

Chapter 2

Expression and Purification of a Matrix Metalloprotease Transmembrane Domain in *Escherichia coli*

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Abstract

Membrane tethered matrix metalloproteases are bound to the plasma membrane by a glycosylphosphatidylinositol-anchor or a transmembrane domain. To date, most studies of membrane-bound matrix metalloprotease have focused on the globular catalytic and protein–protein interaction domains of these enzymes. However, the transmembrane domains have been poorly studied even though they are known to mediate intracellular signaling via interaction with various cellular proteins. The expression and purification of the transmembrane domain of these proteins can be challenging due to their hydrophobic nature. In this chapter we describe the purification of a transmembrane domain for a membrane-bound matrix metalloprotease expressed in *E. coli* and its initial characterization by NMR spectroscopy.

Key words Membrane-anchored MMP, Matrix metalloproteases, NMR spectroscopy, Transmembrane domain, Protein expression, Isotopically labeled

1 Introduction

Matrix metalloproteases comprise a large family of zinc-dependent endopeptidases that function at neutral pH to degrade extracellular matrix proteins, cleave cell surface receptors, release apoptotic ligands, and activate chemokines and cytokines [1–3]. Matrix metalloproteases (MMPs) participate in a wide range of cellular processes including tissue remodeling, cell proliferation, cell migration, differentiation, angiogenesis, apoptosis, and the immune response [4–9]. Aside from these normal physiological roles, MMPs have been implicated in a large number of pathological disorders such as arthritis, Alzheimer’s disease, atherosclerosis, vascular disease, central nervous system disease, liver cirrhosis, and various cancers [10, 11].

MMP activity is regulated at multiple levels, including biosynthesis (transcription/translation) [12], zymogen activation [10, 12], compartmentalization [10, 13, 14], and inactivation [14–16]. Several MMPs are active during development and normal

physiology and may play a role in homeostasis [7, 9, 17–20], while others play a role in tissue injury and infection [4, 7]. In chronically inflamed tissues and most cancers MMPs contribute to various pathological processes, including tissue degradation, tumor progression and invasion [21–27].

MMPs are expressed and transported through the cell as inactive zymogens and secreted or anchored to the plasma membrane, confining their activity to the extracellular environment or the cell surface [8]. Evidence suggests that secreted MMPs bind to specific cell-surface receptors, membrane-anchored proteins and cell-associated extracellular matrix (ECM) molecules, and function pericellularly at focused locations [14]. MMPs also function in the cell nucleus (MMP2, 3, 9, 13 and MT1-MMP), cytoplasm (MMP1, 2, 26, 23), and various organelles, where their localization is facilitated by interactions with other proteins, proteoglycan core proteins and/or their glycosaminoglycan chains, and other molecules [1, 10, 13, 28–31].

Unlike the majority of MMPs that function as soluble proteases, several are anchored to the cell membrane [32–34]. The membrane tethered MMPs include the membrane-type MMPs (MT-MMPs) and MMP23, a membrane anchored MMP containing a domain architecture that differs significantly from that of the MT-MMPs [35]. There are six MT-MMPs denoted MT1-MMP (MMP14), MT2-MMP (MMP15), MT3-MMP (MMP16), MT4-MMP (MMP17), MT5-MMP (MMP24), and MT6-MMP (MMP25). MT4-MMP and MT6-MMP are tethered to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor while the remaining MT-MMPs are anchored via a transmembrane (TM) domain [32]. The MT-MMPs contain a common domain architecture consisting of an N-terminal signal peptide followed by a pro-domain, a catalytic domain, a hinge (linker-1) region, a hemopexin-like (Hpx) domain, and a stalk region (linker-2). Linker-2 is followed by a TM domain or GPI-anchor and a C-terminal cytoplasmic tail (Fig. 1) [32]. In contrast, the domain architecture of MMP23 comprises an N-terminal signal sequence followed by a cytoplasmic tail, a membrane-anchoring TM domain, a linker region, a pro-domain, a catalytic domain, a small cysteine-rich toxin-like domain (TxD), and a C-terminal immunoglobulin-like cell adhesion molecule (IgCAM) domain (Fig. 1) [35].

To date, studies of membrane-anchored MMPs have mostly focused on the characterization of the catalytic and globular protein–protein interaction domains. However, evidence indicates that the single helical TM domain and unstructured cytoplasmic domain of these enzymes can play a key role in the function of membrane-anchored MMPs. For example, the formation of MT1-MMP homo-dimers mediated by both the Hpx [36] and TM domains [37] is required for proMMP2 activation on the cell surface [36] and degradation of collagen [38].

