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Enhanced expression and purification of membrane proteins by SUMO fusion in *Escherichia coli*

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Abstract

Severe acute respiratory syndrome coronavirus (SARS-CoV) membrane protein and 5-lipoxygenase-activating protein (FLAP) are among a large number of membrane proteins that are poorly expressed when traditional expression systems and methods are employed. Therefore to efficiently express difficult membrane proteins, molecular biologists will have to develop novel or innovative expression systems. To this end, we have expressed the SARS-CoV M and FLAP proteins in *Escherichia coli* by utilizing a novel gene fusion expression system that takes advantage of the natural chaperoning properties of the SUMO (small ubiquitin-related modifier) tag. These chaperoning properties facilitate proper protein folding, which enhances the solubility and biological activity of the purified protein. In addition to these advantages, we found that SUMO Protease 1, can cleave the SUMO fusion high specificity to generate native protein. Herein, we demonstrate that the expression of FLAP and SARS-CoV membrane proteins are greatly enhanced by SUMO fusions in *E. coli*.

Abbreviations: FLAP – 5-lipoxygenase-activating protein; FPLC – Fast Performance Liquid Chromatography; IPTG – isopropyl-β-D-*thiogalactopyranoside;* M protein of SARS-CoV – membrane protein of SARS coronavirus; Ni-NTA – nickel-nitrilotriacetic acid; PMSF – phenylmethylsulfonyl fluoride; SARS – Severe Acute Respiratory Syndrome; SARS-CoV – SARS coronavirus.

Introduction

To date, the Protein Data Bank (PDB, http://www.rcsb.org/pdb) has accumulated nearly 20,000 independent protein structures, of which only 101 are membrane proteins. Since approximately one-third of the total proteins are membrane proteins, one can conclude that the structures of membrane proteins are particularly difficult to solve [1]. Specifically, the major "bottleneck" for structural and functional studies of membrane proteins is efficient expression and purification. Therefore, to express and purify the remaining membrane proteins, molecular biologists will have to think outside the box and develop novel expression systems. Herein, we apply a novel expression system to two membrane proteins, SARS-CoV membrane (M) protein and FLAP that are poorly expressed when traditional expression systems and methods are employed.

Severe Acute Respiratory Syndrome (SARS) is a respiratory illness caused by SARS coronavirus (SARS-CoV), which only recently has been reported in Asia, North America, and Europe [2]. The SARS-CoV M protein is a 25-kDa glycoprotein, which is the most abundant structural protein with three membrane spanning domains naturally located on the exterior surface of the virus [2]. It is known that the membrane proteins of coronavirus play a key role in virus assembly [3]. When expressed in combination with viral envelope (E) proteins, the M protein is retained in the budding compartment and is incorporated into virus-like particles (VLPs). Mutations in the C-terminal residue of M proteins have devastating effects on the formation of VLPs, suggesting that the C-terminus of the M protein interacts with the E protein to form VLPs [3]. However, because the SARS-CoV M protein is very difficult to express in traditional systems, the exact structures and functions of the protein are unknown. Expression of intact and correctly folded M protein is essential for the development of SARS vaccines since the VLPs are believed to be strong antigens of the virus [2, 3].

5-Lipoxygenase-activating protein (FLAP) is an 18-kDa integral membrane protein that is essential for cellular leukotriene (LT) synthesis, and it is the therapeutic target of LT biosynthesis inhibitors [4]. It is known that FLAP activates 5-lipoxygenase (5-LO) by transferring arachidonic acid to 5-LO, but the mechanism by which FLAP activates 5-LO and the structure of the protein has not been determined. Effective expression and purification of FLAP is a prerequisite for further functional and structural studies.

