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Endotoxin-free purification of recombinant membrane scaffold protein expressed in *Escherichia coli*

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ABSTRACT

Membrane scaffold protein (MSP) is a versatile protein that can be used to study diverse membrane proteins. MSP is strongly expressed in *E. coli*; however, applications of MSP in *in vivo* studies remain limited because of contamination with large amounts of endotoxins. Endotoxins cannot be easily removed from MSP following standard purification protocols for His6-tagged proteins, washing with detergents, or Q-Sepharose anion exchange chromatography, regardless of whether the expression host is *E. coli* BL21(DE3) or ClearColi BL21(DE3). Furthermore, the concentrations of MSP-bound endotoxins were not reduced during nanodisc formation, such that the assembled nanodiscs still contained significant amounts of endotoxins. We hypothesized that the structural properties of MSP that are responsible for membrane scaffolding mediated the strong binding between MSP and the endotoxins. We showed that partial denaturation of MSP with 2 M urea effectively disrupted MSP-endotoxin interactions. MSP-bound endotoxins were successfully removed *via* Q-Sepharose chromatography following urea treatment. The combined treatment with urea and Q-Sepharose resulted in ~80-fold reduction in the specific endotoxin level relative to that of conventional Ni-NTA chromatography combined with detergent treatment. The low endotoxin level of 2.0 EU/mg MSP obtained in this study makes it suitable for applications in animal studies.

1. Introduction

The membrane scaffold protein (MSP), which is derived from apolipoprotein A-1 (Apo A-1), is highly useful for studying membrane proteins and has diverse biotechnological applications [1–7]. MSPs are used to form nano-sized discoidal lipid bilayer structures called nanodiscs, based on its ability to enclose lipid tails. Nanodiscs can provide a native-like environment to membrane proteins even in aqueous solution [8]. Membrane proteins inserted in the lipid bilayer of nanodiscs remain stable and active [9], making it possible to investigate the characteristics and reaction mechanisms of membrane proteins in their native-like states. Furthermore, nanodisc structures can be useful in a variety of biotechnological applications [3,9–11].

MSPs can be easily produced in large quantities through recombinant expression in *Escherichia coli* [8–12]. However, similar to recombinant expression of other proteins in *E. coli*, contamination with endotoxins is one of the biggest problems related to the purification of MSPs [13]. Endotoxins are lipopolysaccharide (LPS) components of the outer cell membrane of Gram-negative bacteria and are composed of hydrophobic lipids, hydrophilic core polysaccharide chains, and hydrophilic O-antigenic side chains [14]. Endotoxins can remain bound to proteins even during cell disruption, thereby limiting the applications of the target proteins in cell culture and animal studies [15] although it is believed that high density lipoprotein (HDL) and nanodisc have great potential as therapeutic agents and drug delivery systems [16]. Small amounts of endotoxins can induce side effects, such as fever, tissue damage, or even death [14,15]. In the present study, we showed that the MSPs expressed in *E. coli* were still contaminated with exceptionally high levels of endotoxins following purification *via* conventional Ni-nitrilotriacetic acid (Ni-NTA) chromatography. The intrinsic affinity between MPS and endotoxin molecules is likely to be mediated by the lipophilic moiety of endotoxins and the capability of MSPs to enclose lipid acyl tails.

ClearColi is an FDA (Food and Drug Administration)-approved *E. coli* strain that has been developed to reduce the side effects of endotoxins [17]. ClearColi cells have genetically modified lipopolysaccharides (LPSs) in the outer cellular membrane to prevent the endotoxic response in human cells [17]. In ClearColi cells, the six acyl chains of LPS, which trigger the endotoxic response, were replaced with modified LPS lacking the two secondary acyl chains (lipid IV_A). In

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addition, the oligosaccharide chain was removed to facilitate easier removal of the modified LPS in downstream processes. Nevertheless, the modified LPS in ClearColi can still act as an endotoxic activator in mammalian hosts other than human cells. Although the modified LPS is endotoxically inactive in humans, ClearColi returns false positive results in the *Limulus* amebocyte lysate (LAL) assay, a commonly used FDA-approved method for endotoxin detection. ClearColi cells grow more slowly compared to ordinary *E. coli* strains. Thus, an efficient method to remove MSP-bound endotoxins is necessary regardless of whether it is produced in *E. coli* BL21(DE3) or ClearColi (DE3).

