg, MD). The Geneclean II kit used for the purification of the restriction products was purchased from BIO 101 (Vista, CA). Chromozym X (*N*-methoxycarbonyl-D-norleucyl-glycyl-L-arginine-4-nitranilide acetate) was obtained from Boehringer Mannheim (Indianapolis, IN).

The coupled silica-2-(trifluoromethyl)-10*H*-(3′-aminopropyl)phenothiazine hydrochloride (TAPP) affinity particles prepared as described earlier (Hentz et al., 1996) were packed into an HR 5/10 glass column (Pharmacia Biotech; Uppsala, Sweden). SDS-PAGE of the purified proteins was carried out on a Phast System (Pharmacia Biotech; Uppsala, Sweden). The activity of the purified GST was determined in a Gilford Stasar III spectrophotometer (Gilford Instrument Laboratories; Oberlin, OH) following the procedure of Habig et al. (1974). Absorbance

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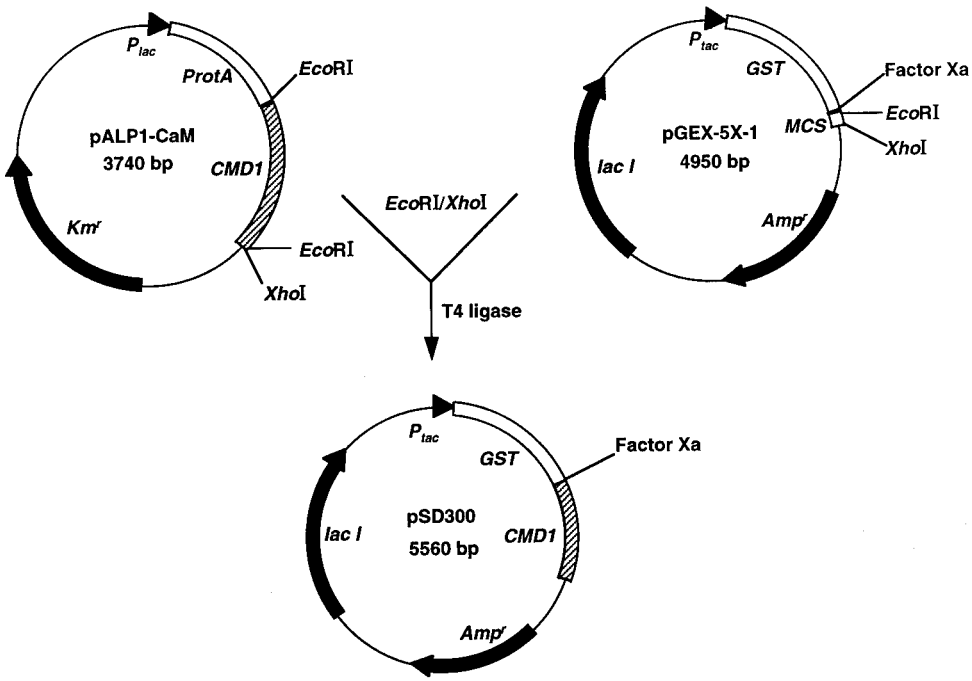

Figure 1. Construction of the pSD300 plasmid for expression of the GST-CaM fusion protein in *E. coli* (genes are not shown to size).

spectra for protease assay were taken on a hewllet Packard 8453 diode array spectrophotometer (Palo Alto, CA).

Construction of the GST-CaM fusion protein was performed by subjecting the plasmid pALP1CaM (Stirling et al., 1992) containing the calmodulin gene to restriction with *Xho*I and *Eco*RI. The same enzymes were used for the digestion of the pGEX-5X-1 plasmid that contains the gene that encodes the GST protein and factor Xa recognition site. Restriction products were purified by employing the Geneclean method and were subsequently placed in a tube and ligated with T4 ligase overnight in a water bath at 4 °C to yield plasmid pSD300. The presence of GST and CaM genes in pSD300 was confirmed by restriction analysis using *Eco*RI and *Xho*I on a 1% agarose gel.

