Protein Expression and Purification 66 (2009) 143–148

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

High-yield expression and purification of a monotopic membrane glycosyltransferase

Hanna M. Eriksson [a,b,*], Karina Persson [c], Shuguang Zhang [b], Åke Wieslander [a,*]

[a] Center for Biomembrane Research, Department of Biochemistry & Biophysics, Stockholm University, SE-10691 Stockholm, Sweden
[b] Center for Biomedical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA
[c] Department of Odontology, Umeå University, SE-901 85 Umeå, Sweden

ARTICLE INFO

Article history:
Received 16 December 2008
and in revised form 19 March 2009
Available online 28 March 2009

Keywords:
Membrane protein
Monotopic
Overexpression
Optimization
Size-exclusion chromatography
Glycosyltransferase
Escherichia coli

ABSTRACT

Membrane proteins are essential to many cellular processes. However, the systematic study of membrane protein structure has been hindered by the difficulty in obtaining large quantities of these proteins. Protein overexpression using *Escherichia coli* is commonly used to produce large quantities of protein, but usually yields very little membrane protein. Furthermore, optimization of the expressing conditions, as well as the choice of detergent and other buffer components, is thought to be crucial for increasing the yield of stable and homogeneous protein. Herein we report high-yield expression and purification of a membrane-associated monotopic protein, the glycosyltransferase monoglucosyldiacylglycerol synthase (alMGS), in *E. coli*. Systematic optimization of protein expression was achieved through controlling a few basic expression parameters, including temperature and growth media, and the purifications were monitored using a fast and efficient size-exclusion chromatography (SEC) screening method. The latter method was shown to be a powerful tool for fast screening and for finding the optimal protein-stabilizing conditions. For alMGS it was found that the concentration of detergent was just as important as the type of detergent, and a low concentration of *n*-dodecyl-β-D-maltoside (DDM) (~1× critical micelle concentration) was the best for keeping the protein stable and homogeneous. By using these simply methods to optimize the conditions for alMGS expression and purification, the final expression level increase by two orders of magnitude, reaching 170 mg of pure protein per litre culture.

Introduction

Membrane proteins are involved in a number of essential cellular processes, including signalling, transport, energy conversions, cell adhesion, vision, smell, taste and mechano- and thermal transductions. Many of them are therefore key targets for pharmaceuticals [1–3]. However, the low purified protein yields usually obtained from membrane protein overexpression are a limiting factor for functional and structural studies, where common problems include the formation of inclusion bodies and protein aggregates. This may explain why less than 1% of the structures deposited in the Protein Data Bank database [4] are membrane proteins [5], although membrane proteins are predicted to constitute up to 30% of the encoded proteins in many genomes [6,7]. That said, it is thought that overall protein yields may be improved *via* the optimization of growth- and purification conditions.

Membrane proteins may be categorized as monotopic, bitopic and polytopic, depending on the mode by which the protein interacts with the membrane. The monotopic proteins only interact with one of the monolayer leaflets of the bilayer, while bitopic and polytopic proteins have one or more segments spanning the full membrane bilayer, respectively [8]. One may anticipate that membrane interface-associated proteins (monotopic) would be easier to experimentally handle since they are not as deeply embedded into the membrane. As a result, they would not need as high a detergent concentration for solubilization and stabilization, as compared to bi- and polytopic membrane proteins. Furthermore, monotopic proteins would also be expected to be more easy to solublize (less prone to aggregate) and, thus yield higher expression levels than transmembrane proteins. Currently only a dozen monotopic protein structures are available, which could be an indication that difficulties producing and studying them exist, or they are hard to crystallize. Comparing the expression levels in *Escherichia coli* of some monotopic and glycosyltransferase membrane proteins, as well as a few soluble glycosyltransferases,

* Corresponding authors. Address: Center for Biomembrane Research, Department of Biochemistry & Biophysics, Stockholm University, SE-10691 Stockholm, Sweden. Fax: +46 8 15 36 79.
E-mail addresses: hanna.eriksson@dbb.su.se (H.M. Eriksson), ake@dbb.su.se (Å. Wieslander).

1046-5928/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2009.03.008

it was found that the yields are always lower than 15 mg/l culture, and often in the range of 5–12 mg/l culture [9–16].

Of utmost importance to improving the final protein yield is the increase in the overall expression level of the membrane protein itself. It has been shown that through screening growth conditions, such as temperature, induction time and level, media etc., higher yields can usually be obtained [17,18]. Next, finding optimal conditions for the detergent solubilization, to obtain stable and functional proteins, is not trivial [18–20]. It is commonly thought that the concentration of detergent should be significantly higher than the critical micelle concentration (CMC), e.g. ~10× CMC [21], in order to effectively liberate the membrane proteins from the membrane while retaining a functional state. The physiochemical properties of detergents vary considerably, and therefore a screening procedure is usually needed to find a suitable detergent for the solubilization. The properties of a high-quality solubilizing detergent might not be optimal for the stabilization of the protein, resulting in additional screening of detergents.