Immobilized metal affinity chromatography (IMAC) techniques, such as Ni-NTA chromatography, are widely used for the purification of His6-tagged recombinant proteins expressed in *E. coli* [18–21]. Ni-NTA chromatography usually yields acceptable purities for subsequent biochemical analysis, but additional chromatography is necessary to remove endotoxins. Anion exchange chromatography is frequently used to remove endotoxins [22,23]. The Q-Sepharose column maintains a strong positive charge in a broad pH range because of the presence of choline residues. The lipid A region of endotoxins is high phosphorylated and is a negatively charged domain anchored to the lipid bilayer [24]. As a result, endotoxins exhibit strong affinity to the choline residues of Q-Sepharose [25]. Adsorption and desorption of endotoxins to and from Q-Sepharose can be easily regulated by controlling the ionic strength of the buffer. However, the nature of MSP-endotoxin interaction appears to be different from other protein-endotoxin interactions because MSP-bound endotoxins cannot be removed *via* Q-Sepharose treatment. Because urea unfolds proteins enabling refolding of target proteins [26–29] and was reported useful in purifying ApoA-1 in ion exchange chromatography [30], we hypothesize that urea-mediated partial unfolding of MSP may facilitate easier removal of endotoxins from MSP *via* ion-exchange chromatography.

2. Materials and methods

2.1. MSP expression in E. coli BL21(DE3) and ClearColi BL21(DE3)

The pMSP1E3D1 was a gift from Stephen Sligar (Addgene plasmid # 20066). ClearColi BL21(DE3) was purchased from Lucigen (Madison, WI, USA). Two *E. coli* strains, namely, *E. coli* BL21(DE3) and ClearColi BL21(DE3), were transformed with pET-28a-MSP1E3D1 and pre-cultured overnight in 10 ml of autoclaved Luria-Bertani (LB) medium at 37 °C with shaking at 250 rpm. Next, 18 ml of the seed culture was inoculated to 1.8 l of LB medium and cultured at 37 °C with shaking at 150 rpm for 2.5 h. Afterwards, 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to the culture upon reaching an OD_{600} of 0.5–0.8. After 1 h of incubation, the temperature was decreased to 25 °C, and cultures were incubated with shaking at 120 rpm for 4 h. Cells were collected by centrifugation at 8000 *g* for 5 min. After removing the supernatant, cell pellets were stored at −80 °C overnight.

2.2. MSP purification via His6-tag immobilized metal affinity chromatography

MSPs expressed in *E. coli* strains were purified *via* IMAC. The following six buffers were prepared: buffer (1) 40 mM Tris-Cl, 300 mM NaCl, and 1% Triton, pH 8.0; buffer (2) 40 mM Tris-Cl, 300 mM NaCl, and 1% (v/v) Triton X-100, pH 8.0; buffer (3) 40 mM Tris-Cl and 300 mM NaCl, pH 8.0; buffer (4) 40 mM Tris-Cl, 300 mM NaCl, and 15 mM imidazole, pH 8.0; buffer (5) 40 mM Tris-Cl, 300 mM NaCl, and 300 mM imidazole, pH 7.4 buffer; and (6) 40 mM Tris-Cl and 100 mM NaCl, pH 7.4. Cell pellets were resuspended in buffer (1) supplemented with 5 μg/ml AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride) and subsequently disrupted *via* sonication. Disrupted cells were centrifuged at 13,000*g* for 40 min. Next, 1 ml of Ni-NTA agarose beads (Qiagen, Hilden, Germany) was loaded in 20 ml of the chromatography column (Bio-Rad, CA, USA) and pre-equilibrated with three column-volumes (CV) of the buffer (1). After centrifugation, the supernatant was reacted with Ni-NTA beads for 2.5 h at 4 °C by vertical rotation. Beads were then sequentially washed with four CVs of buffers (1) to (4). MSP was eluted by adding 10 ml of buffer (5). Proteins were concentrated with an Amicon Ultra-0.5 10 K centricon (Merck Millipore, Carrigtwohill, Ireland). MSP was desalted, and the buffer was replaced with buffer (6) in the PD-10 desalting column (GE Healthcare, Uppsala, Sweden). Finally, the purified MSP was stored in 10% (v/v) glycerol and 5 μg/ml AEBSF at −80 °C.