For the expression of the GST-CaM fusion protein *E. coli* strain DH5a was transformed (Maniatis et al., 1988) with the newly constructed plasmid pSD300. Expression was induced by IPTG. The purification procedure of the GST-CaM fusion protein was essentially the same as described elsewhere (Hentz et al., 1996). The procedure by Smith and Corcoran (1990) was followed for the site-specific proteolysis of the GST-CaM fusion protein. A volume of 100 μL of the digestion mixture was loaded onto the TAPP affinity column, which was preequilibrated with buffer A (10 mM HEPES−NaOH, 0.5 mM $CaCl_2$, pH 7.0), and the same purification protocol as for the purification of GST-CaM was followed. The GST was eluted with buffer B (10 mM HEPES−NaOH, 0.5 mM $CaCl_2$, 1 M NaCl, pH 7.0), and the CaM and the undigested GST-CaM were eluted with buffer C (10 mM Tris-HCl, 5 mM EGTA, pH 8.0) as confirmed by SDS-PAGE.

The efficiency of GST purification and absence of residual factor Xa activity in the protein sample was determined by employing a protease assay using Chromozym X as a substrate. For this, 1.9 mL of 0.9 mM of the chromogenic substrate Chromozym X in 50 mM Tris-HCl, pH 8.3, 150 mM NaCl, and 10 mM $CaCl_2$ was mixed with 100 μL of the sample containing purified GST at room temperature. The increase in absorbance at 405 nm over a 10-min interval was monitored by using a Hewllet Packard 8453 diode array spectrophotometer.

Results and Discussion

The goal of this work was to develop a model system for the purification of recombinant proteins based on a calmodulin fusion tail. Glutathione-S-transferase (GST) was chosen as the target protein. A GST-CaM fusion protein was constructed as described in the Materials and Methods from plasmid pGEX-5X-1 encoding GST and the blood coagulation factor Xa recognition sequence and from pALP1CaM encoding yeast CaM protein (Figure 1) lacking 12 amino-terminal amino acids (Stirling et al., 1992). A differently prepared GST-CaM was used previously to study on−off switching of enzymatic reactions (Kobatake et al., 1996).

Commonly used stationary phases for the purification of CaM are based on phenyl sepharose columns (Ishii et al., 1995) and immobilized phenothiazine (Jarret, 1986) and mellitin (Kinkaid and Coulson, 1985). Although, in the presence of Ca^{2+}, CaM shows high affinity for all these stationary phases, phenothiazine was selected for our studies because phenyl sepharose based columns bind to many other proteins in addition to calmodulin, and therefore, the expression procedure requires extra steps in order to obtain high-purity product (Roberts et al., 1985). Likewise, mellitin (a polypeptide) is more susceptible to degradation, and therefore, the service life of the affinity column would be shorter. Further, some binding of CaM to the immobilized mellitin has also been detected in the absence of Ca^{2+} (Fleminger et al., 1992), which makes it less desirable for the development of a purification system based on CaM binding. It is our experience that the phenothiazine affinity column was still functional after more than 1 year of use.

Plasmid pSD300 was transformed in *E. coli*, and after expression, the protein was purified on the silica-TAPP affinity column. The presence of the desired fusion protein in the eluent was detected by SDS-PAGE as

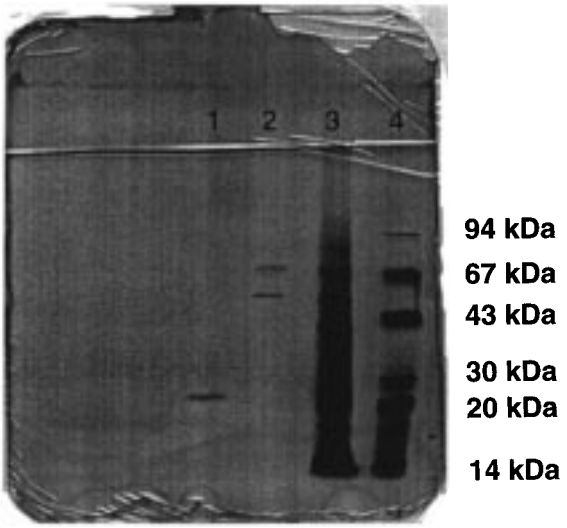

Figure 2. SDS-PAGE developed with silver stain: lane 1, peak eluted with buffer B containing pure GST after digestion of GST-CaM fusion protein with factor Xa and purification on the affinity column; lane 2, peak eluted with buffer C after purification of GST-CaM from crude extract; lane 3, crude cell extract; lane 4, molecular weight marker. The molecular weights (in kDa) corresponding to the protein markers are shown to the right of lane 4.