A novel expression system based on the SUMO (small ubiquitin-related modifier) tag and SUMO protease has been described previously [5]. SUMO has been shown to enhance expression and solubility. In addition, the protease that cleaves the fusion is highly specific and generates native protein with any desired N-terminal residue except for proline. Herein, we demonstrate that SARS-CoV M protein and FLAP are efficiently expressed when directly fused with SUMO, which can then be selectively cleaved to generate high quantities of native protein with a desired N-terminus. Lastly, we compare the level of expression of the SUMO fused membrane proteins with unfused membrane proteins in *Escherichia coli*.

Materials and methods

Construction of 6×His and 6×His-SUMO fusion expression vectors

SARS-CoV membrane protein (M) was separately expressed in *E. coli* as a polyhistidine-SUMO fusion and as a polyhistidine fusion. The SARS-CoV cDNA was derived from mRNA provided by CDC Atlanta. All the expression constructs utilized the pET24d plasmid (Novagen, Madison, WI) as the backbone. The pET24 derivative carrying the SUMO (*Smt3)* gene of *S. cerevisiae*, has been described previously [5]. An N-terminal hexahistidine (6×His) tag was introduced by PCR into the SUMO coding sequence, as well as a unique *Bsa*I site at the C-terminus. By utilizing the Class IIS properties of the *Bsa*I site, the SARS protein coding sequence was inserted in frame with SUMO. PCR primers incorporating this site were used to amplify the SARS-CoV coding sequences from cDNA clones carried in the pTOPO vectors described above. The 3′ primers carried a *Bam*HI site for insertion into the multiple cloning site of pET24d.

The primer pairs used to PCR amplify the SARS-CoV M protein are: Forward: tttGGTCTCaaggtatggcagacaacggtactattacc; Reverse: cgcGGATCCtcactgtactagcaaagcaatattg. Restriction enzyme recognition sites used for cloning are indicated in upper case letters. In addition, a set of parallel vectors that does not carry the SUMO sequence but has an N-terminal 6×His tag and an identical multiple cloning site was created, so that the same PCR fragment can be simultaneously cloned as 6×His-SUMO and 6×His fused versions. The FLAP gene was cloned by PCR from the baculo virus vector described by Mancini *et al.* [4]. The N-terminal FLAP was fused to the C-terminus of SUMO as described for the SARS M protein. The final vector expresses pET24d-6×His-SUMO-FLAP. A control vector that expressed un-fused FLAP (pET24d) was also constructed to examine the protein expression enhancing properties of SUMO. All plasmids were routinely sequenced and transmitted to the *E. coli* bacterial strain BL21 (DE3) for protein

expression. For PCR amplification of the genes of interest, a proofreading polymerase was used (Platinum Taq, Invitrogen, Carlsbad, CA).

Expression of SUMO-fusion proteins in E. coli

FLAP and SARS-CoV M proteins, either fused to 6×His-SUMO or to 6×His, were expressed in *E. coli*. In a typical experiment, a single colony of the *E. coli* BL21 (DE3) strain containing each of the plasmids described above was inoculated into 50 ml of Luria-Bertani (LB) medium. The antibiotic kanamycin was added at 10 μg/ml to the medium. The cells were grown at 37 °C overnight with rotation at 250 rpm. The next morning 25 ml of the overnight culture was transferred into 500 ml of fresh LB medium to permit exponential growth. When the optical density at 600 nm (OD_{600}) reached ~0.6–0.7, protein expression was induced by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and the cells were allowed to grow at 20 °C overnight.

SDS-PAGE was used to check protein expression. Typically, cells from a 1.5 ml of the culture at the times just before expression was induced and after completion of induction were collected by centrifugation at 6000 rpm for 5 min. The cell pellets were suspended in 50 μl of distilled water and the sample was freeze–thawed once to facilitate cell disruption. After the cell lysates were incubated with RNAse and DNAse (each at 40 μg/ml) for 15 min, they were mixed with SDS-PAGE buffer containing 0.1% SDS and 5 mM β-mercaptoethanol and heated at 95 °C for 5 min to denature and reduce proteins, which were separated on the gels with Tris–Glycine running buffer.

