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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

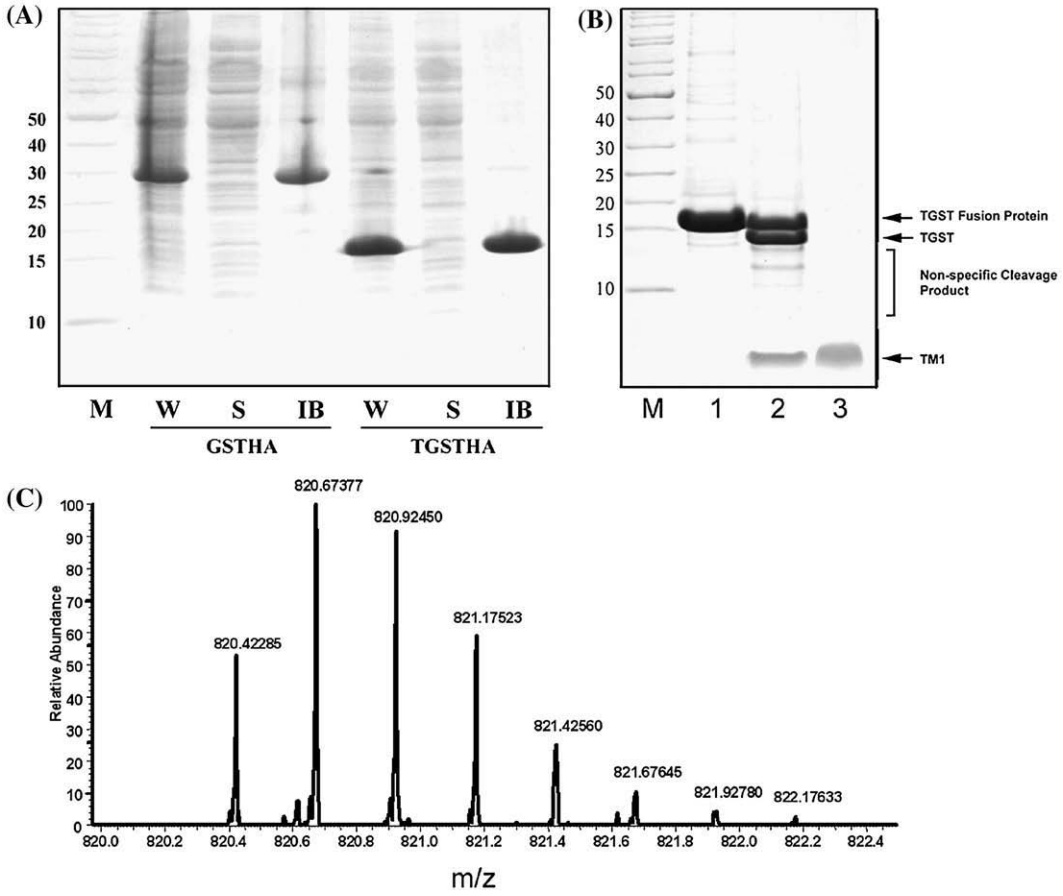

Fig. 1. (A) Expression and distribution of GST and TGST fusion protein. M: marker, W: whole cell, S: soluble fraction, IB: inclusion body. (B) Hydroxylamine cleavage of TGST fusion protein. Lane 1: uncleaved fusion protein; Lane 2: after hydroxylamine cleavage (the weak bands from 10 to 15 kDa were from non specific cleavage); Lane 3: purified CorA-TM1. (C) The ESI FT-ICR mass spectrum of CorA-TM1. Molecular weight of CorA-TM1 is calculated as follows: $4\times820.4228-4\times1.0087=3277.6564$, in which 820.4228 is the monoisotopic m/z of CorA-TM1, 1.0087 is the atomic weight of protons and 4 is the positive charge on CorA-TM1. The charge state of CorA-TM1 was determined based on the separation between two adjacent isotopic peaks (1/4).

inclusion bodies were then solubilized in 6 M guanidine chloride (GuHCl) and the volume adjusted to make the concentration of fusion protein approximately 7 mg/mL as determined by UV absorbance. A hydroxylamine stock solution (4 M hydroxylamine chloride, 50 mM Tris–HCl, pH 8.5) was added to achieve 1.1 M hydroxylamine and a 5 mg/mL protein solution. To reduce methionine oxidation, free methionine and EDTA were added to the reaction solution at concentrations of 50 mM and 5 mM, respectively. The reaction was performed at 45 °C with gentle shaking for 6 h. To stop the reaction, 1/10 volume of glacial acetic acid was added to the reaction solution.