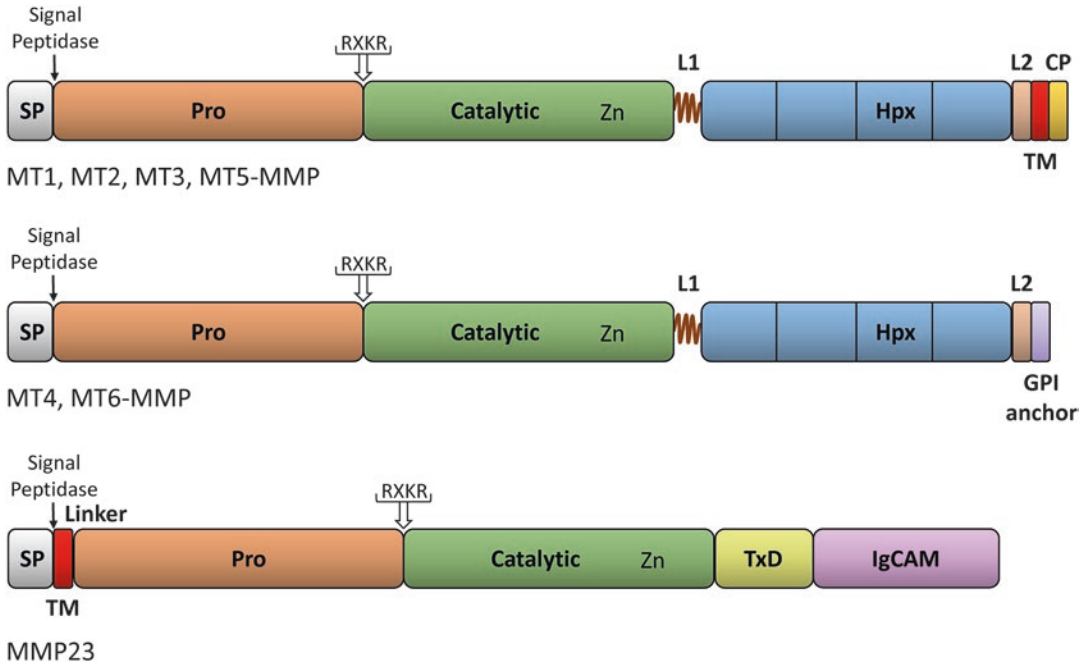


Fig. 1 Structural domains of the membrane-anchored MMPs. MT1-MMP, MT2-MMP, MT3-MM, MT4-MMP, MT5-MMP, and MT6-MMP share a common domain structure consisting of an N-terminal signal peptide (SP) that targets the protease to the cell surface, a pro-domain (Pro), a catalytic domain, a linker domain (L1), a hemopexin (Hpx) domain involved in protein–protein interactions, and a second linker region (L2). MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP contain a C-terminal single helical transmembrane (TM) domain followed by a cytoplasmic (CP) domain while MT4-MMP and MT6-MMP possess a C-terminal GPI anchor. MMP23 differs in its domain structure, possessing an N-terminal signal peptide followed by a single type-II helical transmembrane domain, a pro-domain, a catalytic domain, a cysteine-rich toxin-like (TxD) domain, and a protein–protein interaction IgCAM domain. The pro-domain (Pro) in most membrane-anchored MMPs (except MMP23) contains an unpaired cysteine sulfhydryl group that interacts with the active site zinc atom and maintains the enzyme in a latent inactive form (the cysteine switch). The MT-MMPs are transported in their inactive latent form to the cell surface, where they are cleaved at the recognition motif (RXKR) within the pro-domain by a furin-like protease, resulting in activation of the enzyme

Previous studies have highlighted the importance of the cytoplasmic domain of membrane-anchored MMPs in mediating various intracellular interactions. For example, the 20 amino acid residue cytoplasmic tail of MT1-MMP has been reported to interact with a number of proteins and is also the target of posttranslational modifications. Interaction of the MT1-MMP cytoplasmic domain (CPD) with FIH-1 (Factor inhibiting HIF-1) triggers a cascade of protein–protein interactions resulting in activation of HIF-1 α thereby regulating ATP production in macrophages during normoxia [39]. While interaction of phosphorylated Tyr573 in the MT1-MMP CPD with p130Cas regulates Rac1 signaling in osteoclast formation [40, 41]. The MT1-MMP CPD has also been

shown to interact with p27RF-Rho (LAMTOR1) which regulates RhoA activation [42]. Other studies have shown that the MT1-MMP CPD is required for its localization to focal adhesions via interactions with the focal adhesion kinase (FAK)-p130Cas [43] and an eight amino acid residue loop structure, MT-Loop, located in the CPD is required to degrade underlying matrices in an efficient manner [44]. MT1-MMP has a half-life of less than 30 min on the cell surface and is endocytosed in a clathrin- and caveolae-dependent manner. The clathrin-dependent endocytosis of MT1-MMP is attributed to the interaction of residues LLY⁵⁷³ with adapter protein 2, a component of clathrin-coated pits [45]. Endocytosed MT1-MMP is also recycled to the cell surface and a sequence (DKV⁵⁸²) within the CPD is required for recycling [46]. Other studies have shown that the last three residues (EWV⁶⁴⁵) in the CPD of MT5-MMP play a similar role in its recycling to the cell surface [47]. These residues act as a PDZ binding motif and bind to Mint-3 which contains two PDZ domains. While our recent studies have shown that the pro-domain of MMP23, containing a TM and cytoplasmic domain, interacts with the voltage-gated potassium ion channel Kv1.3 and regulates the trafficking of this ion channel to the cell surface [35, 48].

Considering the important role played by the TM and cytoplasmic domains of membrane-anchored MMPs, very few studies have been undertaken to define their structure and dynamics. In this chapter we focus on the expression and purification of a truncated protein containing the transmembrane and cytoplasmic domains of a membrane-anchored MMP (MMP23) for the characterization of these domains by NMR spectroscopy.