Western blot

Proteins separated by a SDS-PAGE were transferred onto nitrocellulose membranes at 42 V (~150 mA) for ~2.5 h. Membranes were then incubated with 30 ml of TTBS buffer, containing 5% milk (pH 8.0) for 1 h at room temperature. The expressed proteins were probed with either monoclonal anti-His or polyclonal anti-M antibodies (obtained from rabbits immunized against SUMO–SARS-CoV M protein fusions, both made by LifeSensors Inc, USA), by incubating overnight at 4 °C with 1:1000 dilution of the primary antibodies. After the membranes were washed with TTBS buffer for 5 min, they were incubated with secondary antibodies (Peroxidase-conjugated goat anti-rabbit IgG, Rockland Immunochemicals, as diluted to 1000×) for 45 min. The membranes were finally washed with TTBS for 10 min before the Western blot substrates were applied (Roche, Mannheim, Germany) and visualized by autoradiography.

Purification of SUMO-fused proteins

Sample preparation

After the cells were harvested from culture medium by centrifugation (5000 *g* for 10 min at 4 °C), cell pellets were suspended (at ratio of 1 mg to 2 ml) in lysis buffer (50 mM phosphate buffer, pH 8.0, containing 0.3 M NaCl, 10 mM imidazole, 1%Triton X100 and 1 mM PMSF). Cells were broken by sonication (at 50% output for 5 × 30 s pulses with 1 min intervals between each cycle) at 4 °C with tube jacketed in wet ice. After the cell lysate was incubated with DNase and RNase (each at 0.1 mg/ml) for 15 min, the supernatant (soluble proteins) was obtained by centrifugation (10,000 *g* for 20 min at 4 °C). The pellets containing inclusion bodies were washed three times in buffer (PBS containing 25% sucrose, 5 mM EDTA and 1% Trition X100, pH 7.5), with centrifugation as described above. The washed inclusion bodies were then resuspended in denaturing lysis buffer (Novagen) that contained 50 mM CAPS (pH 11.0), 0.3% *N*-lauroyl sarcosine, and 1 mM DTT to extract insoluble proteins by incubation for 30 min at room temperature with shaking. The supernatant (extracted from inclusion bodies) was obtained by centrifugation (20,000 rpm for 20 min, 4 °C). Protein concentrations were determined using the Bradford color-reaction assay (Bio-Rad) and absorbance at 595 nm with bovine serum albumin as standards, according to the manufacturer's instruction.

Purification of His tagged SUMO-fusion

In this study, the SUMO-fusion proteins extracted from *E. coli* inclusion bodies were purified using Nickel affinity chromatography under denaturing conditions. BioLogic Duo-Flow FPLC system

(Bio-Rad) was used for high-throughput fractionations. Typically, ~30 ml of the extract (from inclusion bodies in 2 l cultured medium) was incubated with ~10 ml of Ni-NTA superflow resin (Qiagen, Valencia, CA) at 4 °C for 1 h with shaking for effective binding of the 6×His tagged proteins to the resin. The mixture was then loaded into an empty column and the flow-through (F/T) sample was collected. The F/T sample was re-applied to the column to minimize losses of the target proteins. Subsequently, the resin was continually washed by the Washing Buffer (20 mM imidazole, 0.3% *N*-lauroyl sarcosine, 50 mM CAPS buffer, and 0.3 M NaCl, pH 11) until UV_{280} reached or fell below the base line (UV value = 0). The 6×His tagged SUMO-fusion proteins were eluted using the elution buffer that contains the same components as in the wash buffer, except that the concentration of imidazole was increased to 300 mM. The proteins with high UV_{280} values were collected in 4 ml fractions. The proteins in fractions with high UV values were checked on SDS gels and pooled.

