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Chemical cleavage of fusion proteins for high-level production of transmembrane peptides and protein domains containing conserved methionines

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Abstract

Due to their high hydrophobicity, it is a challenge to obtain high yields of transmembrane peptides for structural and functional characterization. In the present work, a robust method is developed for the expression, purification and reconstitution of transmembrane peptides, especially for those containing conserved methionines. By using a truncated and mutated glutathione-*S*-transferase construct as the carrier protein and hydroxylamine (which specifically cleaves the peptide bond between Asn and Gly) as the cleavage reagent, 10 mg of the first transmembrane helix of CorA, a Mg^{2+} transporter from *Mycobacterium tuberculosis*, can be conveniently obtained with high purity from 1 L of M9 minimal media under optimized conditions. The biophysical properties of the peptide were studied by circular dichroism and nuclear magnetic resonance spectroscopy, and the results show that this CorA peptide is well folded in detergent micelles and the secondary structure is very similar to that in recent crystal structures. In addition, this CorA construct is oligomeric in perfluoro-octanoic acid micelles. The compatibility with the transmembrane peptides containing conserved methionines, the high yield and the simple process make the present method competitive with other commonly used methods to produce such peptides for structural and functional studies.

Keywords: Hydroxylamine; Transmembrane peptide; Fusion protein expression; Glutathione-*S*-transferase; Membrane protein

1. Introduction

Many important membrane proteins have a small molecular weight. In the *Mycobacterium tuberculosis* genome 60% of the putative membrane proteins have a molecular weight less than 40 kDa [1]. Furthermore, there is growing evidence that the membrane and water-soluble domains of membrane proteins often function independently [2–7]. Consequently, there are many hydrophobic peptides and proteins that are important to express in high yields for structural and functional studies. However, the high hydrophobicity makes their expression and purification much more difficult than for water-soluble proteins [1,8–10].

Considering that small hydrophobic peptides/proteins are usually unstable and toxic to the host, the most common way to biosynthesize these proteins and peptides is to express the target as a fusion protein that can enhance the target's expression level [4,9–14]. A challenge is to achieve site-directed proteolysis of the fusion protein followed by efficient purification. Enzymatic

Abbreviations: CD, circular dichroism; CorA-TM1, the first transmembrane helix of CorA; CSI, chemical shift index; DPC, dodecyl phosphocholine; ESI-TOF mass, electrospray ionization time-of-flight mass; CNBr, cyanogen bromide; GST, glutathione-*S*-transferase; GuHCl, guanidine chloride; HSQC, heteronuclear single quantum coherence; IPTG, isopropyl-β-D-thiogalactopyranoside; LIC, ligation independent cloning; MBP, maltose binding protein; NMR, nuclear magnetic resonance; PFO, perfluoro-octanoic acid; TEV, tobacco etch virus protease; TMPs, transmembrane peptides; TGST, truncated glutathione-*S*-transferase; SDS, sodium dodecyl sulfate

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cleavage is the most common method due to mild cleavage conditions, however, the requirements that the fusion protein must be soluble and that the designed cleavage site must be accessible to the protease makes this approach inappropriate in many cases. Especially challenging are those cases involving hydrophobic peptides since the fusion protein containing a highly hydrophobic tail is not stable and prone to aggregation during expression. Using maltose binding protein (MBP) as the carrier protein is an efficient way to avoid aggregation of small peptides/proteins, but the proteolysis is not always successful [13]. For chemical cleavage, the reaction is performed under denaturing conditions in which the solubility of the protein and accessibility of the cleavage site would not be limiting. The most widely used chemical cleavage reagent is cyanogen bromide (CNBr). Although very successful in many cases [4,11,12,15,16], the main limitation is that there must be no conserved methionines in the target sequence. However, amino acid sequence analysis of transmembrane peptides (TMPs) has shown that the frequency of methionines in TMPs is substantially higher than that in helices of soluble proteins [17], and some conserved methionines play critical roles in the structure and/or function of membrane proteins [18,19]. As another chemical cleavage reagent, hydroxylamine specifically hydrolyses the peptide bond between Asn and Gly [20,21], which make it an alternative method for the production of TMPs containing conserved methionines.