2 Materials

2.1 Expression of Isotopically Labeled Peptides in *E. coli* in M9 Minimal Media

1. *E. coli* BL21(DE3) or *E. coli* BL21(DE3)pLysS chemically competent cells (Novagen).
2. pET32a expression vector (Novagen) containing cDNA for your peptide of interest covering the TM and cytoplasmic domains (TM/CPD) of your membrane-anchored MMP and an N-terminal 3C protease recognition site codon optimized for expression in *E. coli* (see **Note 1**).
3. Luria-Bertani (LB) broth: 10 g tryptone, 10 g NaCl, 5 g yeast extract per liter of deionized water. Adjust the pH to 7.0 with 0.5 N NaOH and autoclave. Add ampicillin to a final concentration of 100 µg/ml immediately prior to use.
4. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
5. 50% (w/v) glucose: 125 g of D-glucose per 250 ml of deionized water. Sterilize by passing through a 0.2 µm filter and store at room temperature.

6. ^{13}C -labeled D-glucose (99% ^{13}C): This can be obtained from a number of suppliers including Cambridge Isotope Labs and Sigma-Aldrich.
7. 1 M MgSO_4 : 24.7 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per 100 ml of deionized water. Sterilize by autoclaving and store at room temperature.
8. ^{15}N -labeled ammonium chloride (NH_4Cl , 99% ^{15}N): This can be obtained from a number of suppliers including Cambridge Isotope Labs and Sigma-Aldrich.
9. Vitamin solution (5 mg/ml): 0.5 g thiamine hydrochloride and 0.5 g nicotinic acid per 100 ml of deionized water. Filter-sterilize and store at 4 °C.
10. Trace element solution (1,000 \times): 0.60 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.60 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.08 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.07 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 2 mg of boric acid, 25 mg of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, and 0.50 g of EDTA per 100 ml of deionized water [49] (*see Note 2*).
11. M9 salts (5 \times): 2.5 g NaCl, 15 g anhydrous KH_2PO_4 , 34 g anhydrous Na_2HPO_4 per liter of deionized water. Autoclave to sterilize (*see Note 3*).
12. Antibiotic for plasmid selection (1,000 \times): 100 mg/ml ampicillin in deionized water (*see Note 4*).
13. M9 minimal medium: 780 ml of deionized water, 200 ml of 5 \times M9 salts, 6 ml of vitamins, 12 ml of D-glucose (50% w/v), 1 ml of antibiotic (1,000 \times), 1 ml of trace elements (1000 \times), 3 ml of 1 M MgSO_4 and 1 g of $^{15}\text{NH}_4\text{Cl}$ (*see Note 5*).
14. Filter-sterilize M9 minimal medium and add ampicillin to a final concentration of 100 $\mu\text{g}/\text{ml}$ immediately prior to use.

2.2 Expression of Isotopically Labeled Peptides in *E. coli* in Autoinduction Media

This method was originally described by Studier [50].

1. 20 \times N: 14.2 g of Na_2SO_4 , 136 g of KH_2PO_4 , and 142 g of Na_2HPO_4 per liter of deionized water. Add each in sequence and stir until dissolved. Sterilize by autoclaving and store at room temperature.
2. 50 \times 5052: Dissolve 250 g of glycerol in 730 ml of deionized water then add 25 g of D-glucose and 100 g of α -lactose. Add each component in sequence and stir until dissolved. Lactose can take several hours to dissolve at room temperature. Sterilize by autoclaving and store at room temperature (*see Note 6*).
3. NG medium: 47 ml of sterile water, 300 μl of vitamins, 50 μl of 1 M MgSO_4 , 50 μl of trace elements (1,000 \times), 500 μl of D-glucose (50% w/v), 2.5 ml of 20 \times N, 50 μl of antibiotic, and 125 mg of $^{15}\text{NH}_4\text{Cl}$ (*see Note 7*).

4. N-5052 medium: 922 ml of sterile water, 6 ml of vitamins, 1 ml of MgSO_4 , 1 ml of trace elements (1,000 \times), 20 ml of 50 \times 5052, 50 ml of 20 \times N, 1 ml of antibiotic (1,000 \times), and 2.5 g of $^{15}\text{NH}_4\text{Cl}$.
5. Additional components for preparing the autoinduction medium include 1 M MgSO_4 , $^{15}\text{NH}_4\text{Cl}$, vitamins, trace element solution (1,000 \times), and antibiotics (1,000 \times) as described in Subheading 2.1.

2.3 Immobilized Metal Ion Affinity Chromatography (IMAC) Purification of Expressed Peptide

1. BugBuster protein extraction reagent (Merck Millipore).
2. cOmplete, EDTA-free protease inhibitor cocktail tablets (Sigma-Aldrich) (*see Note 8*).
3. Human Rhinovirus (HRV) 3C protease (Novagen) (*see Note 9*).
4. 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) (Critical micelle concentration of 8–10 mM) (*see Note 10*).
5. Lysis buffer: BugBuster containing 10 mM CHAPS, 1.0 mg/ml lysozyme, 5 $\mu\text{g}/\text{ml}$ DNaseI, and 1 \times cOmplete EDTA-free protease inhibitor cocktail.
6. Ni^{2+} affinity column: 5 ml HiTRAP chelating HP column charged with nickel (GE Healthcare Life Sciences) (*see Note 11*).
7. NTA charging solution: 250 mM NiSO_4 in deionized water.
8. NTA buffer A: 20 mM Tris–HCl buffer, pH 8.0 containing 150 mM NaCl, 10 mM CHAPS and 5 mM imidazole.
9. NTA buffer B: 20 mM Tris–HCl buffer, pH 8.0 containing 150 mM NaCl, 10 mM CHAPS and 35 mM imidazole.
10. NTA buffer C: 20 mM Tris–HCl buffer, pH 8.0 containing 150 mM NaCl, 10 mM CHAPS and 350 mM imidazole.