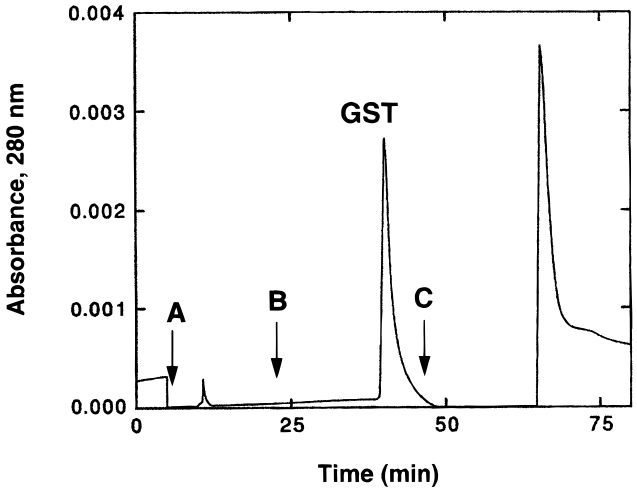

Figure 3. Chromatogram of a 100-μL injection of the protein mixture after digestion of the GST-CaM fusion protein with factor Xa. A, B, and C correspond to the addition of buffers A, B, and C, respectively, on the column.

shown in Figure 2. A band that corresponds to the GST-CaM fusion protein at 45 kDa (lane 2) was present. Another band at 67 kDa attributed to a contaminant (see below) was also present.

The separation and purification of GST from CaM was accomplished by site-specific proteolysis using highly pure factor Xa as described in the Materials and Methods. The elution with buffer A (Figure 3) rendered a very small peak, which is probably due to factor Xa that is unretained by the column. The content of the peak eluted with buffer B was determined by SDS-PAGE (Figure 2), and it showed the presence of only one band at 28 kDa (lane 1). An amount of 15 μg of pure GST per 500 μL of cell lysate was obtained. To confirm the quantitative removal of factor Xa from the final preparation of GST, we performed a peptide cleavage assay using the chromogenic substrate Chromozym X. Factor Xa catalyzes the hydrolysis of Chromozym X as a result of which formation of 4-nitroaniline is observed at 405 nm. Our experiments did not show any increase in absorbance at this wavelength over a 10-min period when purified protein sample was added to the substrate, thus confirming that GST has been obtained in a pure fashion. The activity of the purified GST was determined by using CDNB and glutathione as substrates. This implies that the factor Xa recognition sequence of the CaM fusion protein is assesible to the protease. By following this procedure, the 67-kDa impurity that was present after the purification of the GST-CaM fusion protein was absent in the final GST preparation. It seems possible that this high-molecular-weight protein is naturally expressed in bacteria and has affinity for either the immobilized phenothiazine or calmodulin. The same impurity was detected during the purification of recombinant calmodulin (unpublished data). We can also exclude the possibility of it being any of the calmodulin-binding proteins since the genes of calmodulin and calmodulin-associated proteins are not present within the bacterial genome. On the other hand, it is possible that this protein is a chaperone associating with calmodulin. We tried to elute the contaminant protein from the phenothiazine column with an ATP-containing buffer after washing the column with buffer B, but this did not result in elimination of the contaminant protein. Irrespective of its nature, this 67-kDa protein is not present in the purified GST preparation because, after the endopeptidase treatment, the contaminating protein is retained on the column.

In summary, we have demonstrated that fusion to the N-terminus of calmodulin can be successfully used for the affinity purification of the model protein GST on the TAPP column. It was further demonstrated that, after ligation of the GST to CaM, purification, and digestion with factor Xa, the enzyme maintains catalytic activity. This calmodulin-based affinity tail system can be extended to the separation of other recombinant proteins of interest.

Acknowledgment

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