A PagP fusion protein system for the expression of intrinsically disordered proteins in *E. coli*

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ABSTRACT

PagP, a beta-barrel membrane protein found in Gram-negative bacteria, expresses robustly in inclusion bodies when its signal sequence is removed. We have developed a new fusion protein expression system based on PagP and demonstrated its utility in the expression of the unstructured N-terminal region of human cardiac troponin I (residues 1-71). A yield of 100 mg fusion protein per liter M9 minimal media was obtained. The troponin I fragment was removed from PagP using cyanogen bromide cleavage at methionine residues followed by nickel affinity chromatography. We further demonstrate that optimal cleavage requires complete reduction of methionine residues prior to cyanogen bromide treatment, and this is effectively accomplished using potassium iodide under acidic conditions. The PagP-based fusion protein system is more effective at targeting proteins into inclusion bodies than a commercially available system that uses ketosteroid isomerase; it thus represents an important advance for producing large quantities of unfolded peptides or proteins in *E. coli*.

Key words: fusion protein expression; cyanogen bromide cleavage

Abbreviations: KSI, ketosteroid isomerase; PagP, PhoPQ-activated gene P; CNBr, cyanogen bromide; Gdn-HCl, guanidine hydrochloride; NMR, nuclear magnetic resonance

INTRODUCTION

Eukaryotic proteins frequently contain long unstructured sequences that do not fold into stable tertiary structures. These flexible and exposed regions often contain protein binding motifs or post-translational modification sites and play a vital role in defining the protein's physiologic context and function. However, producing large quantities of unstructured proteins can be challenging, since they are prone to degradation in live cells.

Cardiac troponin I is a 210-residue protein that does not fold independently into any stable domain. Instead, it forms an elongated structure with many helical regions that interact with troponin C, troponin T, and actin-tropomyosin [1]. Residues 1-33 of troponin I are unique to the cardiac isoform and known to be important in regulating cardiac contractility via phosphorylation [2]; but the region is unstructured and flexible, and it was excluded in the X-ray crystal structure determination of the cardiac troponin complex [1]. Our initial attempts to express residues 1-71 of human cardiac troponin I (cTnI[1-71]) in *E. coli* resulted in negligible expression, suggesting intracellular degradation.

Expression into insoluble inclusion bodies circumvents intracellular degradation. One commercially available system, the pET31b vector, expresses the protein of interest fused to ketosteroid isomerase (KSI) [3], a hydrophobic but soluble protein that misfolds into inclusion bodies when overexpressed in *E. coli*. We used the pET31b system to express cTnI[1-71] fused to KSI.

We then fused cTnI[1-71] to PagP, a β-barrel integral membrane protein that accumulates in inclusion bodies when expressed in *E. coli* [4]. β-barrel membrane proteins are known to form inclusion bodies in *E. coli* when expressed without an N-terminal signal sequence [5]. The PagP fusion system compared favorably with the KSI-based system, thus representing a novel alternative for producing large quantities of unstructured protein.

MATERIALS AND METHODS

Bacterial strains and chemical reagents

pET31b vector was purchased from EMD4Biosciences. All primers were synthesized by Integrated DNA Technologies, Inc. All DNA modifying enzymes were purchased from Fermentas. DNA purification kits were purchased from Qiagen. *E. coli* Rosetta(DE3) cells were purchased from EMD4Biosciences. XL10 Gold Ultracompetent cells were purchased from Agilent. Ni-NTA affinity columns were purchased from Qiagen. Cyanogen bromide (CNBr) was purchased from Sigma.

Cloning

See Figures 1A and 1B for the protein expression constructs produced.

The expression vector, pETPagP, was created by PCR-amplifying PagP (residues 1-161 without the signal sequence) from the pETCrcAHΔS plasmid [4] using the primers, 5'-GGTAAAG **CAT ATG** AAC GCA GAT GAG TGG AT and 5'-TCC **CTC GAG CAG CAT CTG** AAA CTG AAA GCG CAT CCA, with the engineered restriction enzyme sites for NdeI, XhoI, and AlwNI, respectively, shown in bold (the AlwNI site is CAG XXX CTG, where X can be A, C, T, or G). The amplified fragment was cloned into pET31b using the NdeI and XhoI restriction enzyme sites, replacing KSI with PagP.

cTnI[1-71] was PCR-amplified from a prior cTnI-containing plasmid using the primers, 5'-G GTT **CAG ATG CTG** GCC GAT GGT AGC AGC GAT GCG and 5'-C CTC **CAG CAT CTG** CTT CTC TCC GCG CCG CTC CTC C, with the AlwNI restriction enzyme sites shown in bold. PCR-amplified cTnI[1-71] was inserted into pET31b or pETPagP using the AlwNI cut sites to yield cTnI[1-71]-pET31b and cTnI[1-71]-pETPagP, respectively.