2.4 HPLC Purification of Peptides

1. 0.2 μm PTFE syringe filters.
2. C8 reversed-phase semi-preparative HPLC column: 100 \AA , C8, 5 μm (Phenomenex).
3. Trifluoroacetic acid (TFA).
4. Acetonitrile (HPLC grade).
5. Solvent A: 0.1% TFA (v/v). Add 1 ml of TFA to 1 L of deionized water.
6. Solvent B: acetonitrile–water (80:20) + 0.085% TFA. To 700 ml of acetonitrile add 300 ml of deionized water and 0.85 ml TFA.
7. Sample buffer: 20 mM sodium citrate, pH 5.0, containing 20 mM TCEP and 10 mM CHAPS.
8. Liquid nitrogen.

2.5 NMR Sample Preparation

1. 5 mm Shigemi tube (Shigemi Inc.).
2. Deuterated dodecylphosphocholine (d_{38} -DPC) (Sigma-Aldrich).
3. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP).
4. Sodium azide (NaN_3).
5. Deuterium oxide (D_2O).
6. NMR buffer: 20 mM sodium citrate pH 5.0, 100 mM deuterated DPC, 20 mM TCEP, 0.02% NaN_3 in 90% H_2O /10% D_2O .
7. 1,4-Dioxane (NMR reference standard) (Sigma-Aldrich) (*see Note 12*).

3 Methods

The truncated membrane-anchored MMP containing the TM and CPD domains of the protein (the TM/CPD protein) can be overexpressed as a recombinant protein in *E. coli* BL21(DE3) following induction by the addition of IPTG. The production of uniformly isotopically labeled protein is achieved using minimal media containing ^{15}N -labeled NH_4Cl and ^{13}C -labeled glucose as the sole nitrogen and carbon sources, respectively.

3.1 Protein Overexpression of Isotopically Labeled Protein in M9 Minimal Media

1. Inoculate 2 ml of LB medium containing the appropriate antibiotic with a single colony of freshly transformed *E. coli* BL21(DE3)pLysS cells harboring the pET32a expression vector containing the TM/CPD cDNA (*see Notes 13 and 14*).
2. Grow overnight at 37 °C with shaking at approximately 200 rpm.
3. Inoculate 2 ml of M9 minimal medium with 20 μl of overnight culture. Grow at 37 °C for 6–8 h until the culture is visibly turbid (*see Note 15*).
4. Inoculate 48 ml of M9 minimal medium with the entire 2 ml of culture and grow overnight at 37 °C (*see Note 16*).
5. Inoculate 500 ml of M9 minimal medium in a 2 L Erlenmeyer flask (*see Note 17*) with 25 ml of overnight starter culture. Grow at 37 °C until the optical density at 600 nm (OD_{600}) is approximately 0.5–1.0 (about 2–3 h although it may take longer to reach this OD_{600}).
6. Add IPTG to a final concentration of 1 mM to induce the expression of your target protein.
7. Grow at 37 °C for 3–5 h or 20 °C overnight (*see Note 18*).
8. Centrifuge at $5,000 \times g$ for 20 min at 4 °C to pellet the cells.
9. Decant the supernatant and store the pellet at –20 °C.

3.2 Protein Overexpression of Isotopically Labeled Peptides in Autoinduction Medium (See Notes 19 and 20)

Autoinduction typically produces significantly higher cell masses and yields of expressed protein compared to conventional induction protocols using IPTG (Fig. 2) [50].

1. Inoculate 2 ml of LB medium containing the appropriate antibiotic with a single colony from a fresh plate of *E. coli* BL21(DE3) cells harboring an expression plasmid containing your peptide (or protein) of interest.
2. Grow overnight at 37 °C with constant shaking at approximately 200 rpm.
3. Inoculate 2 ml of NG minimal medium with 20 μ l of overnight culture.
4. Grow at 37 °C for 6–8 h until the culture is visibly turbid.
5. Inoculate 48 ml of NG minimal medium in a sterile 250 ml Erlenmeyer flask with the entire 2 ml of culture and grow overnight at 37 °C (see Note 21).
6. Inoculate 500 ml of N-5052 autoinduction medium in a sterile 2 L Erlenmeyer flask (see Note 17) with 25 ml of overnight starter culture and grow for 24–48 h at 30 °C with constant shaking at approximately 200 rpm (see Note 22).
7. Centrifuge for 20 min at $5,500 \times g$ and 4 °C to pellet the cells.
8. Decant the supernatant and store the pellet at –20 °C.

3.3 Purification of Isotopically Labeled His₆-Tagged TM/CPD Peptide

1. Weigh the frozen pellet and add 10 ml of lysis buffer per 1 g of pellet.
2. Place on a rotary mixer and gently resuspend the pellet over 30–60 min.
3. Lyse the cells by using either sonication (see Note 23) or a French press.
4. Centrifuge the lysate at $16,000 \times g$ for 20 min at 4 °C.