Monoglucosyldiacylglycerol synthase (alMGS[1]; 45 kDa), is a monotopic protein that is associated with the cytosolic side of the membrane by both hydrophobic and electrostatic interactions [12,22–24]. It catalyzes the first step in the glucolipid synthesis in the mycoplasma *Acholeplasma laidlawii* [25] resulting in a mono-glucose lipid, GlcDAG. The glycosyltransferase alMGS is predicted to have two Rossman-fold like domains (GT-B) [22,24] with a few predicted attachment segments to the membrane [26], and belongs to the Carbohydrate Active Enzymes (CAZy) GT-4 family (http://www.cazy.org/) [27,28]. There are only a few solved structures from this family, although it is one of the largest CAZy families (~9400 CAZy Entries, March 2009).

Previously, the expression level of alMGS was in the order of 2 mg/l [12]. The overall aim of this study was to optimize the expression and purification conditions to obtain higher yield of pure and active protein, which can be used for structural and functional studies. We report here a simple optimization procedure, which resulted in nearly two orders of magnitude increase in yield of the purified protein.

Materials and methods

Cloning and expression hosts

Isolation of the gene for the enzyme monoglycosyldiacylglycerol synthase (alMGS) from *A. laidlawii* has been described previously [12,22]. It was obtained by PCR amplification from chromosomal DNA of *A. laidlawii* strain A-EF22 and the gene was ligated into the pET-15b vector (Novagen Inc.) containing an N-terminal His_6-tag, followed by a thrombin cleavage site before the alMGS gene. The calculated molecular mass of the expressed protein is 48,000 Da. *E. coli* strains BL21 (DE3) pLysS (Invitrogen), BL21-AI™ Invitrogen), Overexpress™ C43 (DE3) (Lucigen) and Overexpress™ C41 (DE3) (Lucigen) were screened as expression hosts for the alMGS containing plasmid. All cultures contained 100 μg/ml carbenicillin, and were inoculated with 1% (v/v) overnight culture of the alMGS clone unless otherwise noted. The cells giving the highest amount of active protein per optical density (600 nm) unit were used for further optimization experiments.

Culture media optimization

The following media for liquid cultures were evaluated: 1× Terrific broth (TB), 2× TB, 1× Luria broth (LB), 2× LB, 2× YT and 1× Superbroth. TB medium (1×) contained 12 g/l bacto-tryptone (Difco), 24 g/l bacto-yeast extract (Difco), 4 ml glycerol, 2.3 g/l KH_2PO_4 and 12.5 g/l K_2HPO_4. The sterile-filtered salts were added after heat sterilization. LB medium (1×) contained 10 g/l bacto-tryptone, 5 g/l bacto-yeast, 5 g/l NaCl and 1 ml 1 M NaOH. Two times of TB and 2× LB media contained the double amounts of the components listed above. YT medium (2×) contained 16 g/l bacto-tryptone, 10 g/l bacto-yeast extract and 5 g/l NaCl. Superbroth medium (1×) contained 32 g/l bacto-tryptone, 20 g/l bacto-yeast extract, 5 g/l NaCl and 5 ml 1 M NaOH. The media optimal for alMGS expression in strain BL21-AI were screened for growth at 37 °C and the medium giving the highest optical density at 600 nm (OD_{600}), and largest wet-pellet weight, was used for further studies.

Expression time and temperature

To further optimize the expression levels of alMGS in expression strain BL21-AI, the effect of different temperatures and induction times were screened. Most experiments were performed by growing the cultures at 37 °C until the OD_{600} reached approximately 0.7, after which they were transferred to lower temperatures (16, 22 and 30 °C), and thereafter induced when reaching OD_{600} ~1 with 1 mM IPTG and 0.2% L-arabinose. Control cultures were kept at 37 or 30 °C throughout the experiment. The optimal induction time was determined by removing samples hourly for one to eight hours after induction, plus at 21 h after induction. The OD_{600} of all samples was measured and for the 21 h sample the wet-pellet weight was determined. The samples were further analyzed by SDS–PAGE and activity assay, and the conditions yielding largest amount of active protein were used for further studies.

Solubilization optimization

Optimum detergent conditions for solubilization of the alMGS protein from the membrane were determined for a set of detergents at ~10× CMC concentration, except for CHAPS, which was both analyzed at ~2× CMC and ~10× CMC. Detergents investigated were: 15 mM CHAPS, 80 mM CHAPS, 24 mM CYMAL®-5, 10 mM LDAO, 1.7 mM DDM, 15 mM FC-12, 1.2 mM FC-14 and 180 mM OG. Control experiment without detergent was also performed. The samples were analyzed with SDS–PAGE and activity assay, and the optimal detergent for solubilization of alMGS was used for further studies.

Overexpression and isolation

His_6-alMGS was overexpressed in *E. coli* BL21-AI™ cells. A 250 ml culture in 1× TB was grown in a 2 l baffled flask, to ensure sufficient aeration, at 37 °C and 250 rpm until OD_{600} ~0.7. The culture was then transferred to 22 °C, and at OD_{600} ~1 protein expression was induced with 0.2% L-arabinose and 1 mM IPTG. After 21–22 h at 22 °C, the cells were collected by centrifugation at 6000*g* at 4 °C for 20 min. The cells were washed once with 100 mM HEPES, pH 8 and collected by centrifugation as above. The pellet was weighed and stored at −80 °C until use.