One advantage of using the AlwNI cut site in pET31b is that it is possible to clone in tandem copies of insert. This was accomplished by performing the ligation reaction of the insert on its own in the absence of vector to create a ladder of tandem products, gel-purifying the products, and then ligating them into the vector [3]. Tandem copies of cTnI were thus inserted into pET31b and pETPagP to produce cTnI[1-71]- cTnI[1-71]-pET31b and cTnI[1-71]- cTnI[1-71]-pETPagP, respectively. Producing these constructs was exceedingly difficult, and longer constructs with more than two tandem sequences could not be produced. This is likely due to circularization of the insert, which is known to occur at >150bp DNA length [6]. To minimize this phenomenon, the ligation reaction was carried out at room temperature for only one hour, in contrast to overnight at 4°C as described previously by Kuliopoulos and Walsh[3]. The greater tendency towards self-circularization in the cTnI construct can be explained by the larger size of the cTnI construct, 222bp, compared to 51bp used in the Kuliopoulos paper.

In light of the difficulties associated with making tandem cTnI[1-71] sequences, we decided to use the single copy cTnI[1-71]-pETPagP construct for further protein expression and purification (see Figure 1c for the amino acid sequence). The C-terminal AlwNI site was mutated from CAG ATG CTG (Gln-Met-Leu) to CAG CGG CTG (Gln-Arg-Leu), so that CNBr cleavage would remove cTnI[1-71] from PagP, but leave the C-terminal His-tag attached for future purification steps (see Figures 1B and 1C), since it would not interfere with our future proposed NMR experiments.

Protein expression and purification

Protein expression plasmids were transformed into Rosetta(DE3) competent cells. Several colonies were selected and grown in 10 mL LB broth with 100 mg/L ampicillin. After the cells had grown beyond A600=1.0 they were added to 1 L M9 minimal media containing glucose 10 g/L, thiamine 100 mg/L, biotin 1 mg/L, and ampicillin 100 mg/L. At A_{600}~0.8, the cells were induced with 1 mM IPTG and incubated for an additional 6 hours before harvesting. All growths were performed at 37 °C.

About 9.4 g cells (wet weight) from 2 L culture were resuspended in 20 mL chilled 50 mM Tris, pH 8.0 containing 1 mM MgSO4 and 10 μg/mL DNase. After thorough resuspension, the sample was mixed for 10 min with 20 mg lysozyme and 200 mg deoxycholic acid pre-dissolved in 1mL buffer each. Cell clumps were broken up using a glass Kontes Duall tissue grinder. The cell lysate was spun at 15 000 rpm for 10min. The insoluble fraction was then thoroughly mixed in 20mL 1% Triton X-100 and 0.5mM EDTA pH 8.0 buffer for 1 hour. Again, the insoluble fraction was collected by a repeat 15 000 rpm spin and the pellet washed three times with distilled water.

The pellet was dissolved in binding buffer for nickel column chromatography under denaturing conditions (6 M Gdn-HCl, 50 mM sodium phosphate, 0.5 M sodium chloride, 10 mM imidazole, pH 8.0). The sample was vigorously stirred overnight and respun at 15 000 rpm to remove undissolved protein. The protein sample was then applied to the nickel column equilibrated with the binding buffer. The elution buffer was identical to the binding buffer with the exception that the imidazole concentration was 200 mM.

CNBr cleavage and purification

To 15 mL of 4 mg/mL PagP-cTnI[1-71] eluted from the nickel column, HCl was added to 0.3 M (to completely acidify the buffer). 100 mg potassium iodide was added and the sample incubated overnight at room temperature. The sample was then diluted ten-fold with 100 mM Tris-HCl, pH 8.0 to precipitate the protein, which was spun down and then redissolved in 30 mL 70% formic acid. 400 mg CNBr were added directly to the solution, and the reaction was allowed to proceed overnight at room temperature.