Cleavage of SUMO fusions

The purified SUMO-fusion proteins were refolded by extensive dialysis for 2 days at 4 °C against a buffer (20 mM Tris–HCl, pH 8.0, 10% glycerol), based on the published method [6]. During the dialysis, the buffer (~1 l) was changed more than 4 times to effectively remove the detergent and imidazole. SUMO Protease 1 was previously produced in our laboratory and stored at −80 °C in storage buffer (5% glycerol, 75 mM Tris pH 8.0, 0.5 mM DTT and 1 mM EDTA) [5]. A unit of protease activity is defined as the amount of the SUMO protease that cleaves 100 μg of SUMO–Met–GFP fusion substrate at 25 °C in 1 h in the buffer containing 20 mM Tris–HCl, pH 8.0 and 5 mM β-mercaptoethanol [5]. In this study, the dialyzed SUMO-membrane proteins were added with the SUMO Protease 1 at a ratio of 1 unit of the enzyme to 100 μg of the substrate, and incubated in the buffer as described above at 30 °C for 1 h, since the SUMO membrane protein fusion is difficult to cleave. It was necessary to remove the *N*-lauroyl sarcosine from the purified SUMO–M proteins before adding the SUMO protease for the cleavage of the fusion since the detergent inhibited the enzyme activity and no detergents was needed for maintaining the SUMO–M proteins in soluble form; the detergent was added back to the sample only when the cleavage was completed.

Subtraction of SUMO and SUMO protease for final purification of target proteins

Since both SUMO and SUMO Protease 1 had 6×His tags, and the cleaved membrane proteins from SUMO fusions did not, the cleaved SUMO fusion samples could be re-applied to the Nickel column to obtain the purified membrane proteins by subtracting the unwanted 6×His-tagged proteins. After SUMO Protease 1 cleaved the SUMO fusion, the sample was loaded onto a Nickel column with Ni-NTA resin. Most of the membrane proteins without 6×His tags were eluted out in the flow-through (unbounded) fractions and the rest were recovered by washing the resin using PBS. The eluted and washed proteins appearing in the fractions with high UV_{280} values were pooled as the final purified sample.

Results

Enhanced expression of the membrane proteins by SUMO-fusion

When fused with 6×His-tags (without SUMO), FLAP was so poorly expressed in *E. coli* that it could not be detected on a Coomassie blue stained SDS-gel (Figure 1, left panel) and only a faint band (~18 kDa) was observed on a Western blot probed with an anti-His antibody (Figure 1). In contrast, when FLAP was fused with SUMO, the expression of the protein was dramatically increased. Although two intense bands with molecular weights of ~25 and ~36–38 kDa were detected in the induced SUMO–M fusion samples when probed by the anti-His antibody (Figure 1, right panel), only one band (~36–38 kDa) was observed on the SDS-gel (Figure 1, left panel). Since the molecular weights of FLAP and SUMO are 18 and 11.5 kDa, respectively (SUMO runs as a ~18–20 kDa band with SDS-PAGE), the expressed protein (~36–38 kDa) detected here was consistent with intact SUMO–FLAP fusion, while the less intense band (~25 kDa) could be a degradation product of the