The frozen cell pellet was thawed and resuspended in 30 ml lysis buffer per gram of cells, containing 50 mM HEPES, pH 8, 1 mg/ml lysozyme, 0.1 mg/ml DNase and 1 Complete™ Protease Inhibitor Cocktail tablet (EDTA-free) (Roche Applied Science); per 50 ml liquid. The cell suspension was incubated for 20 min at 4 °C before

[1] *Abbreviations used:* alMGS, monoglucosyldiacylglycerol synthase from *Acholeplasma laidlawii*; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulphonate; CMC, critical micelle concentration; CYMAL®-5, 5-cyclohexyl-1-pentyl-β-D-maltoside; DAG, 1,2-dioleoyl-sn-glycerol; DDM, *n*-dodecyl-β-D-maltoside; FC-12, Fos-choline®-12; FC-14, Fos-choline®-14; GlcDAG, monoglucosyldiacylglycerol; GT, glycosyltransferase; LDAO, *n*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide; OG, *n*-octyl-β-D-glucoside; PC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; PG, 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)].

sonicated on ice. The membranes were collected by ultracentrifugation at 135,000*g* at 4 °C for 60 min, and solubilized in 100 ml solubilization buffer (50 mM HEPES, pH 8, 20 mM $MgCl_2$, 20% Glycerol, 500 mM NaCl, 15 mM CHAPS, Complete™ Protease Inhibitor cocktail (EDTA-free) and 3 mM TCEP), per gram of cells. The suspension was incubated at 4 °C for 2 h with continuous stirring. The supernatant was cleared by 30 min centrifugation at 22,000*g* at 4 °C, prior to application to the His-affinity column.

Small scale buffer optimization method

The protein was expressed and isolated as described above. Part of the isolated membrane-bound fraction of alMGS was added to 100 μl of Ni Sepharose™ 6 Fast Flow beads (GE Healthcare) and incubated for 30 min for batch purification. The beads were then washed with 30 bed volumes buffer W (50 mM HEPES, pH 8, 20 mM $MgCl_2$, 20% glycerol, 500 mM NaCl and 1 mM TCEP) containing 20 mM imidazole and the detergent of interest. The protein was eluted by addition of 3× 200 μl of buffer W containing 250 mM imidazole and the detergent of interest. The fractions containing the target protein were concentrated 5- to 10-fold, to ~1 mg/ml using an Amicon® Centricon® YM-3 centrifugal filter device (Millipore). For screening of the optimal types and concentrations of detergents, as well as salt concentration, a small size (3 ml) size-exclusion chromatography (SEC) column was used. All buffers contained 50 mM HEPES, pH 8, 10 mM $MgCl_2$, 10% glycerol, 500 mM NaCl, 1 mM TCEP, unless otherwise noted. An ÄKTApurifier™ 10 (GE Healthcare) was used for the SEC analysis; a method was programmed using UNICORN™ v5.11 software (GE Healthcare) for a 3 ml Superdex 200 5/150 GL SEC column. The program started with a pump wash, followed by a two-column volume (CV) equilibration step with the buffer of interest. Thereafter the His-purified protein was injected, using a 10 μl loop. Fractions of 50 μl were collected in a 96-multiwell plate from 0.6 ml through 3.0 ml of the elution run, resulting in 48 fractions. The flow rate was set to 0.3 ml/min. Using this program, one sample can be screened in just 40 min, including pump wash and column equilibration. Analysis by dot-blot assay and/or SDS–PAGE was performed to estimate the amount of protein in each fraction. The intensity recorded from each spot from the dot-blot could be inserted as an additional curve in a chromatogram in UNICORN™, and it was smoothed over two-column volumes.

Optimized large scale purification procedure

The protein was expressed and isolated as described above. The initial purification step was histidine-affinity chromatography. Imidazole was added to the clarified supernatant, prior to the his-affinity step, to a final concentration of 10 mM. The detergent CHAPS (at 15 mM) was exchanged for DDM by addition of 1 mM DDM to the supernatant, which was incubated at 4 °C for 60 min before loading on a HisTrap™ FF Crude column (GE Healthcare). The ÄKTApurifier™ 10 (GE Healthcare) was used for the purification steps. The column was washed extensively with at least 20 column volumes (CV) of buffer A (50 mM HEPES, pH 8, 20 mM $MgCl_2$, 20% glycerol, 500 mM NaCl, 0.1 mM DDM, 1 mM TCEP and 20 mM imidazole) to facilitate the detergent exchange. The protein was eluted by a step-gradient of 30–50% buffer B (50 mM HEPES, pH 8, 20 mM $MgCl_2$, 20% glycerol, 500 mM NaCl, 0.1 mM DDM, 1 mM TCEP and 500 mM imidazole). Collected fractions were analyzed with SDS–PAGE and/or Western blot. Fractions containing alMGS were combined and concentrated 5- to 10-fold using an Amicon® Ultra-15 30 k centrifugal filter device (Millipore). The protein sample was then applied to a 24 ml size-exclusion column Sephadex™ 200 10/300 GL (GE Healthcare), and eluted with a final buffer (50 mM HEPES, pH 8, 10 mM $MgCl_2$, 10% glycerol, 500 mM NaCl, 0.1 mM DDM and 1 mM TCEP). Collected fractions were analyzed with SDS–PAGE and activity assay. Fractions containing alMGS were combined and concentrated as above.