Following CNBr cleavage, the reaction mixture was dialyzed against water 4 times to remove the CNBr, and to the post-dialysis CNBr-containing waste solutions, 0.1 M NaOH (or 0.1 M excess relative to formic acid) and then bleach were added to react any remaining residual CNBr. Gdn-HCl was added to the dialysate up to 6 M, as well as 50 mM Tris, pH 8.0, and the sample was again purified on the nickel column under denaturing conditions, yielding pure cTnI[1-71]-His_6 protein. Although the cTnI[1-71]-His_6 construct was water soluble, some of it would precipitate in the presence of PagP CNBr cleavage products, which is why the second nickel column was also run under denaturing conditions.

The purified cTnI[1-71]-His_6 protein was exchanged into water by dialysis, lyophilized, and stored at 4 °C.

RESULTS

Protein expression

cTnI[1-71] was initially cloned into the pET31b vector as described in the Materials and Methods section, singly or as a tandem repeat to produce KSI-cTnI[1-71] and KSI-cTnI[1-71]-cTnI[1-71], respectively (see Figure 1A). In cells overexpressing KSI from the original pET31b vector, a single band is seen on an SDS-PAGE gel at roughly the expected 13 kDa (Figure 2A, lane 2). Expression of KSI-cTnI[1-71] yields a single band at an apparent molecular weight of 30 kDa, slightly higher than the expected 23 kDa (lane 3). Surprisingly, expression of KSI-cTnI[1-71]-cTnI[1-71] did not yield a higher molecular weight band on SDS-PAGE, but rather showed lower bands at about 28 kDa and 17 kDa (lane 4), suggesting intracellular degradation of the overexpressed products.

cTnI[1-71] was then cloned into pETPagP to produce PagP-cTnI[1-71] and PagP-cTnI[1-71]-cTnI[1-71] (see Figure 1B). Overexpresssion of PagP on its own leads to a robust band at 20 kDa as expected (see Figure 2B, lane 2). There is an additional band at about 23 kDa corresponding to the folded β-barrel [7]. PagP is able to fold in SDS in the presence of glycerol and β-mercaptoethanol [8], which are both present in the SDS-PAGE sample loading buffer. Similarly, expression of PagP-cTnI[1-71] gives double bands at about 31 kDa and 34 kDa (lane 3), and expression of PagP-cTnI[1-71]-cTnI[1-71] gives bands at about 37 kDa and 41 kDa (lane 4). In contrast to KSI, there is no indication of protein degradation products by SDS-PAGE.

Protein purification

PagP-cTnI[1-71]-His_6 protein was expressed as shown in Figure 3, lane 1. The major band in the whole cell lysate corresponds to PagP-cTnI[1-71]-His_6, indicating robust expression. (Note that PagP-cTnI[1-71]-His_6 ran predominantly as a single band on this gel, in contrast to Figure 2B. The folding of PagP is dependent on many factors, such as the presence of detergents, lipids, alcohols, and thiols. The lysis buffer used for the whole cell lysate contains 1% deoxycholic acid, and PagP is unfolded in this detergent.) None of the fusion protein was visible in the soluble fraction (lane 2). The insoluble pellet was white and almost entirely soluble in 6M Gdn-HCl. The pellet contained almost pure PagP-cTnI[1-71]-His_6 protein (lane 3), as the SDS-PAGE bands before and after nickel affinity purification (lane 4) appear exactly the same. (The extra bands at about 34, 50, and 60 kDa correspond to folded monomer, unfolded dimer, and folded dimer.) This is consistent with a recent study that showed it was possible to crystallize PagP solubilized directly from inclusion bodies for X-ray diffraction studies [9].

After the first nickel affinity purification, the protein yield was calculated using a molar extinction coefficient ε_{280} = 85.4 mM^{-1} cm^{-1} (ProtParam from www.expasy.ch), indicating a yield of about 100 mg fusion protein per L M9 minimal media culture.