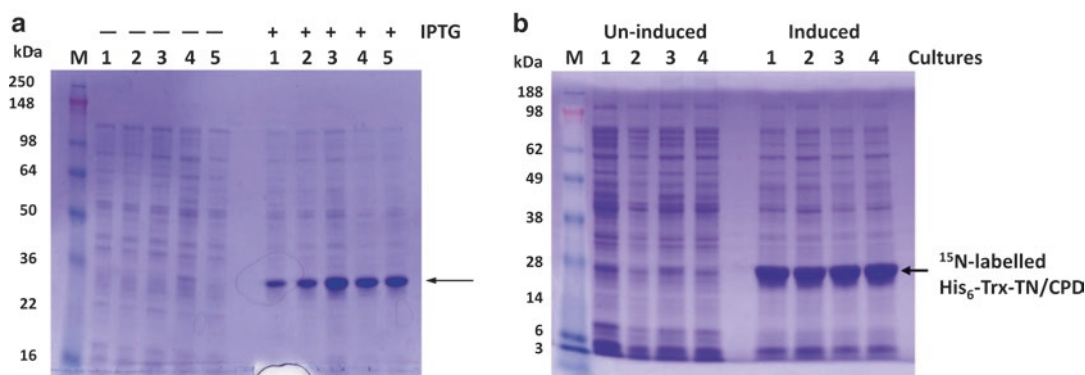


Fig. 2 Expression of ¹⁵N-labeled His₆-tagged Trx-TM/CPD. Protein expression was induced by (a) the addition of 1 mM IPTG and the culture was grown for 3 h at 37 °C or (b) autoinduction at 28 °C over 48 h

5. Collect the supernatant and load onto a 5 ml nickel affinity column equilibrated with NTA buffer A (*see Note 24*). Collect the flow-through and take a 50 μ l sample for analysis by SDS-PAGE.
6. Extensively wash the column with 50 ml of NTA buffer A followed by 50 ml of NTA buffer B (*see Note 25*). Collect the flow-through and take a 50 μ l sample for analysis by SDS-PAGE.
7. Elute the His₆-tagged peptide using a linear gradient of 0–100% NTA buffer C over 30 min at a flow rate of 2 ml/min and collecting 5 ml fractions. Take a 50 μ l sample of each fraction for analysis by SDS-PAGE.
8. Analyse samples taken during the purification of the TM/CPD protein by SDS-PAGE (Fig. 3).
9. Pool fractions containing the protein and store at 4 °C.
10. Add 3C protease to the pooled fractions and incubate with constant stirring at 4 °C to cleave the His₆-tagged protein (*see Note 26*).

3.4 Reverse-Phase HPLC Purification of TM/CPD Peptide

1. Add an equal volume of solvent A and then filter the cleaved peptide through a 0.22 μ m membrane.
2. Load onto a semi-preparative C8 reverse-phase HPLC column at a flow-rate of 1 ml/min.
3. The column is developed at 0% solvent B for 5 min followed by a gradient of 0–40% solvent B over 10 min and 40–80% solvent B over 30 min. Monitor the absorbance at 214 nm and collect eluting peaks.
4. Freeze the peak fractions in liquid nitrogen and lyophilize.

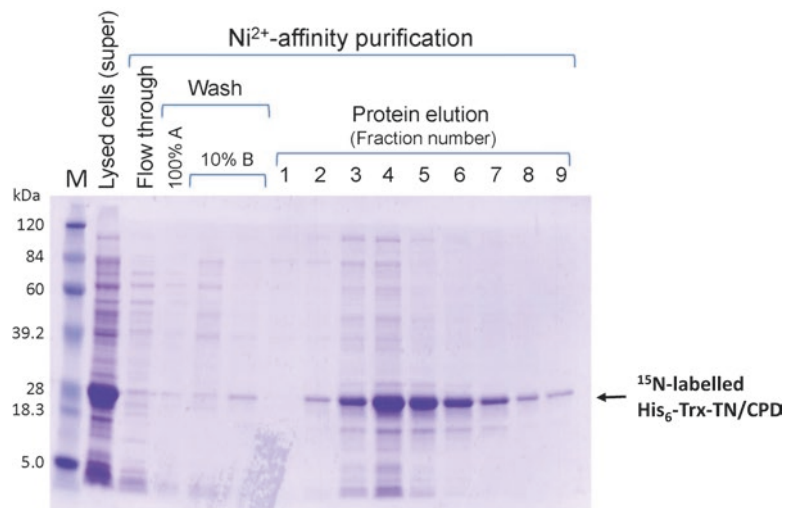


Fig. 3 Ni²⁺-affinity (NTA) purification of the His₆-tagged TRX-TM/CPD protein. A and B refer to NTA buffers A and B, respectively

5. Dissolve the dried powder for each fraction in 0.5 ml of sample buffer and analyse by SDS-PAGE.
6. Pool peak fractions containing the TM/CPD peptide, add an equivalent volume of solvent A and then load onto the same semi-preparative C8 reverse-phase HPLC column.
7. Develop the column with a gradient of 0–50% solvent B over 5 min followed by 50–70% solvent B over 40 min. Monitor the absorbance at 214 nm and collect eluting peaks.
8. Freeze the peak fractions in liquid nitrogen, lyophilize, and store dried powder at $-20\text{ }^{\circ}\text{C}$ (*see Note 27*).
9. Dissolve a small amount of dried powder for each peak fraction in sample buffer and analyse by SDS-PAGE (Fig. 4a) (*see Note 28*).
10. Determine the purity and mass of the purified TM/CPD peptide by analytical C8 reverse-phase HPLC and MALDI-TOF mass spectrometry.