Analysis

SDS–PAGE was performed with 4–12% NuPAGE® Bis–Tris gels (Invitrogen) and NuPAGE® MES running buffer (Invitrogen). Molecular mass was indicated by a LMW marker (GE Healthcare). All gels were stained with SimplyBlue™ SafeStain (Invitrogen).

Activity assay for alMGS was performed by mixing 20 μl of alMGS with 25 μl of mixed micelles (20% DAG, 30% PC and 50% PG (mole/mole) (all lipids from Avanti Polar Lipids, Inc.) in 50 mM HEPES, pH 8, 10 mM $MgCl_2$, 10% glycerol and 35 mM CHAPS), 2.5 μl assay buffer (200 mM HEPES, pH 8, 40 mM $MgCl_2$ and 40% glycerol) and 2 μl 25 mM DTT. The mixture was incubated on ice for 30 min before the reaction was started with 5.5 μl [C^{14}]-UDP-Glc mix (3 μl [C^{14}]-UDP-Glc (75 nCi) (GE Healthcare) and 2.5 μl ddH_2O) and incubated at 30 °C for 30 min. Enzyme activity was stopped by the addition of 350 μl chloroform:methanol 2:1 (v/v) and 150 μl 0.9% NaCl. After vortexing and centrifugation, the solvent phase was withdrawn and 25 μl of each sample was mixed with 1 ml of OptiPhase SuperMix scintillation liquid (PerkinElmer) in 24-well plates, and the amount of the produced radiolabeled GlcDAG lipid was determined with a Wallac MicroBeta® TriLux scintillation counter (PerkinElmer).

Western blot analysis was performed by transferring the proteins from the 4–12% NuPAGE® Bis–Tris gel to a nitrocellulose membrane. The membrane was blocked with 5% powder-milk and 0.1% Tween 20 in PBS. The primary antibody was a mouse anti-His monoclonal IgG antibody (Novagen) diluted 1:5000, and the secondary an ImmunoPure goat anti-mouse IgG (H + L), HRP-conjugated (Pierce) diluted 1:5000. The membrane was developed using ECL Plus Western blotting detection reagents (GE Healthcare), and detected with a FluorChem (Alpha Innotech Corp.) For the dot-blot analysis, 1 μl samples were loaded directly on the nitrocellulose membrane. After drying, the membrane was immersed in blocking solution and the remaining part of the analysis was performed following the same protocol as for the Western blot.

Protein quantification was carried out with Bradford assay [29], or by absorbance at 280 nm using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific). The two methods were found to yield similar results with respect to quantification.

Results and discussion

Overexpression optimization

Bacterial strain

The BL21-AI™ cells gave the highest amount of active protein per OD_{600} unit, as determined by the activity assay (Table 1). In BL21-AI™, the gene for the expressed protein is tightly regulated, and very little activity was observed for cultures not induced (Table 1). A tightly regulated system is advantageous for overexpression of membrane proteins, which may be harmful for the cell when in large concentrations [30]. The alternative strain BL21-pLysS yielded equally high expression levels as BL21-AI™, but here also the non-induced cells showed similar activity (Table 1), i.e. the promoter was "leaking". Therefore, BL21-AI™ was the preferred choice for further investigation.

Growth conditions

Cultures were grown at a minimum shaking of 250 rpm, with a starting temperature of 37 °C. When OD_{600} reached ~0.7 they were

Table 1
Summary of overexpression conditions screened for alMGS.

Conditions	Activity[a] (cpm)	OD_{600}	Pellet amount[b] (g/l)	SDS–PAGE[c]
Strains				
BL21-AI_{ind}[d]	2098 (421)[e]	3		
BL21-p$LysS_{ind}$[d]	2224 (1936)[e]	2		
$C41_{ind}$[d]	336 (584)[e]	2		
$C43_{ind}$[d]	211 (321)[e]	2		
Medium				
1× TB		12	24	
2× TB		12	29	
1× LB		3	7	
2× LB		4	14	
2× YT		6	11	
1× Superbroth		7	13	
Temperature				
37–22 °C[f]	17275	14	24	+++
37–16 °C[f]	11636	12	29	+++
Induction time				
3 h[g]	2261	3		+
8 h[g]	15513	8		++
21 h[g]	17275	14	24	+++

[a] CPM GlcDAG product, whole cell extract (blank value 27).
[b] Amount of wet-weight pellet in grams per litre of culture.
[c] Yield estimated from SDS–PAGE.
[d] 1× LB, 37 °C.
[e] Non-induced cells.
[f] 1× TB.
[g] 1× TB, 37–22 °C.

Table 2
Overview of the detergents used for solubilization of alMGS from the membrane.