To optimize cleavage conditions, a series of reactions were performed using temperature, pH and reaction times as variables. After exhaustive dialysis, the pellet was collected by centrifugation and applied to SDS-PAGE. The specific cleavage efficiency and the percentage of non specific cleavage were estimated through image analysis by using Scion Image (Scion Inc.) software. For estimating the specific cleavage efficiency the sum of the optical densities of bands corresponding to the desired cleavage reaction products was divided by the total optical density of the lane, and the non specific cleavage efficiency was estimated in a similar way, except that the reaction products corresponding to non specific cleavage products were divided by the total optical density of the lane.

2.4. Purification of CorA-TM1

The reaction solution was dialyzed against water overnight and the precipitate collected. After resolubilization in a solution containing 6 M GuHCl and 0.2% DPC (dodecyl phosphocholine) at room temperature, the sample was loaded onto Ni^{2+}-NTA agarose resin (Qiagene). After incubation overnight, the flow through was collected and exhaustively dialyzed against water containing 10 mM β-mercaptoethanol for two days. The precipitate was collected, lyophilized and stored at −20 °C. For identification of the final product, the peptide was solubilized in acetonitrile/H_2O (50/50, v/v) and the accurate mass was determined using a 14.5 Tesla Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer through direct diffusion electrospray ionization (ESI).

2.5. Circular dichroism (CD) experiment

The stored CorA-TM1 was solubilized in a buffer containing 170 mM sodium dodecyl sulfate (SDS), 5 mM acetic acid, pH 4.5. The concentration of CorA-TM1 was adjusted to 20 μM determined by UV absorbance based on a predicted molar absorption coefficient [26]. CD experiments were performed using an AVIV 202 CD spectrometer with 0.1 cm quartz cuvettes. Spectra were recorded from 260 nm to 200 nm in 0.5 nm steps with a 1 s integration time at 25 °C. Each curve represents the average of at least four scans and a blank was subtracted from each of the spectra. The spectra were plotted as the molar (per residue) circular dichroism ($\Delta\varepsilon$, $M^{-1}\cdot cm^{-1}$).

2.6. Nuclear magnetic resonance (NMR) experiments

NMR samples were prepared by solubilization of CorA-TM1 in 170 mM SDS, 10 mM DTT, 100 mM acetic acid, pH 4.5 and 10% D_2O. The concentration of peptide was approximately 1 mM determined by UV absorbance. The 2D (^1H-^{15}N heteronuclear single quantum coherence, ^1H-^{15}N HSQC [27]) and 3D (HNCA [28] and CBCA(CO)NH [29] for sequential resonance assignment) experiments were performed at 30 °C on a VARIAN Inova 720 MHz spectrometer at the National High Magnetic Field Laboratory.

2.7. PFO-PAGE

The PFO-PAGE was performed as in a previous report [30]. The peptide was solubilized in perfluoro-octanoic acid (PFO) sample buffer (4% PFO, 100 mM Tris–HCl, pH 8.0) at a concentration of approximately 1 mg/mL, and 1 μL of the solubilized peptide solution was loaded. The proteins in the low molecular weight kit (Amersham Pharmacia Biotech Inc.) were used as standard proteins and loaded onto the gel in separate lanes (5 mg/mL, 1 μL per lane). The gel was stained with silver nitrate.