CNBr cleavage

Complete CNBr cleavage of PagP-cTnI[1-71]-His_6 yields fragments at 0.8, 2, 8, and 10 kDa (see Figure 1C for amino acid sequence). Although this is what is observed by SDS-PAGE (Figure 3, lane 5), at least trace amounts of partial cleavage fragments are always produced. Purification of the cleavage reaction by nickel affinity chromatography under denaturing conditions yields the expected product at 10 kDa (lane 7). However, when this "pure" fraction is dialyzed to remove Gdn-HCl, trace amounts of precipitate form, corresponding to partial cleavage products with an intact C-terminal His-tag (lane 6). This provided a convenient method of removing partial cleavage products. However, there was one partial cleavage product that contained an additional six amino acid residues from the PagP fusion construct. Even though the SDS-PAGE indicated 100% cleavage, MALDI-TOF mass spectrometry indicated a small percentage (<1% to 30%, depending on the prep) of this uncleaved product (indistinguishable from the fully cleaved product by SDS-PAGE). Thus, optimizing conditions for CNBr cleavage was important for the purity of the final product (see Discussion below).

The final yield of the cleaved cTnI[1-71]-His_6 peptide was 16 mg from a 1 L M9 minimal media growth, corresponding to 3.4 mg protein per gram of wet weight cells.

DISCUSSION

Incomplete CNBr cleavage was due to two factors. First of all, it was impossible to perfectly solubilize PagP-cTnI[1-71]-His_6. Originally, we used the protocol of Rodriguez *et al*. [10], dissolving the protein in 6M Gdn-HCl with 0.1M HCl for CNBr cleavage. However, we found that 70% formic acid, the more conventional solution for CNBr cleavage, was a more potent denaturant and solvent than concentrated guanidine. There is a theoretical risk of formylation of serine and threonine side chains, but we could not detect this subsequently with MALDI-TOF mass spectrometry or NMR spectroscopy. It is possible that any esters formed during the cleavage reaction were hydrolyzed during the subsequent dialysis steps.

It is tempting to eliminate the first nickel affinity purification step and to proceed directly to the CNBr cleavage following inclusion body solubilization. However, even though the fusion protein appears pure by SDS-PAGE at this stage, there are still non-protein contaminants like lipids and nucleic acids. This makes the inclusion bodies more difficult to solubilize than the purified protein, even with 70% formic acid. Hence, the first nickel affinity purification step can be eliminated, but the efficiency of the CNBr cleavage reaction can be compromised due to incomplete solubilization.

The other factor contributing to incomplete CNBr cleavage was oxidation of methionine residues to their sulfoxide or sulfone forms [11]. Thus, it was necessary to introduce a reduction step prior to CNBr cleavage, either with β-mercaptoethanol or potassium iodide [12]. We found that pre-treatment with potassium iodide led to a more complete cleavage than β-mercaptoethanol. It is worthwhile to note that potassium iodide reacts with CNBr to form iodine, which could potentially react with tyrosine [13] or tryptophan [14]. Also, high concentrations of β-mercaptoethanol seemed to interfere with CNBr cleavage. Thus, reducing agents (either potassium iodide or β-mercaptoethanol) were removed from the mixture prior to CNBr cleavage. Since there are no cysteine residues in PagP-cTnI[1-71]-His_6, the need for reducing agent could not be explained by cysteine disulfide bridge reduction. Cleavage of PagP-cTnI[1-71]-His_6 was >99% in formic acid with potassium iodide pre-treatment, as indicated by MALDI-TOF mass spectrometry of the final product (data not shown).

Using a fusion tag to enhance protein expression is a common practice. While there have been comparisons of the efficacy of various soluble protein tags [15] [16], we are not aware of any published comparisons of tags that intentionally direct proteins into inclusion bodies. Compared to KSI, PagP was better at overproducing cTnI[1-71] in inclusion bodies. This cannot be strictly a function of protein hydrophobicity. The amino acid sequence of PagP is, in fact, less hydrophobic than that of KSI, despite the fact that PagP is an integral membrane protein. The predominantly β-barrel structure must be advantageous for keeping proteins in inclusion bodies, as this is a universal feature of β-barrel membrane proteins. Another advantage of β-barrel membrane proteins as fusion partners is that they are fairly hydrophilic in nature, as the interior of the β-barrel is not exposed to the membrane and is composed of mainly hydrophilic residues. This can be important in solubilizing the fusion construct from inclusion bodies, since poor solubilization can decrease yields and reduce CNBr cleavage efficiency [10] (see above).