Detergents	SDS–PAGE[a]	Activity[b] (cpm)
15 mM CHAPS	++++	14,492
80 mM CHAPS	++++	495
24 mM Cymal-5	+++	2294
10 mM LDAO	+++	927
1.7 mM DDM	+	15,247
15 mM FC-12	++	8664
1.2 mM FC-14	+	14,379
180 mM OG	+++	31
No detergent	+	15,800

[a] Estimated amount of soluble protein.
[b] CPM of GlcDAG product per mg total protein in supernatant after solubilization (blank value 27).

transferred to 22 °C. As protein overexpression can lead to a toxic condition, lowering the temperature and thereby expressing the protein slower over a longer time has been shown to be helpful. Hence, cultures were transferred to 22 °C to cool down before the induction of the protein synthesis. The lower temperature increased the amounts of active alMGS protein compared to higher temperatures (data not shown). However at 16 °C, the lowest temperature investigated, the cell growth rate was slower, resulting in less active protein. Thus, for this expression system the optimum temperature, to obtain high yields of active protein, was 22 °C (Table 1). Initial culture temperatures of 37 °C were used instead of 30 °C in order to obtain more cells before the induction of protein expression, which was made with 1 mM IPTG and 0.2% L-arabinose at OD_{600} ~0.8–1 for alMGS expression, in the BL21-AI™ cells. By keeping the cells at 22 °C for 21–22 h after induction, the amount of expressed protein increased substantially (Table 1).

Optimal growth medium conditions leading to the highest amount of active protein was 1× TB medium supplemented with 100 μg/ml carbencillin. For the growth of large cultures, the final OD_{600} was usually near 20, and ~30 g wet-weight pellet was obtained per litre of culture. A rich medium such as TB allowed the cells to grow continuously throughout the long expression time at low temperature. A less rich medium such as LB resulted in much a lower optical density. The optimized conditions are summarized in Table 1.

This work shows that, *via* a very simple method, the amount of membrane protein produced can be increased several times by the optimization of a few basic parameters such as temperature and media conditions. Membrane proteins are much more sensitive after removal from their natural environment and therefore it is crucial for successful further studies to obtain large amounts of active protein in the initial steps. Compared to other proteins such as glycosyltransferases and monotopic proteins [9–16], the levels of purified alMGS in this study was about 10 times higher than what has been reported before. GT-4 family proteins may potentially be particular easy to work with and give high expression levels, but in fact, the 3D structure is solved for only 8 out of approximately 9400 GT-4 proteins (http://www.cazy.org/) [27,28], which makes this assumption less plausible. Also, when comparing to other monotopic proteins, only eleven protein structures are so far reported [5], which contradicts the suggestion that monotopic membrane proteins are intrinsically easier to express and purify than bi- and polytopic membrane proteins.

Solubilization optimization

As the protein is tightly bound to the membrane, detergent is necessary for solubilizing the membrane-associated alMGS protein. A number of detergent types and concentrations were screened to find the one that solubilized the protein most efficiently from the membrane, and at the same time kept it active. To this end, 15 mM CHAPS (at ~2× CMC) was found to be the best overall choice (Table 2). It was not the single best detergent to solubilize the protein, however, 15 mM CHAPS was the only detergent that also kept the protein active: as determined using a relevant activity assay. FC-14 also kept the protein active, but it was an inferior solubilizing agent at 10× CMC. Higher concentrations of FC-14 (e.g. 5.2 mM (0.2%, ~40× CMC)), were more efficient for solubilizing alMGS, but at the same time the activity analysis showed that this concentration of FC-14 inactivated the protein and also made it precipitate in solution within one day (data not shown).

Small scale buffer screening using SEC

The fast and efficient SEC screening method was successfully used to find some detergents that resulted in a stable and monodisperse protein (Fig. 1a). The first peak in the chromatogram, at 1 ml, is the void volume of the column and should be interpreted as protein aggregate. The buffer containing 0.1 mM DDM gave only one major peak with the mass for a monomer of the protein, i.e. no aggregates were formed and only one form of the protein existed in the sample. It is evident from the chromatogram in Fig. 1a, that 15 mM CHAPS gave more protein than 0.1 mM DDM, but it also resulted in a quite large aggregate peak. The substantial difference between the curves in the chromatogram of the 0.1 (~1× CMC) and 0.4 (~4× CMC) mM solutions of DDM, were investigated further by a second experiment analyzing 0.1, 0.2, 0.3 and 0.4 mM DDM (Fig. 1b). The results were consistent, and 0.1 mM DDM was the optimal detergent and concentration giving the highest yield of a homogenous protein. The shift seen in retention volume (mass) of the major peak is caused by detergent, which interacts with the protein and thereby increases the determined species mass that now represents the protein-detergent complex. The shift depends on the micelle mass of the detergent [31], however the protein is still a monomer as indicated in Fig. 1b. The biological function of purified alMGS was confirmed by activity assay analy-