3. Results and discussion

3.1. Expression of fusion protein

Initially the CorA-TM1 gene was inserted into a vector in which GST acts as the carrier protein, followed by a TEV cleavage site between GST and the inserted peptide. However, as shown in Fig. 1A, although the fusion protein is over-expressed, the fusion protein formed inclusion bodies that could not be refolded and cleaved by TEV. Although GST is a good carrier protein for expressing small soluble proteins with high yield, its capability to enhance the solubility of the target protein is poor [31], resulting in difficulties for proteolysis. However, chemical cleavage of the fusion protein is possible. As the most widely used chemical cleavage reagent, CNBr is very successful in some cases, however, this method cannot be used here since our CorA-TM1 construct has two highly conserved methionines: one is in the Mg^{2+} transporter signature sequence "GMN" and the other is in the highly conserved "MPEL" sequence in the interhelical loop [32]. Hydroxylamine, however, hydrolyzes the Asn–Gly bond, and has been used successfully to produce small soluble peptides, such as insulin-like growth factor I [21,33] and an antimicrobial peptide [34]. Through site-directed mutation, a hydroxylamine cleavage site was introduced just before the target peptide sequence, such that after cleavage there is only one additional residue, a Gly, at the N-terminus of CorA-TM1.

Expression of a fusion protein containing a large carrier protein can be a waste of material, especially when isotopic labeling is performed. A smaller carrier protein is better for increasing the final yield of the target peptide. The GST fold contains an N-terminal GSH binding domain and a C-terminal substrate binding domain. To decrease its molecular weight while preserving its function as a carrier protein, GST was truncated and only the N-terminal GSH binding domain (1–83) was left. In this way, the mass fraction of the target peptide in the fusion protein can be almost doubled, and the theoretical yield of target peptide can be increased substantially if the yields of the two fusion proteins are similar. As shown in Fig. 1A, like the full length GST fusion protein, the truncated GST fusion protein was exclusively expressed in the inclusion body fraction with even higher expression level. Accordingly, pTGSTHA was selected to express the CorA-TM1 in the following experiments. Since the purity in inclusion bodies is already high (after washing twice with Triton X-100, the purity is usually over 90%), for our experiments, we did not purify the fusion protein any more. Usually 200–400 mg of fusion protein was obtained from 1 L LB or M9 culture in shaking flasks.

Table 1
Optimization of the cleavage reaction[a]

		37 °C			45 °C		
pH		8.5	8.75	9	8.5	8.75	9
20 hours	specific cleavage	30%	40%	46%	49%	49%	48%
	non specific cleavage	11%	9%	10%	17%	20%	20%
6 hours	specific cleavage	24%	28%	30%	46%	49%	47%
	non specific cleavage	3%	2%	3%	5%	5%	6%

[a] In experimental condition optimization, two temperatures (37°C and 45°C), two reaction times (6 and 20 h) and three pH values (8.5, 8.75 and 9.0) have been tested. All the data were from image analysis of an SDS-PAGE gel in a typical experiment by using Scion Image (Scion Inc.) software. The specific cleavage is the percentage of cleavage which occurred at the desired cleavage site, and the non specific cleavage is the percentage of cleavage that occurred at other sites in the fusion protein.

3.2. Optimization of the experimental conditions for cleavage reaction and CorA-TM1 purification

In previous reports, it has been shown that the reaction conditions, especially temperature and pH, could substantially influence the cleavage reaction [21]. In addition, different reaction conditions have been used in different reports [21,33,34]. To optimize the experimental conditions for fusion protein cleavage by hydroxylamine, the reaction conditions were screened and the results are listed in Table 1. Consistent with the previous report, the cleavage efficiency is temperature dependent: increasing the temperature from 37 °C to 45 °C increases the cleavage efficiency up to 100%. Although it was reported that high pH was advantageous for the reaction [21], here, pH has little effect on the reaction at high temperature. More importantly, we found that long reaction times decrease the reaction specificity. It had been reported that under extreme conditions, hydroxylamine could cleave any peptide bond following Asn *(19)*. In our preliminary experiments, it was found that even under mild conditions (low temperature, low pH and short reaction time), there was frequent cleavage between N79 and M80 in the TGST fragment resulting in a significant contamination of the final product. To eliminate this undesired cleavage, N79 was mutated to a histidine, hence the use of the mutant TGST fusion. Although there are four additional Asn residues in the fusion protein: two in TGST, one in the linker between TGST and CorA-TM1, and one in CorA-TM1, the undesired cleavage at these sites was low, compared with the specific cleavage between Asn and Gly under the optimized reaction conditions (Fig. 1B).