To demonstrate the chemical cleavage approach, we present here the production of a TMP from CorA, the only constitutively expressed Mg^{2+} transporter in most bacteria responsible for both the influx and efflux of magnesium ions across the membrane. Functional studies have shown that mutations in the first transmembrane helix (CorA-TM1), especially in the universally conserved "GMN" signature sequence abolish transport activity [19]. In the recent crystal structures, it has been shown that CorA has two transmembrane helices per monomer and forms a pentameric structure. The five CorA-TM1 helices line the pore for ion transport [22–24]. Although very similar in general features, one of the significant discrepancies among the crystal structures is the orientation of the "GMN" residues at the entrance of the pore, which is thought to form a selectivity filter and cation dehydration mechanism. Furthermore, the transmembrane domain has been suggested to function quite independently from the rest of the protein in that the structures from the crystallization of the water-soluble domain and full length protein are superimposable [22]. Data has been obtained in our lab on Mg^{2+}, Co^{2+} and inhibitor binding to the transmembrane domain of this protein (Hu, Qin, Sharma, Cross and Gao, unpublished results). Therefore, a detailed structural study of CorA-TM1 and this transmembrane domain would be valuable.

In the present work, a method is refined for using hydroxylamine to cleave a fusion protein to produce the CorA-TM1 of *M. tuberculosis* for structural and functional studies. By using this protocol, combined with a truncated and mutated version of glutathione-*S*-transferase (TGST) as the carrier protein, 10 mg of highly purified, isotopically labeled CorA-TM1 is obtained from 1 L M9 minimal media culture. The biophysical and biochemical characterizations indicate that CorA-TM1 is well folded and forms oligomers in detergent micelles that are suitable for further structural and functional investigation. This robust method is anticipated to be suitable for the production of other TMPs, especially for those containing conserved methionines.

Scheme 1. (A) Construction of expression plasmids for GST (pGSTHA) and TGST (1–83) (pTGSTHA) fusion protein. The shaded NG site is the hydroxylamine cleavage site, and the cleavage point is indicated by the arrow. The underlined residues are the TEV cleavage site. Note that in the sequence of TGST, N79 was mutated to H to remove the main undesired cleavage site by hydroxylamine. (B) The sequence of CorA-TM1. The conserved methionines are labeled with stars.

2. Materials and methods

2.1. Gene clone and plasmid construction

The plasmid used in the present work was modified from a GST fusion protein expression plasmid in which a ligation independent cloning (LIC) site follows the gene for GST [25]. A tobacco etch virus protease (TEV) cleavage site was inserted between the GST and LIC sites. Through site-directed mutation, a hydroxylamine cleavage site, NG, was introduced exactly before the LIC site, the resulting plasmid was named pGSTHA. The gene encoding the first transmembrane helix of CorA (CorA-TM1) from *M. tuberculosis* was amplified by PCR and inserted into the plasmid through LIC. To truncate GST, a pair of primers (F: 5′- GGT GGT GGC GAC CAT CCT CC -3′, R: 5′- ACC ACC CAA CAT GTT GTG CTT GTC -3′) were phosphorylated by T4 polynucleotide kinase (New England Biolabs Inc.), and then used for PCR amplification. After digestion by DpnI for 2 h at 37 °C, the PCR product was ligated by T4 DNA ligase and transformed into *Escherichia coli* strain DH5α. To remove the potential hydroxylamine cleavage site in the sequence of TGST, N79 was mutated to H with the primers (F: 5′- GCT GAC AAG CAC CAC ATG TTG GGT GGT G -3′, R: 5′- C ACC ACC CAA CAT GTG GTG CTT GTC AGC -3′) by using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene Inc.). The resulting plasmid was named pTGSTHA. The plasmids used in the present work and the sequence of CorA-TM1 are shown in Scheme 1. All the constructs have been verified by sequencing.

2.2. Expression of fusion protein

The plasmids encoding the fusion proteins were transformed into *E. coli* strain BL21 (DE3)-RP codon plus for expression. Usually, a single clone was picked and inoculated into 3 mL of rich media (LB) culture with 100 μg/mL ampicilin, and grown overnight at 37 °C. The 3 mL culture was then added to 1 L LB media, and the culture was grown to OD_{600}=0.6 at 37 °C. The culture was then cooled to 30 °C, and the expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 16–20 h at 30 °C with shaking. In M9 media, the bacteria from a 3 mL overnight culture were collected by centrifugation and washed with M9 media once before inoculating a 1 L M9 culture. Other procedures were the same as those for the LB culture.

2.3. Fusion protein purification and hydroxylamine cleavage

Cells were collected by centrifugation at 4000 ×*g* for 10 min, and then washed once with a buffer containing 20 mM Tris–HCl, pH 8.0. Chilled cells were lysed by French Press in a solution containing 50 mM NaCl, 20 mM Tris–HCl at pH 8.0. After centrifugation (10,000 ×*g*) for 20 min, the supernatant was discarded. The pellet was washed with 0.2% Triton X-100 in the above buffer twice, then with water another two times to remove residual Triton X-100. The