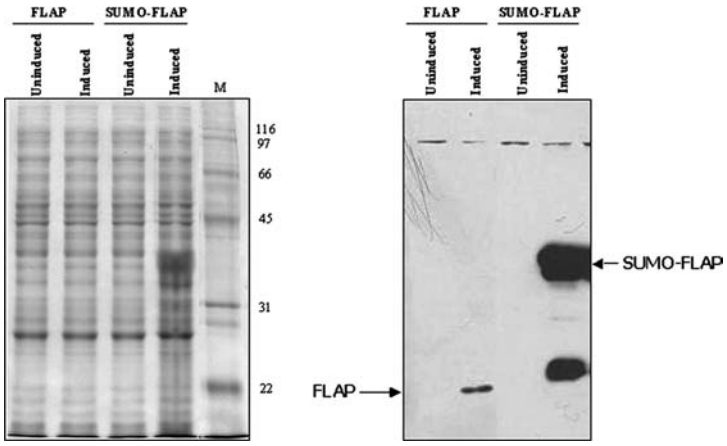

Figure 1. Enhanced expression of FLAP by SUMO fusion in *E. coli*. Left panel: 10% SDS-gel; right panel: Western blot. Samples were the whole cell lysate extracted from the *E. coli* expressed the recombinant FLAP proteins fused with either His tag (indicated as FLAP) or His-tag-SUMO (as SUMO–FLAP). Proportional volumes of the samples (~12 μl for the SDS gel and ~2 μl for the Western blot) were loaded and electrophoresed on the SDS gels, which were either stained with Coomassie blue or transferred to a membranes for the Western blot probed with the anti-His monoclonal antibody. M: molecular weight markers. Arrows highlight observed positions of expressed proteins.

fusion. In addition, a very faint band appeared close to the top of the Western blot image in all the samples (Figure 1, right panel), indicating that the anti-His antibody had non-specific reactions with the bacteria proteins.

Figure 2 shows the Western blot of the reactions of the unfused 6×His-M protein and the two clones expressing His-SUMO–M protein with the anti-His antibodies. Only soluble fractions (supernatant of the cell lysates) were analyzed in this experiment. When expressed with only a His-tag (without SUMO), the M protein was poorly expressed, because only a faint band (~25 kDa) was observed. In contrast, a few highly intense bands of the SUMO–M fusion samples were detected (Figure 2), of which the major band (~43 kDa) was consistent with the expected size of the SUMO–M fusion, as the combination of SUMO (~18 kDa) and FLAP (~25 kDa). Although higher level aggregated forms associated with immunological reactions in the SUMO–M protein samples were observed (Figure 2) as compared to those in Figure 3 (see below), it is apparent that SUMO fusion dramatically enhanced the expression of the membrane protein (Figure 2).

Purification of SARS-CoV M protein

As described above, a typical procedure for purification of the SARS-CoV M protein from the inclusion body of the *E. coli* involves three steps: purification of the SUMO fusion, cleavage of the fusion, and subtraction for final purified M proteins.

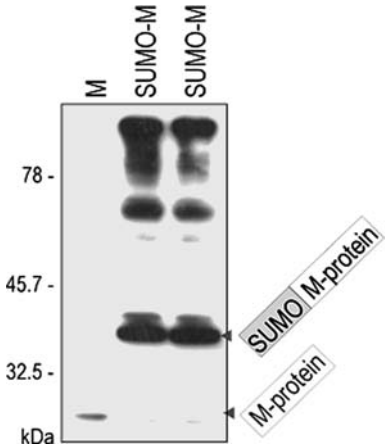

Figure 2. Enhanced expression of SARS-CoV M protein by SUMO fusion in *E. coli*. The Western blot was from a 10% SDS gel separating the SARS-CoV M proteins expressed with His-tag fusion (M) and with two clones of His tag-SUMO fusions (SUMO–M), respectively. Samples contained the supernatant extracted from the whole cell lysates prepared without adding any detergent except 0.1% SDS in the final SDS-PAGE sample buffer before loading the gels. Proportional volumes (~4 μl) of the samples were loaded on the gel, which were transferred to the membrane and then probed by the anti-His antibody. Positions of molecular weights are indicated and arrows highlight observed positions of expressed protein bands. Membrane protein aggregation is often observed in SDS-PAGE conditions.