There are two types of possible side-reactions in the use of hydroxylamine. One is methionine oxidation, which can be minimized by the addition of free methionine as a scavenger in the cleavage reaction [21]. The other is the formation of hydroxamate on the side chain of Asn or Gln, which can be minimized, but not completely eliminated by using lower pH, lower temperature, and a lower concentration of hydroxylamine [21]. By considering the specific and non specific cleavage efficiency, and side-reactions, the optimized experimental conditions were observed to be: 1.1 M hydroxylamine in 4.3 M GuHCl, 50 mM methionine, 5 mM EDTA, 50 mM Tris–HCl, at pH 8.5 and 45 °C for 6 h. As shown in Fig. 1B, approximately 50% of the TGST fusion protein was

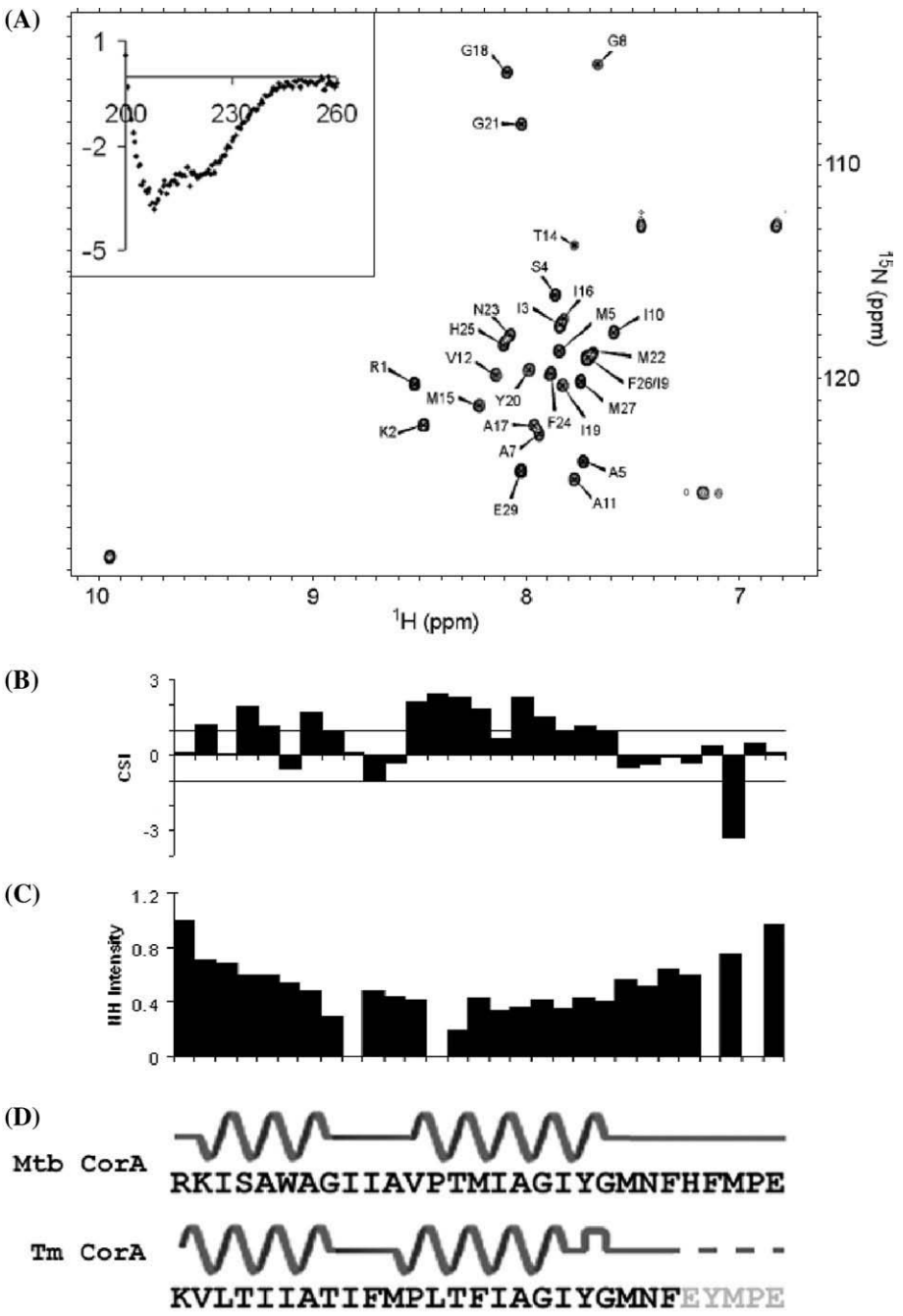

Fig. 2. Initial structural characterization of CorA-TM1. (A) ^{1}H-^{15}N HSQC of 1 mM CorA-TM1 in 170 mM SDS micelles, pH 4.5 at 30 °C and CD spectrum (inserted figure) under similar conditions. The units in CD spectra are wavelength (nm) and molar circular dichroism, $\Delta\varepsilon$ ($M^{-1}\cdot cm^{-1}$) for abscissa and ordinate, respectively. (B) Chemical shift index of C_α. (C) NH peak intensities in the HSQC spectrum (prolines and overlapped residues are not included). (D) Comparison between the secondary structure of TM1 from *M. tuberculosis* CorA and that from the *T. maritima* CorA crystal structure. The grey color in the sequence of *T. maritima* CorA represents the unresolved residues in the crystal structure.

cleaved under these experimental conditions. The moderate cleavage efficiency in our experiments could be due to the aggregation of the fusion protein with the released target peptide. Some aggregation was noticed in our reactions and the contents of this aggregation were