2.3. MSP purification via Q-Sepharose anion exchange chromatography

2.3.1. Standard Q-Sepharose anion exchange chromatography

An anion exchange buffer containing 40 mM Tris-Cl (pH 7.4) was prepared. Q-Sepharose (GE Healthcare, Uppsala, Sweden) was pre-equilibrated with buffer containing 100 mM NaCl. MSP solution (4 ml) was purified *via* Ni-NTA chromatography, loaded onto the columns, and reacted with the beads for 6 h at 4 °C with vertical rotation. After removing unbound proteins, the remaining proteins were eluted with four CVs of buffer (6). Next, for each NaCl concentration, 4 ml of buffer containing 150–500 mM NaCl was used twice to desorb MPS from Q-Sepharose. The eluted MSP was concentrated, and the buffer was replaced with buffer (6) (40 mM Tris-Cl buffer and 100 mM NaCl, pH 7.4) on an Amicon Ultra-0.5 10 K centricon.

2.3.2. Urea pretreatment and Q-Sepharose anion exchange chromatography

Urea (5 M) was dissolved in buffer (6) (40 mM Tris-Cl buffer and 100 mM NaCl, pH 7.4). The resulting solution was mixed with the MSP solution and purified *via* Ni-NTA chromatography to yield a final urea concentration of 2 M. Q-Sepharose beads were pre-equilibrated with buffer (6) containing 2 M urea, after which the MSP solution in the urea-containing buffer (6) was loaded onto the column. The mixture was incubated at 4 °C for 6 h with vertical rotation. After removing unbound proteins, remaining proteins were eluted with four CVs of the buffer containing 2 M urea. Next, 4 ml of the buffer containing 150–500 mM NaCl were used twice for each NaCl concentration to desorb MSPs from Q-Sepharose. Thus, 2 M urea was present in the desorption buffers with varying concentrations of NaCl. The eluted MSP was concentrated, and the buffer was replaced with buffer (6) (40 mM Tris-Cl buffer and 100 mM NaCl, pH 7.4) on an Amicon Ultra-0.5 10 K centricon.

2.4. LAL endotoxin assay

Endotoxin levels was determined *via* the *Limulus* amebocyte lysate (LAL) test. The test was performed using Limulus Amebocyte Lysate QCL-1000™ (Lonza Walkersville, Inc., MD, USA), which contains lyophilized LAL, *E. coli* 0111:B4 endotoxin, chromogenic substrate powders, and endotoxin-free LAL reagent water. The lyophilized LAL, *E. coli* endotoxins, and chromogenic substrate powders were reconstituted by adding 1.4, 1.0, and 6.5 ml of endotoxin-free LAL reagent water, respectively. To prepare the standard, *E. coli* endotoxins were diluted to 1.0, 0.5, 0.25, and 0.1 EU (Endotoxin Unit) per ml using endotoxin-free LAL reagent water. Several dilutions of the samples were prepared using the LAL reagent water. Only values lower than 1.0 EU/ml were taken into account. For each of the *E. coli* endotoxin standards and samples, 50 μl was loaded onto the 96-well microplate at 37 °C, and 50 μl of LAL solution was added to each well. After incubation at 37 °C for 10 min, 100 μl of chromogenic substrate prewarmed at 37 °C was added to each well, and the microplate was incubated at 37 °C for 6 min. Afterwards, 50 μl of 25% glacial acetic acid was added to each well as a stop reagent. Absorbance was read at 405 nm using a spectrophotometer, and endotoxin levels were calculated based on the standard curve.