Protein expression in inclusion bodies followed by chemical cleavage is a useful method for producing large quantities of intrinsically disordered proteins. Nickel affinity chromatography and cyanogen bromide cleavage are natural choices for purification and fusion partner removal, respectively, since both of these are best carried out under denaturing conditions. These methods can also be used to produce structured proteins, as long as a protocol for refolding the protein exists. Maximizing protein yield is particularly important for biophysical studies by NMR spectroscopy, which typically require tens of milligrams of isotopically enriched protein. In this regard, the PagP fusion protein system represents an important advance.

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Figure legends

Figure 1. A) Fusion constructs containing KSI and cTnI[1-71]. B) Fusion constructs containing PagP and cTnI[1-71]. The key methionine residues between fusion partners and the His-tag are shown. (CNBr cleavage occurs after each methionine residue.) Note that there are also Met residues within the primary sequence of KSI and PagP that are internal CNBr cleavage sites. C) Amino acid sequence of PagP-cTnI[1-71]. The native PagP sequence is comprised of residues 2-162 and cTnI[1-71], residues 166-236, or the fusion construct. Methionine residues are highlighted in bold and red. The amino acids corresponding to the AlwNI restriction sites are italicized, *QML* (residues 163-165) and *Q(M/R)L* (residues 237-239). Residue 238 was mutated from methionine to arginine for the PagP-cTnI[1-71]-His_6 construct so that the C-terminal His-tag would survive CNBr cleavage.

Figure 2. A) Whole cell lysates of KSI-construct-expressing cells in LB media. Samples were run on 15% Tris-Glycine SDS-PAGE gels and stained with Coomassie Brilliant Blue. Lane 1, pre-induction; Lane 2, KSI construct, 4 hours post-induction; Lane 3, KSI-cTnI[1-71]; Lane 4, KSI-cTnI[1-71]-cTnI[1-71].
B) Whole cell lysates of PagP-construct-expressing cells in LB media. Samples were run on 15% Tris-Glycine SDS-PAGE gels and stained with Coomassie Brilliant Blue. Lane 1, pre-induction; Lane 2, PagP construct, 4 hours post-induction; Lane 3, PagP-cTnI[1-71]; Lane 4, PagP-cTnI[1-71]-cTnI[1-71].

Figure 3. Expression and cleavage of PagP-cTnI[1-71]-His_6, and isolation of final cTnI[1-71]-His_6. Samples were run on 15% Tris-Glycine SDS-PAGE gels and stained with Coomassie Brilliant Blue. Lane 1, whole cell lysate from M9 minimal media, lysed as described in Materials and Methods; Lane 2, soluble fraction; Lane 3, insoluble fraction; lane 4, insoluble fraction purified on Ni column in 6M Gdn-HCl; lane 5, post-cyanogen bromide cleavage of PagP-cTnI[1-71]-His_6; lane 6, insoluble incomplete cleavage products that precipitated after Gdn-HCl (from the second Ni column) removal by dialysis; lane 7, soluble purified cTnI[1-71]-His_6, MW by mass spectrometry, 9617.5.

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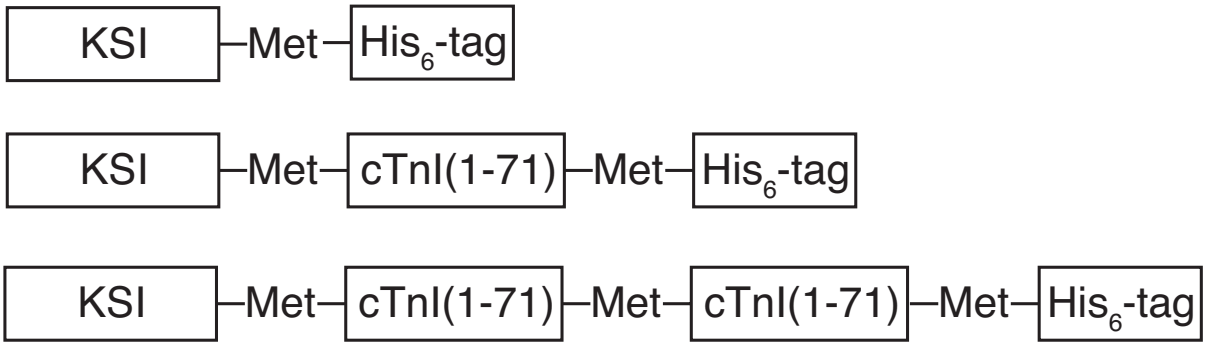