3.5 NMR Sample Preparation

1. Dissolve the dried TM/CPD peptide in 550 μl of NMR buffer (*see Notes 29 and 30*).
2. Vortex briefly for about 30 s to promote the incorporation of the TM/CPD peptide into the DPC micelles.
3. Take a 50 μl sample to determine the concentration of the protein and for analysis by circular dichroism (CD) spectroscopy (Fig. 4b) (*see Note 31*).
4. Transfer the remaining 500 μl of material to a 5 mm NMR tube (*see Note 32*). Store at $4\text{ }^{\circ}\text{C}$ prior to NMR data acquisition.

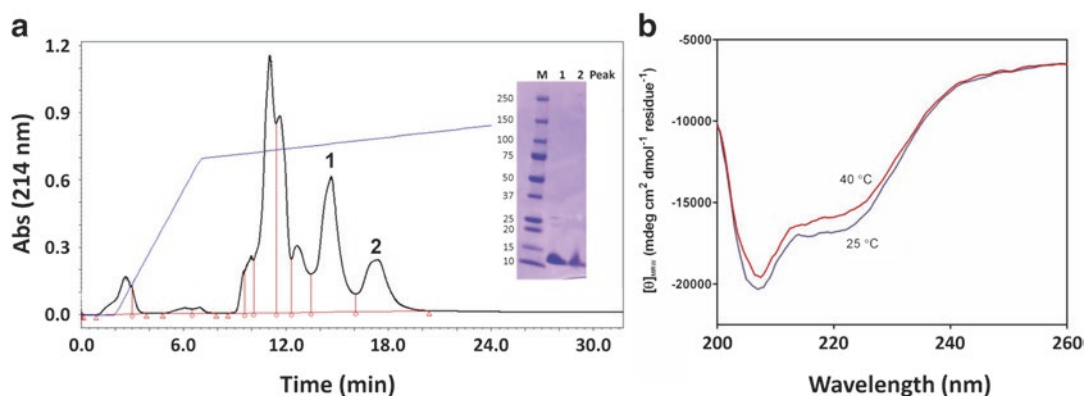


Fig. 4 The purified TM/CPD protein. **(a)** C8 reverse-phase HPLC chromatogram for the purification of the TM/CPD protein. The *blue line* indicates the percentage of solvent B in the mobile phase. *Inset*: SDS-PAGE analysis of HPLC peaks 1 and 2 showing the presence of the TM/CPD protein in both fractions. Native PAGE gel analysis shows peak 2 contains the dimeric form of the TM/CPD protein (data not shown). **(b)** CD spectrum recorded at 25 and 40 °C for the TM/CPD protein prepared in the NMR buffer which contains DPC micelles. The spectrum indicates that part of the protein adopts a helical structure (minima at 208 and 222 nm)

3.6 NMR Screening

1. Record a 1D ^1H spectrum and a 2D ^1H - ^{15}N -TROSY spectrum of the TM/CPD protein at different temperatures to determine the optimal temperature to acquire NMR spectra (*see* **Notes 33** and **34**).
2. Process the NMR data using TopSpin (Bruker), NMRView (One Moon Scientific Inc.) [51], NMRPipe/NMRDraw [52], or another suitable software package.
3. Examine the spectra (i.e., spectral dispersion, line widths, peak intensities and number of resonance peaks compared to number expected based on the amino acid sequence) to determine whether they are of sufficient quality for structure determination (Fig. 5).

4 Notes

1. The pET32a expression vector contains an ampicillin resistance gene and N-terminal His₆-tag and thioredoxin fusion tag. There are a wide range of commercially available expression vectors that contain other selectable markers for expression in the presence of an antibiotic (e.g., kanamycin, chloramphenicol), protease recognition sites (e.g., tobacco etch virus (TEV) protease, thrombin, enterokinase, and factor Xa), and one or more N- or C-terminal fusion tags. Fusion tags can facilitate detection on Western blots (e.g., FLAG, S-tag, c-myc, HSV, glutathione S-transferase (GST), His₆), solubility (e.g., thioredoxin (Trx), SUMO, maltose binding protein (MBP), NusA), and purification (e.g., His₆, GST, MBP) of the target protein.
2. Trace metals are required for maximal growth in minimal media. Each salt should be added in the order outlined in **item 9** in Subheading 2.1 after the previous salt has fully dissolved into the solution. Filter-sterilize and store in the dark at room temperature.
3. M9 salts can be stored at room temperature for up to 6 months after autoclaving.
4. The majority of T7-based expression vectors use either ampicillin or kanamycin (35 mg/ml in deionized water) for plasmid selection.
5. $^{15}\text{NH}_4\text{Cl}$ and U^{13}C D-glucose can be substituted as the nitrogen and carbon source instead of unlabeled NH_4Cl and D-glucose for the expression of uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled peptides (or proteins).
6. Uniformly labeled ^{13}C D-glucose can be added instead of D-glucose for the expression of ^{13}C -labeled peptides.
7. $^{13}\text{C}/^{15}\text{N}$ -labeled peptides (or proteins) can be produced if ^{13}C -glycerol is used as the carbon source instead of unlabeled glycerol.