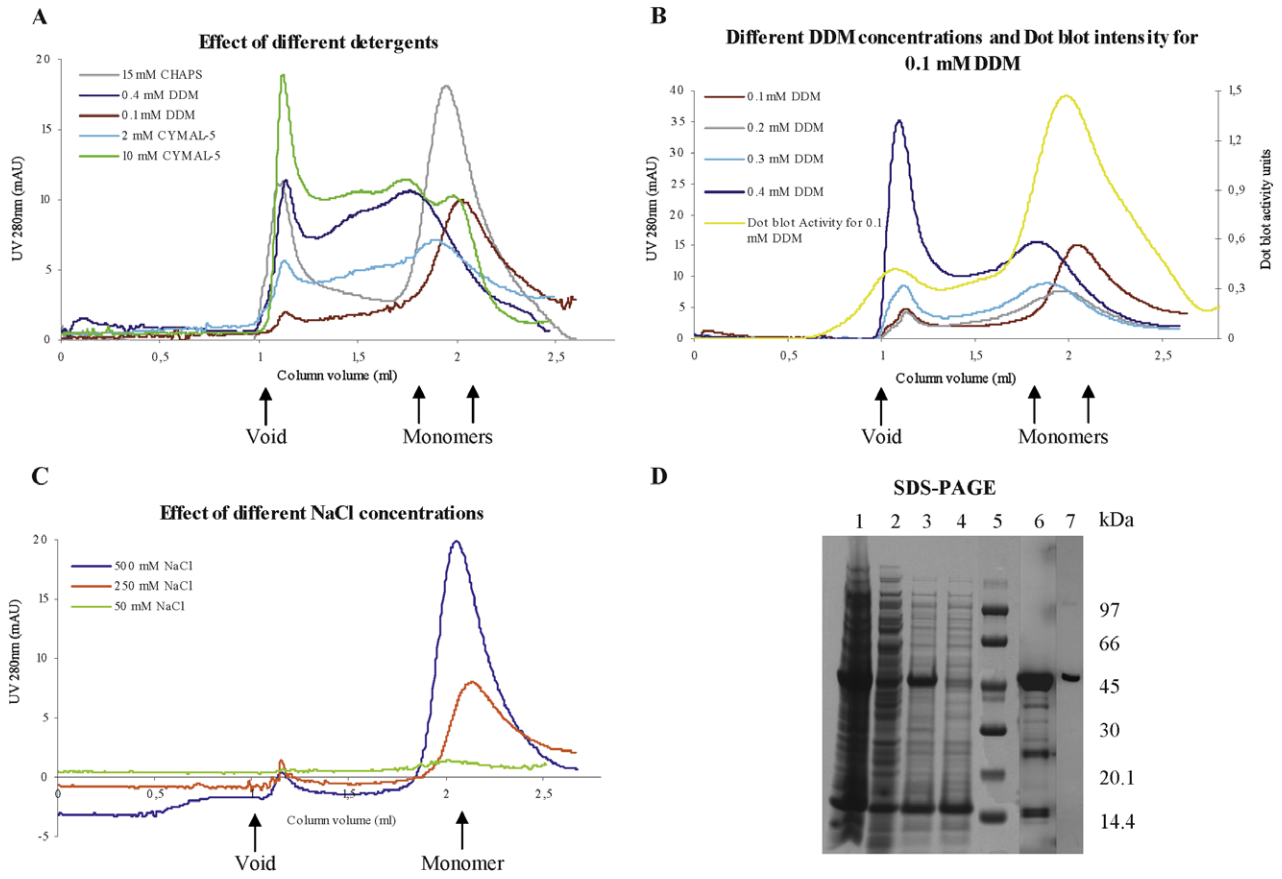

Fig. 1. Screening conditions by SEC. (A–C) Size-exclusion chromatogram of solubilized alMGS using a Superdex 200 5/150 GL column. Arrows indicate void and monomer retention volumes. (A) The effects of different detergents on alMGS. The peak profile shows the monodispersity of the protein, a single monomer peak is desirable. (B) Effect of different detergent concentrations, 0.1–0.4 mM DDM is analysed. The yellow curve indicates the dot-blot intensity for the 0.1 mM sample. (C) NaCl concentrations screened with permanently low (0.1 mM) DDM concentration. (D) SDS–PAGE of purification steps for alMGS with 15 mM CHAPS in the solubilization buffer, and 0.1 mM DDM in the final buffer: (1) lysed cells, (2) supernatant after ultra-centrifugation, (3) sample loaded on His-column, (4) flow-through from His-column, (5) LMW marker, (6) His-pool and (7) SEC-pool.

sis (data not shown) of the pooled monomer peak fractions from the 0.1 mM DDM run. The result of the dot-blot analysis of the 0.1 mM DDM sample (Fig. 1b) confirmed that all the protein in the main peak was indeed alMGS, since the dot-blot intensity (His-tag specific antibody) coincided with the recorded absorbance at 280 nm in the chromatogram obtained from the SEC run. This rapid dot-blot analysis saves time by quickly identifying unfavourable buffer components without the need to purify the protein in larger scale for SDS–PAGE analysis.

Most of the detergents were tested in low concentrations with respect to their CMC [31]. For example, CYMAL®-5 at 2 mM and DDM at 0.1 mM are about 1× CMC, and 0.4 mM DDM, 10 mM CYMAL®-5 and 15 mM CHAPS are about 2–5× CMC (literature values, Anatrace Inc., http://www.anatrace.com). Note that the specific CMC in the buffers used in the present study are not exactly known, and that the values given by Anatrace are from measurements in water or 0.15–0.2 M NaCl solutions. Our results show that for alMGS the concentration of detergent was as important as the type of detergent (Table 2 and Fig. 1). We conclude that a low concentration, around 1× CMC, is optimal for keeping the protein stable and homogeneous. This rather low detergent concentration may be explained by the fact that only parts of alMGS are hydrophobic, since the membrane association area is limited [26].