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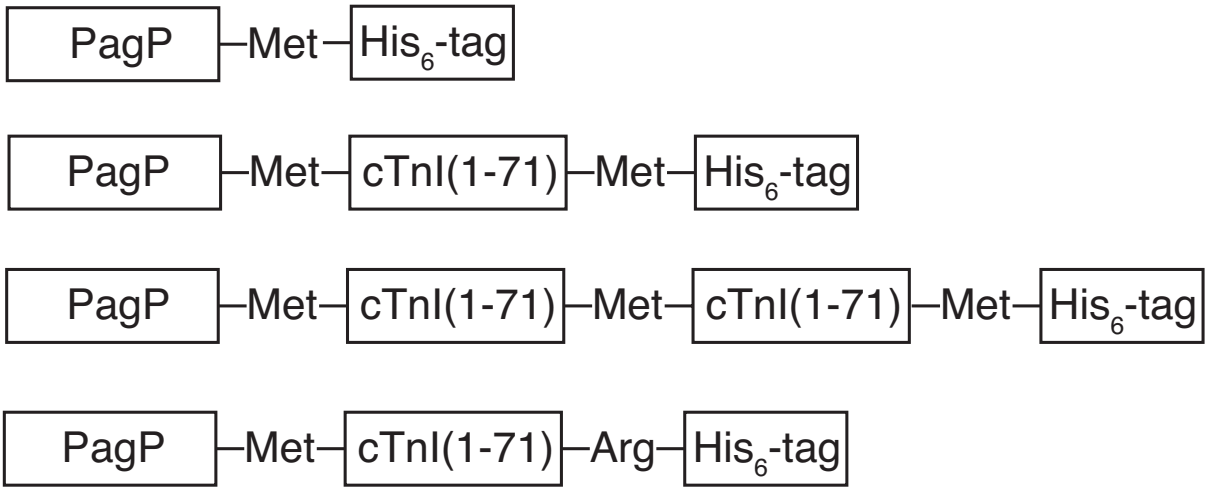

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         110         120         130         140         150
NFHLGLGFTA GVTARDNWNY IPLPVLLPLA SVGYGPVTFQ MTYIPGTYNN

         160         170         180         190         200
GNVYFAWMRF QFQMLADGSS DAAREPRPAP APIRRRSSNY RAYATEPHAK

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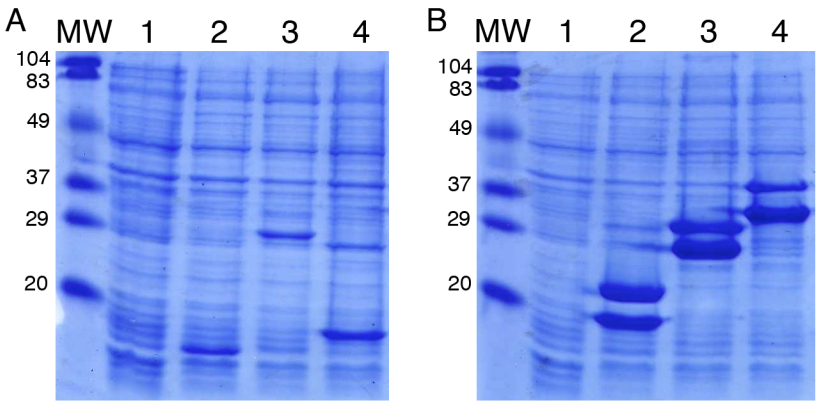
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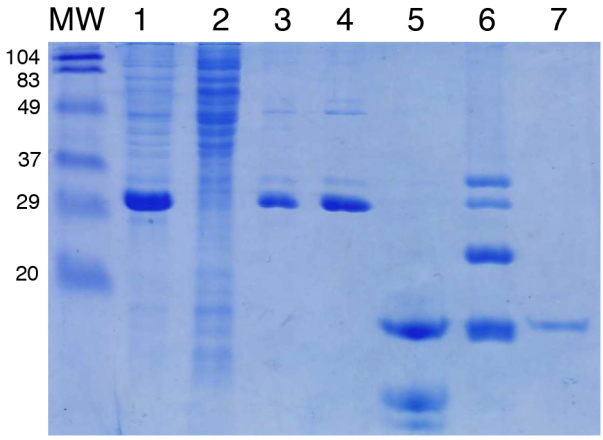
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