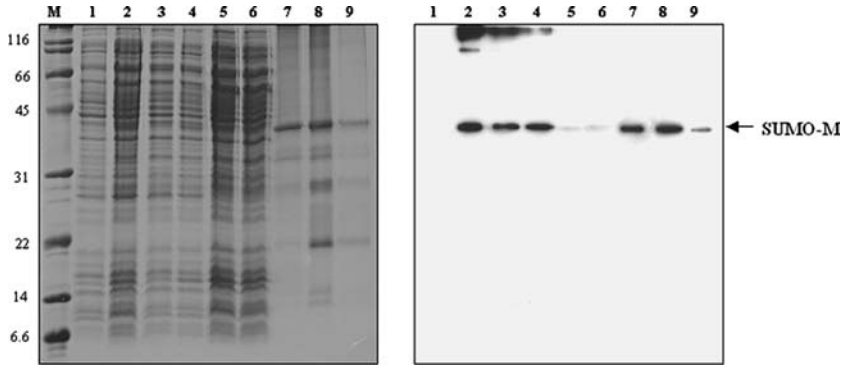

Figure 3. Detection of expressed and purified SARS-CoV M proteins. Left panel: 15% SDS-gel; right panel: Western blot probed by anti His-tag antibody. Proteins expressed with 6×His-SUMO fusion in *E. coli* were extracted with the solubilization buffer and the SUMO–M protein with His tags from inclusion bodies were purified using the Nickel affinity chromatography under denaturing conditions. Proportional volumes of the samples (~12 μl for the SDS-gel and 2 μl for the Western blot) were loaded and detected by Coomassie blue staining (left panel) and the Western blot (right panel). Lanes: 1 – Uninduced whole cell lysate (control); 2 – Induced whole cell lysate; 3 – Supernatant; 4 – Extract from inclusion bodies; 5 – Pooled flow-through fractions (unbounded) from the Nickel column; 6 – Pooled wash fractions; 7, 8 and 9 – Fractions 1, 2 and 3, respectively, in the UV peak containing the three fractions with top values in fraction 2, in which the target proteins were eluted by the Elution buffer containing 300 mM imidazole. Arrow highlights the SUMO–M protein (~43 kDa).

Figure 3 shows the detection of the proteins in crude and purified samples at different steps of purification of the SUMO–M protein fusion. Briefly, the expressed protein (~43 kDa) was detected from the induced *E. coli* cells, and the protein was confirmed to be the SUMO–M fusion by the reaction with anti-His antibodies (Figure 3, Lane 2). Approximately, 40% of the total expressed proteins were found in the supernatant of the cell lysate (soluble fraction) and ~60% were recovered from inclusion bodies (Figure 3, Lanes 3 and 4). Even though detergents were used in sample preparation to solubilize proteins, some aggregated forms of the SUMO–M proteins in the crude samples were found as bands accumulated at the top of gel when detected by the Western blot (Figure 3, right panel lanes 2–4). Most of the proteins without 6×His tags, along with minor amounts of the target proteins, were eluted in the flow-through (unbounded fractions) and washed out by using the washing buffer containing 20 mM imidazole (Figure 3, Lanes 5–6). Finally, the His-tagged SUMO–M proteins were efficiently eluted with elution buffer containing 300 mM imidazole (Figure 3, Lanes 7–9). In this experiment, the eluted fusion proteins were in an isolated peak with high UV_{280} values containing three 4-ml fractions. Although some unwanted impurities were present in the eluted samples, the most intense band (~43 kDa) detected on the SDS-gel was the SUMO–M protein, which was recognized by the anti-His antibody (Figure 3, Lanes 7–9).

The dialyzed SUMO–M protein fusion sample was subjected to cleavage by SUMO Protease 1. Under the conditions described in the Materials and methods, the SUMO fusion was not completely cleaved by SUMO Protease 1, indicating that the fusion will require modified reaction conditions for optimal cleavage. Nevertheless, we could cleave at least 50% of the total amount of the SUMO fusion under the experimental conditions. The degraded components of the cleaved SUMO fusion were clearly shown on the SDS-gel, as the intensity of SUMO–M protein band (~43 kDa) was reduced, and two new bands corresponding to the expected molecular weights of SUMO (~18–20 kDa) and the M protein (~25 kDa) appeared.