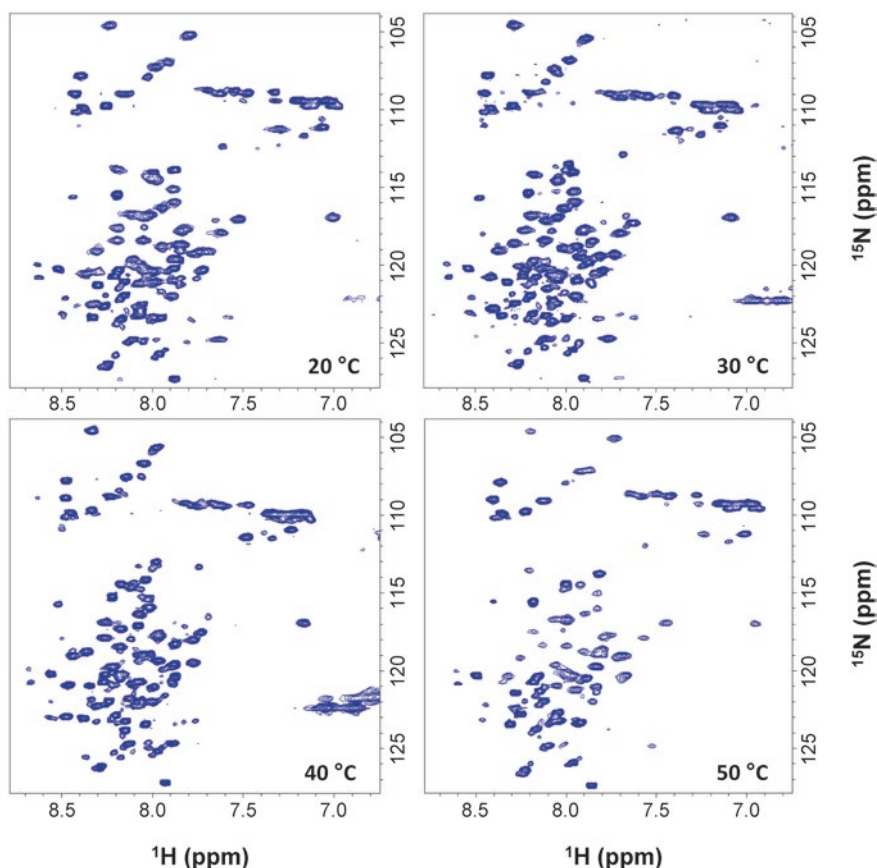


Fig. 5 NMR spectra for the TM/CPD protein in DPC micelles. 2D $^1\text{H}/^{15}\text{N}$ TROSY spectra were acquired at 20, 30, 40, and 50 °C on a Varian Inova 600 MHz NMR spectrometer equipped with a cryogenically cooled triple-resonance probe. Optimal spectral resolution and peak intensities was obtained at 40 °C

8. The cComplete EDTA-free protease inhibitor cocktail comprises tablets that contain inhibitors for serine and cysteine proteases. The cComplete EDTA-free cocktail is recommended since EDTA interferes with the IMAC (immobilized metal affinity chromatography) purification of His₆-tagged fusion proteins.
9. HRV 3C protease is a recombinant cysteine protease used to remove fusion tags from proteins containing the HRV 3C protease cleavage site (Leu-Glu-Val-Phe-Gln*Gly-Pro). This protease exhibits a high degree of specificity and has optimal activity at 4 °C.
10. CHAPS is a zwitterionic detergent that is used for the solubilization of membrane proteins over a wide range of pH (2–12). It has a high critical micelle concentration (CMC) and for this reason can be easily removed by dialysis or reverse-phase HPLC.

11. The HiTrap chelating HP column is prepacked with chelating sepharose high performance agarose. The medium is charged with nickel for Immobilized Metal Ion Affinity Chromatography (IMAC) and subsequent purification of polyhistidine tagged proteins.
12. Chemical shifts are measured relative to the reference compound 1,4-dioxane (3.69 ppm). Other reference compounds that are commonly used include: 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 0.00 ppm) and tetramethylsilane (TMS, 0.00 ppm). D₂O is included as a deuterium lock to correct for drift in the NMR spectrometers magnetic field with time. NaN₃ (sodium azide) is an antimicrobial that is included to prevent microbial growth.
13. It is often convenient to prepare a 50% glycerol stock of *E. coli* cells transformed with the expression vector containing your gene of interest and store frozen at -80 °C. To do this inoculate 5 ml of LB broth containing an appropriate antibiotic with a single colony from an LB agar plate and grow overnight at 37 °C. Take 0.5 ml of overnight culture and add 0.5 ml of sterile glycerol. Freeze on liquid nitrogen and then store at -80 °C. Fresh cultures can be prepared by scraping a small amount of material from the surface of the frozen glycerol stock and using this to inoculate the LB broth.
14. *E. coli* BL21(DE3)pLysS contains the pLysS plasmid which expresses T7 lysozyme that binds to the T7 promoter and represses expression of the target protein (or peptide) in the absence of IPTG. Inhibition of basal expression of proteins (or peptides) prior to induction with IPTG is especially important if the expressed protein (or peptide) is toxic to the cells.
15. This step allows the cells to adapt to M9 minimal medium before inoculating into a large volume of minimal media.
16. This provides a starter culture for inoculating a much larger volume of M9 minimal medium the following day. The volume of this initial starter culture should be approximately 5% of the final larger volume.
17. Good aeration is particularly important during induction. The use of baffled flasks and culture volumes no greater than 25% of the flask volume will help to improve yields.
18. Prior to isotopic labeling, optimal protein expression conditions (i.e., temperature and concentration of IPTG) should be determined using small-scale cultures. Parameters such as temperature, IPTG concentration, OD₆₀₀ at induction, expression strain, and induction time can critically influence the yield and solubility of the expressed protein or peptide.
19. The autoinduction medium contains glucose, glycerol, and lactose as carbon sources. Glucose is the initial primary carbon and energy source promoting culture growth to high cell den-