analyzed by SDS-PAGE, showing a mixture of the uncleaved fusion protein and the released CorA-TM1. After addition of acetic acid to stop the reaction and then the exhaustive dialysis against water, the precipitate was re-solubilized in a solution containing 6 M GuHCl and 0.2% DPC and passed through the Ni^{2+}-NTA agarose resin to remove uncleaved fusion protein and TGST. Then CorA-TM1 in the flow through could be easily obtained with high purity (Fig. 1B). Fig. 1C shows the mass spectrum of the purified CorA-TM1, indicating a +4 charged species reflecting a molecular weight of 3277.6 Da, the same as the calculated value. The simplicity in purification, as well as the high yield of the fusion protein, makes the yield of CorA-TM1 (~10 mg/L in shaking flask culture of M9 media) higher than the yields of peptides with similar molecular

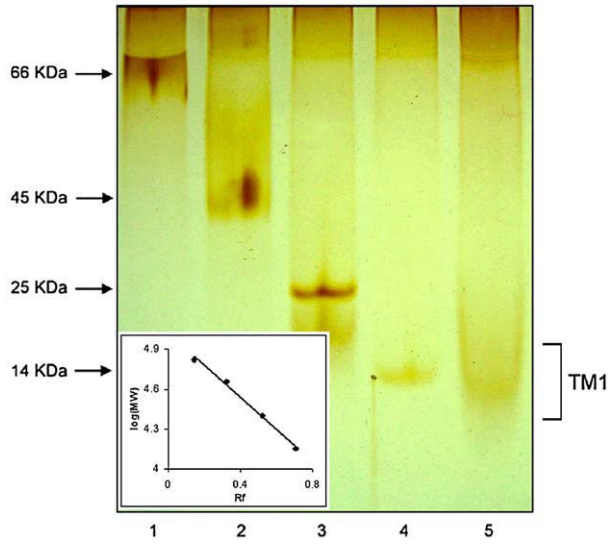

Fig. 3. Analysis of the oligomeric state of CorA-TM1 by PFO-PAGE. The standard proteins used were albumin (lane 1, 66 kDa), ovalbumin (lane 2, 45 kDa), chymotrypsinogen A (lane 3, 25 kDa) and lysozyme (lane 4, 14 kDa). CorA-TM1 was solubilized in sample loading buffer containing 4% PFO at a concentration about 1 mg/mL and 1 μL was loaded on lane 5. The bracket indicates the CorA-TM1 oligomer. The gel was stained by silver nitrate. The calibration curve used for estimation of the oligomer molecular weight is shown in the inserted figure.

weights in other reports. Recently, an efficient method to produce TMPs by using MBP as the carrier protein and TEV as the cleavage reagent has been refined in our lab [35]. This method does work well for some TMPs, but the yield for CorA-TM1 was substantially lower than that reported here. In addition, hydroxylamine is easier to handle and less expensive than TEV.