We also found the SEC method very useful for the screening of other buffer components. Salt concentrations were screened in a step-wise manner, ranging from 50 to 500 mM NaCl in an attempt to reduce the amount of NaCl in the buffer. The other buffer components were kept constant; 50 mM HEPES, pH 8, 10 mM $MgCl_2$, 10% glycerol, 1 mM TCEP and 0.1 mM DDM. We found that 500 mM NaCl was the most favourable concentration for keeping the protein monodispersed at low concentration of the detergent DDM (Fig. 1c). The screening trials showed that the final buffer composition, ideal for the alMGS protein, was 50 mM HEPES, pH 8, 500 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 1 mM TCEP and 0.1 mM DDM. These conditions also resulted in a highly pure protein (Fig. 1d).

The SEC method reveals whether the protein of interest migrates as a monomer or dimer etc., by comparison of the retention volumes. This way of using size-exclusion chromatography is a well established method [32–34], but SEC is usually very time-consuming because of long columns where the sample need to migrate slowly for good separation. The small 3 ml SEC column used in this study was found to be a very convenient and fast screening tool for selecting detergent type and concentration, as well as other buffer components.

Expression levels after optimization

After optimization of both growth and purification conditions, the yield of alMGS was 170 mg pure and concentrated protein per litre of *E. coli* culture. This is nearly two orders of magnitude more than before the optimization process. The protein yields in each step during the purification are summarized in Table 3. The purification was always performed from a smaller volume of cul-

Table 3
Yields in each step in the purification of alMGS.

Purification step	Total protein[a] (mg)[b]	Contaminant removed[c] (%)	Yield[a] (mg)[b]	Purity[d] (%)	Yield/L[e] (mg)[b]
Cell lysis	1100	0			
Membrane fraction	660	40			
Loaded on His-column	580	12			
After His-column	17.5 (13.5)[f]	97	16 (12)[f]	90	460 (340)[f]
After SEC-column	11.5 (6)[f]	34	11.5 (6)[f]	99	330 (170)[f]

[a] Corresponding to a 35 ml culture.
[b] Protein concentration estimated by Nano-drop measurement.
[c] Based on the total protein amounts.
[d] Purity estimated by SDS–PAGE.
[e] Amount per litre of culture, estimated from the amounts per 35 ml culture.
[f] Values in parenthesis are after concentration of protein.

ture; Table 3 shows the results of purification corresponding to a 35 ml culture. The amount of removed contaminating proteins is also given, as calculated from the reduction of total protein.

Conclusions

Membrane proteins normally yield low expression levels in most cell types. By careful optimization of basic parameters, the yields for the monotopic membrane protein alMGS were improved by two orders of magnitude. In these processes, a small-sized SEC column is a valuable and efficient tool for fast buffer and detergent optimization.

Acknowledgement

We thank Dr. L. Kaiser, Dr. P. Carlsson and Prof. L.D. Unsworth for helpful discussions and critical reading of the manuscript, Prof. P. Dedon laboratory (MIT) and Prof. A.J. Grodzinsky laboratory (MIT) for equipment assistance, and Dr. D. McRee for valuable advice and helpful discussion. This work was funded by the Swedish Research Council. H.M.E was supported by fellowships from the Sweden-America foundation, the Sven and Lilly Lawski foundation and the Helge Ax:son Johnson foundation. K.P was supported by the Åke Wiberg foundation.