sities while inhibiting protein expression. Glucose inhibits recombinant protein expression by preventing induction of operons responsible for metabolizing lactose to allolactose, the actual inducer. Once the glucose has been consumed lactose is imported into the host cells and induces the expression of the recombinant protein. This allows the cell culture to reach very high OD₆₀₀ values of ~20 prior to the expression of the target protein. Glycerol is provided in the autoinducing media as a good carbon and energy source that does not prevent glucose depletion during growth, glucose inhibition of protein expression, or the uptake of lactose upon glucose depletion.

20. For autoinduction, do not use an *E. coli* strain harboring a pLysS plasmid if your expression vector contains a T7 lac promoter. The presence of the lac repressor in combination with T7 lysozyme expressed by the pLysS plasmid (which binds to and inactivates the T7 promoter) leads to significantly lower levels of protein expression in the autoinduction medium.
21. This starter culture is used to inoculate 1 L of medium the next day. Increase the size of the starter culture proportionally (i.e., 50 ml of starter culture per 1 L of minimal medium) if you plan to inoculate a larger batch of medium.
22. Grow the cells to saturation when using autoinduction medium. The cultures will grow to a high OD₆₀₀ before induction starts. It may take at least 24 h or more for the cultures to reach saturation when grown at lower temperatures.
23. Sonication should be performed on ice with five rounds of short bursts of 1.0 min at 2.0 min intervals. It is important to avoid frothing or overheating the sample as these may cause protein denaturation.
24. Load the lysate onto the column at a flow-rate of about 2–3 ml/min. The clear lysate can be kept at room temperature at this stage as it contains protease inhibitors and becomes cloudy at lower temperatures due to the presence of the CHAPS detergent.
25. The same flow-rate should be used as for loading the lysate onto the column. Monitor the absorbance at 280 nm and keep washing the column until OD₂₈₀ no longer decreases. It should be noted that imidazole exhibits a significant absorbance at 280 nm.
26. Perform a small-scale pilot trial in order to determine ratio of protease to purified protein to use and the time of incubation. We prefer to use 3C protease since it is a highly specific protease and has an optimal activity to 4 °C. However, expression vectors containing a variety of protease recognition sites (e.g., TEV, thrombin, enterokinase, and Factor Xa) are also available

but it is important that you check to ensure that the protein that you wish to express does not also contain an identical protease cleavage site.

27. Most proteins and peptides are very stable when store for long periods as a lyophilized powder.
28. Place the sealed container containing the lyophilize powder on the bench for 10–20 min to equilibrate to room temperature before opening to avoid the powder absorbing moisture from the atmosphere.
29. The final concentration of the TM/CPD peptide should be at least 1 mM. Concentrations lower than 1 mM are acceptable but will result in weaker NMR signals and an increase in the amount of time required to acquire NMR spectra. If possible concentrate the protein to about 300 μ l using a centrifugal concentrator such as an Amicon Ultra-0.5 Centrifugal Unit (Merck Millipore) with an appropriate molecular weight cut-off. The high concentration of DPC included in the NMR buffer ensures that only one molecule of peptide will be incorporated into each DPC micelle.
30. The buffer and pH used for the NMR buffer will depend on the pI of the TM/CDP peptide, as proteins tend to aggregate close to their pI value where they have neutral charge. If sufficient protein is available screen a range of buffer conditions.
31. Determine the concentration of the TM/CPD protein by measuring the absorbance at 280 nm and using the equation $A = \epsilon bc$ (where A is the absorbance, ϵ is the molar extinction coefficient, b is the path length, and c is the concentration).
32. If the sample volume is less than 500 μ l transfer to a 5 mm Shigemi NMR tube. These NMR tubes can take a minimum volume of 300 μ l.
33. We usually record spectra at 10, 20, 30, 40, and 50 $^{\circ}$ C (depending on the stability of the protein). Single helical transmembrane proteins often exhibit higher quality spectra (improved spectral resolution and peak intensity) at higher temperatures. However, transient structure within the extra-membrane regions (e.g., the cytoplasmic domain) may only be observed at lower temperatures.
34. 2D-TROSY spectra exhibit improved spectral resolution compared to 2D-HSQC (heteronuclear single quantum coherence) spectra due to the large size of the TM/CPD protein-DPC micelle complex (typically \geq 30 kDa). Typical 2D datasets are usually recorded with 1024×256 complex points with 8 to 32 scans per increment (depending on the sample concentration) on a 600 MHz NMR spectrometer. Data acquisition times are typically 30–60 min.

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