3.3. Biophysical and biochemical characterization of CorA-TM1

The biophysical properties of CorA-TM1 in detergent micelles were characterized by CD and solution NMR. As shown in the insert figure in Fig. 2A, the CD spectrum in SDS micelles at pH 4.5 is featured with about 40% helix content estimated by CDPro software. The ^{1}H-^{15}N HSQC NMR spectrum (Fig. 2A) recorded under similar sample conditions (but at 1 mM) showed all 28 expected resonances (the glycine at the N-terminus and two prolines are not included), indicating a homogeneous preparation and a unique conformation in SDS micelles with a ^{1}H chemical shift dispersion of approximately 1 ppm. Based on the C_α chemical shift index (CSI) [36] (Fig. 2B) and probability analysis [37], the secondary structure of CorA-TM1 in SDS micelles is predicted as shown in Fig. 2D. The analysis suggests a broken helix, as is observed in the CorA crystal structures from *Thermotoga maritima* [22]. The proline and glycine residues in this region of the CorA-TM1 sequence may be responsible. The peak intensity analysis (Fig. 2C) shows that the resonance intensities decrease towards the center of the lipid bilayer consistent with increasing low frequency dynamics near the micelle center. Note that the peak intensities of the non-protonated prolines and those of the overlapped peaks are not reported. The break in the helix would need to be confirmed with additional structural restraints and even so may be an artifact of a detergent environment, typical of solution NMR and crystallographic studies.

In the crystal structure of *T. maritima* CorA, five TM1s form the pore-like structure and there are extensive interactions between adjacent TM1 helices. To check whether the purified CorA-TM1 forms an oligomer, CorA-TM1 was characterized by PFO-PAGE, a less denaturing environment for membrane proteins than SDS that can preserve oligomer structure in PFO micelles [30]. CorA-TM1 showed a diffuse band from 12 to 16 kDa on the PFO gel (Fig. 3) based on the range of the R_f values from the gel and the calibration curve shown in the figure insert. This suggests that CorA-TM1 forms an oligomeric structure in PFO micelles since the molecular weight is much greater than that shown by SDS-PAGE. The diffuse band further suggests that the oligomeric state is unstable between two or more oligomeric forms. Potentially at the 1 mM concentration in SDS micelles the CorA-TM1 peptide is also in an oligomeric state.

3.4. Conclusions

CNBr is the most widely used chemical cleavage reagent, and has been successfully used for the production of some hydrophobic peptides. However, the inevitable limitation of CNBr cleavage is that it can't be used in the presence of conserved methionine residues and since methionines are common in transmembrane helices, this is a significant limitation. Hydroxylamine hydrolyzes the peptide bond between asparagine and glycine, which makes the probability of a hydroxylamine cleavage site in any sequence about 1/400 and in transmembrane helices this residue is quite rare. Although non specific cleavages can occur, under controlled conditions the reaction can be made much more specific and the chemical side-reactions can be minimized. The results shown here suggest that hydroxylamine is a good alternative to the widely used CNBr as a cleavage reagent.

In the present work, the isotopically labeled CorA-TM1 from *M. tuberculosis* was expressed in high yield, purified to homogeneity, and characterized by CD, NMR and PFO-PAGE. By using hydroxylamine under mild conditions as the cleavage reagent, half of the CorA-TM1 was released from the fusion protein. The high-level expression of the TGST fusion protein, the high fraction of target peptide in the fusion construct and the simplicity in purification all contribute to the high yield of the target peptide. The biophysical characterization suggested that CorA-TM1 was well folded in detergent micelles and that its secondary structure was similar to that in the *T. maritima* crystal structures. Accordingly, the present method appears to be appropriate for other hydrophobic peptides, especially for those containing conserved methionines.

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