References

[1] A.L. Hopkins, C.R. Groom, The druggable genome, Nat. Rev. Drug Discov. 1 (2002) 727–730.
[2] F. Gardoni, MAGUK proteins: new targets for pharmacological intervention in the glutamatergic synapse, Eur. J. Pharmacol. 585 (2008) 147–152.
[3] V.S. Trubetskoy, T.J. Burke, Engineered polymeric micelles: a novel tool for solubilization and stabilization of membrane protein targets for proteomics and drug discovery, Am. Lab. 37 (2005) 19–22.
[4] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, The protein data bank, Nucleic Acids Res. 28 (2000) 235–242.
[5] S. White, Membrane proteins of known 3D structure, 1998–2009. Available from: <http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html>.
[6] A. Krogh, B. Larsson, G. von Heijne, E.L.L. Sonnhammer, Predicting transmembrane protein topology with a hidden markov model: application to complete genomes, J. Mol. Biol. 305 (2001) 567–580.
[7] E. Wallin, G. Von Heijne, Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms, Protein Sci. 7 (1998) 1029–1038.
[8] G. Blobel, Intracellular protein topogenesis, Proc. Natl. Acad. Sci. USA Biol. Sci. 77 (1980) 1496–1500.
[9] S.E. Bane, J.E. Velasquez, A.S. Robinson, Expression and purification of milligram levels of inactive G-protein coupled receptors in *E. coli*, Protein Expr. Purif. 52 (2007) 348–355.
[10] S. Ha, E. Chang, M.C. Lo, H. Men, P. Park, M. Ge, S. Walker, The kinetic characterization of *Escherichia coli* MurG using synthetic substrate analogues, J. Am. Chem. Soc. 121 (1999) 8415–8426.
[11] T. Imai, K. Kasai, J. Kurita, K. Fukami, M. Tashiro, S. Shimotakahara, Expression and characterization of a pleckstrin homology domain in phospholipase C, PLC-eta 1, Protein Expr. Purif. 56 (2007) 247–252.
[12] L. Li, P. Storm, O.P. Karlsson, S. Berg, A. Wieslander, Irreversible binding and activity control of the 1, 2-diacylglycerol 3-glucosyltransferase from *Acholeplasma laidlawii* at an anionic lipid bilayer surface, Biochemistry 42 (2003) 9677–9686.
[13] H.C. Losey, M.W. Peczuh, Z. Chen, U.S. Eggert, S.D. Dong, I. Pelczer, D. Kahne, C.T. Walsh, Tandem action of glycosyltransferases in the maturation of vancomycin and teicoplanin aglycones: novel glycopeptides, Biochemistry 40 (2001) 4745–4755.
[14] A.L. Lovering, L.H. de Castro, D. Lim, N.C.J. Strynadka, Structural insight into the transglycosylation step of bacterial cell-wall biosynthesis, Science 315 (2007) 1402–1405.
[15] K.U. Wendt, C. Feil, A. Lenhart, K. Poralla, G.E. Schulz, Crystallization and preliminary X-ray crystallographic analysis of squalene-hopene cyclase from *Alicyclobacillus acidocaldarius*, Protein Sci. 6 (1997) 722–724.
[16] M. Barreras, P.L. Abdian, L. Ielpi, Functional characterization of GumK, a membrane-associated {beta}-glucuronosyltransferase from *Xanthomonas campestris* required for xanthan polysaccharide synthesis, Glycobiology 14 (2004) 233–241.
[17] N. Bonander, K. Hedfalk, C. Larsson, P. Mostad, C. Chang, L. Gustafsson, R.M. Bill, Design of improved membrane protein production experiments: quantitation of the host response, Protein Sci. 14 (2005) 1729–1740.
[18] F. Junge, B. Schneider, S. Reckel, D. Schwarz, V. Dotsch, F. Bernhard, Large-scale production of functional membrane proteins, Cell. Mol. Life Sci. 65 (2008) 1729–1755.
[19] G.G. Privé, Detergents for the stabilization and crystallization of membrane proteins, Methods 41 (2007) 388–397.
[20] R.D. Krueger-Koplin, P.L. Sorgen, S.T. Krueger-Koplin, I.O. Rivera-Torres, S.M. Cahill, D.B. Hicks, L. Grinius, T.A. Krulwich, M.E. Girvin, An evaluation of detergents for NMR structural studies of membrane proteins, J. Biomol. NMR 28 (2004) 43–57.
[21] M.C. Wiener, A pedestrian guide to membrane protein crystallization, Methods 34 (2004) 364–372.
[22] S. Berg, M. Edman, L. Li, M. Wikstrom, A. Wieslander, Sequence properties of the 1, 2-diacylglycerol 3-glucosyltransferase from *Acholeplasma laidlawaii* membranes – recognition of a large group of lipid glycosyltransferases in eubacteria and archaea, J. Biol. Chem. 276 (2001) 22056–22063.
[23] O.P. Karlsson, A. Dahlqvist, S. Vikstrom, A. Wieslander, Lipid dependence and basic kinetics of the purified 1, 2-diacylglycerol 3-glucosyltransferase from membranes of *Acholeplasma laidlawii*, J. Biol. Chem. 272 (1997) 929–936.
[24] M. Edman, S. Berg, P. Storm, M. Wikstrom, S. Vikstrom, A. Ohman, A. Wieslander, Structural features of glycosyltransferases synthesizing major bilayer and nonbilayer-prone membrane lipids in *Acholeplasma laidlawii* and *Streptococcus pneumoniae*, J. Biol. Chem. 278 (2003) 8420–8428.
[25] A. Dahlqvist, S. Andersson, A. Wieslander, The enzymatic synthesis of membrane glucolipids in *Acholeplasma laidlawii*, Biochim. Biophys. Acta Biomembr. 1105 (1992) 131–140.
[26] J. Lind, T. Ramo, M.L. Rosén Klement, E. Barany-Wallje, R.M. Epand, R.F. Epand, L. Maler, A. Wieslander, High cationic charge and bilayer interface-binding helices in a regulatory lipid glycosyltransferase, Biochemistry 46 (2007) 5664–5677.
[27] J.A. Campbell, G.J. Davies, V. Bulone, B. Henrissat, A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities, Biochem. J. 326 (1997) 929–939.
[28] P.M. Coutinho, E. Deleury, G.J. Davies, B. Henrissat, An evolving hierarchical family classification for glycosyltransferases, J. Mol. Biol. 328 (2003) 307–317.
[29] M.M. Bradford, Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[30] S. Wagner, From Biogenesis to Overexpression of Membrane Proteins in *Escherichia coli*, Ph.D. Thesis, Department of Biochemistry and Biophysics, Stockholm University, Stockholm, 2008, pp. 1–50.
[31] Anatrace Inc., Detergents and their uses in membrane protein science, Anatrace, Inc., Maumee, OH, 2008.
[32] P. Andrews, The gel-filtration behaviour of proteins related to their molecular weights over a wide range, Biochem. J. 96 (1965) 595–606.
[33] GE HealthCare, Gel filtration – Principles and Methods, GE Healthcare Life Sciences, Uppsala, Sweden, 2007.
[34] E. Stellwagen, Gel filtration, Methods Enzymol. 182 (1990) 317–328.