Lastly, the SARS-CoV M protein was purified by subtracting the components bearing His tags, such as uncleaved SUMO–M fusion, SUMO, and SUMO Protease 1. The M protein (~25 kDa), along with some less-abundant proteins (impurities), was detected in the final purified sample on the SDS-gel (Figure 4, left panel), indicating at least 50% purity of the M protein obtained. Identity of the M protein was confirmed by its reaction with the anti-M antibody (Figure 4, right panel). The uncleaved SUMO–M

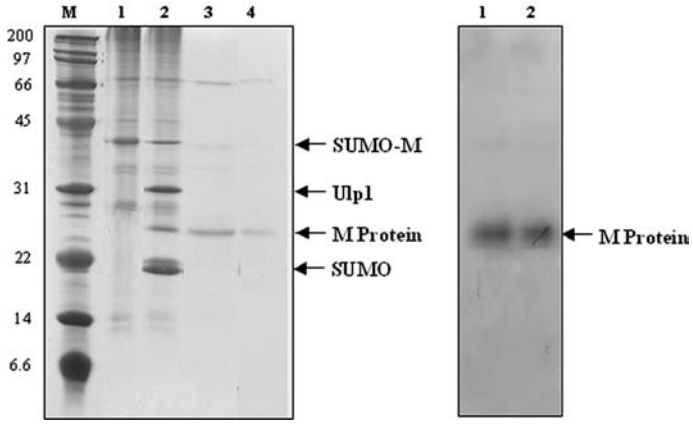

Figure 4. Cleavage of SUMO–M protein fusion by SUMO Protease 1 and purification of SARS-CoV M protein. Left panel: 15% SDS-gel to detect the cleaved SUMO–M fusion and the purified M proteins. Lanes: 1– Purified and dialyzed SUMO–M fusion sample; 2 – SUMO fusion cleaved by SUMO Protease 1; 3 and 4 – 4 μg and 2 μg of the purified M protein sample, respectively. Right panel: Western blot, in which lanes 1 and 2 were the same samples as lane 3 and 4 of the SDS-gels, to confirm the identity of M proteins by probing with an anti-M antibody.

fusion band was not observed in the Western blot, possibly because that the amount of the M protein present in the fusion was not enough for immunological reaction with the anti-M antibody. In this study, we obtained ~15 ml of the eluted M protein sample in which the protein concentration was 0.33 mg/ml, presenting a final yield of ~5.0 mg of the proteins that were purified from the 500 ml *E. coli* culture.

Discussion

Membrane proteins, naturally embedded in the lipid bilayers on cell surfaces, play crucial roles in many cellular and physiological processes. Nevertheless, before such processes are studied, large quantities of pure protein are required for structural studies using crystallography or NMR. To date, most membrane protein structures remain unsolved, due in part to the inefficiencies of existing protein expression systems. For instance, the production of membrane proteins in mammalian cells usually results in non-expression or very low yields of correctly folded proteins [7].

Furthermore, FLAP can be expressed in insect cells with recombinant baculovirus, but the yield is too low for purification [4]. Nevertheless, Grisshammer and co-workers have expressed and purified several important membrane proteins of humans in prokaryotes using a maltose binding protein fusion. This indicates that *E. coli* is a suitable vector for expression of membrane proteins and fusion tags improve the expression [8–12].

Expressing recombinant membrane proteins is quite an arduous task because they are strongly hydrophobic when expressed in host cells, fold incorrectly and aggregate, leading to either rapid degradation or the accumulation of inclusion bodies, respectively. Fortunately, these problems are somewhat alleviated when membrane proteins are expressed with a fusion partner. However, in the past, traditional fusion systems have given variable results of expression and have faced major problems attributed to either the inefficient cleavage of the fusion protein or cleavage within the target protein, both of which compound the difficulties of purification. Therefore, membrane proteins would benefit not only from fusion partners that enhance expression and solubility but also from one that is efficiently and specifically cleaved.

Ubiquitin has been reported to exert chaperoning effects on fused proteins, thus increasing expression of proteins in *E. coli* and yeast [13–15]. SUMO is a ubiquitin-like protein containing approximately 100 amino acids, which is highly conserved in eukaryotes and absent from prokaryotes [16]. We hypothesize that the attachment of a highly stable and compact SUMO structure to the N-terminus of the membrane proteins will facilitate correct protein folding and enhance

solubility and expression. A rationale for the role of SUMO in promoting solubility of insoluble proteins is that the inner SUMO core is a dense hydrophobic globular structure, and the protein surface is hydrophilic and highly water-soluble (similar to amphipathic detergents) [13, 15]. Our laboratory has exploited the chaperoning properties of several ubiquitin-like proteins including SUMO and SUMO proteases in cleaving SUMO fusions to develop a technology that will provide both enhanced expression and robust cleavage of the fusion protein. Specifically, a number of proteins have been expressed with SUMO in *E. coli*, demonstrating that SUMO-fusion dramatically enhances the expression of the proteins and that SUMO Protease 1 cleaves a variety of SUMO fusions with high specificity [5].

In this study, we expressed FLAP and SARS-CoV membrane protein as SUMO fusions in *E. coli* to evaluate the roles of SUMO and SUMO Protease 1 on the production of the membrane proteins. As described above, eukaryotic cells do not produce sufficient quantities of heterogonous membrane proteins; therefore, we have chosen to work in *E. coli*. Moreover, the expression of recombinant proteins in *E. coli* is well established [9, 11, 12]. In addition, *E. coli* is easier to grow *in vitro*, less expensive, and produces recombinant proteins more rapidly as compared to alternative systems. In this study, SUMO dramatically enhanced the expression of the FLAP and SARS-CoV M proteins in *E. coli* (Figures 1–3).

When SUMO-fusions are expressed as 6×His-SUMO, rapid purification is possible by using Nickel affinity chromatography. This method is particularly useful for small molecular weight proteins or peptides since they are easily lost during purification using non-affinity chromatographic methods [1]. In this study, the SUMO–M fusion that was recovered from inclusion bodies (insoluble protein aggregates) was efficiently purified under denaturing conditions (Figures 3 and 4).

Another advantage of the SUMO fusion technology is the utility of SUMO Protease 1, which is remarkably robust and highly specific. Previous work has shown that the enzyme efficiently and selectively cleaves many SUMO fusions over a wide range of conditions, including a broad range of pH (5.5–10.5) and temperature (4–37 °C). Except for proline, any N-terminal amino acid can be efficiently cleaved by SUMO Protease 1 [5]. Furthermore, SUMO Protease 1 is highly robust, since it can cleave in the presence of 300 mM imidazole, 2 M urea and 100 mM Gu-HCL [5]. In this study, we found that the SUMO–SARS-CoV M protein can be specifically cleaved by SUMO Protease 1 (see Figure 4). Incomplete cleavage of SUMO fusions could occur when the proteins are resistant to the enzyme, or the reaction conditions are not favorable. This problem is especially pronounced with membrane proteins, whose cleavage in the presence of detergents may be desirable to allow proper folding of the protein. On the other hand, detergents may decrease the hydrolytic activities of the enzyme. Nevertheless, we obtained >50% of the total amount of fusion proteins cleaved under the conditions typically used for non-membrane proteins (Figure 4).

In summary, the data provided here demonstrates that fusion with SUMO is able to enhance expression of membrane proteins in *E. coli*. The SUMO fusion technology could be widely applied to the production of a variety of structurally diverse membrane proteins in *E. coli*. After the SUMO tag is removed by SUMO Protease 1, target proteins with high purity and quantity can be used in functional assays or structural studies, such as crystallography and NMR.

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