Process Biochemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Using self-cleavable ternary fusion pattern for efficient preparation of Bacteriorhodopsin

Haihong Huang, Bin Yang, Baosheng Ge*, Jun Lao, Shitan Zhou, Fang Huang*

State Key Laboratory of Heavy Oil Processing and Center for Bioengineering and Biotechnology, China University of Petroleum (Huadong), Qingdao 266580, PR China

ARTICLE INFO

Keywords:
Self-cleavable
Intein
Expression
Membrane protein
Bacteriorhodopsin

ABSTRACT

The study of membrane proteins has been notoriously hampered by its low expression level and difficulty in purification and crystallization. Therefore, development of efficient method for preparation of properly folded membrane proteins with milligram yield would be of critical importance. Here, we developed a simple and efficient strategy to obtain high purity of bacterio-opsion (bO), the apoprotein of bacteriorhodopsin (bR), in *Escherichia coli* (*E. coli*) using self-cleavable ternary fusion pattern. This method overexpresses bO as a ternary fusion protein, which can undergo controllable self-cleavage without addition of exiguous enzymes. After self-cleavage, there is no extra amino acid left on the native terminus of the bO. The final yield of bO can reach 270 mg/L with purity over 95%. BR can be obtained by reconstituting the purified bO with retinal in either *n*-Decyl-β-maltopyranoside (DDM) micelles or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicles. Our work provides a simple and efficient way for preparation of membrane proteins, and will be beneficial for their structural and functional studies.

1. Introduction

About 20–25% of the sequenced open reading frames code for membrane proteins and they play critical roles in various biological processes, such as: transportation, intercellular signaling, ion conductance and so on [1,2]. Many membrane proteins are deemed as important drug targets [3]. Despite of their critical importance, studies on membrane proteins have been greatly hampered by their properties, low abundance, hydrophobicity and membrane environment requiring [4,5]. Developing an efficient method for milligram and functional preparation of membrane proteins would be beneficial for their structural and functional studies and related pharmaceutical development [6,7].

To achieve milligram production, membrane proteins are normally heterologous expressed using *E. coli*, yeast, insects or mammalian cell lines and cell-free expression systems due to the low abundance in their native environments [8,9]. Compared with other heterologous expression systems, *E. coli* expression system has been widely employed as a powerful and versatile host for high-level protein expression with advantages as easy plasmid construction, feasibility for large-scale production, low cost and short culture time [10,11]. It is found that almost 90% of soluble proteins in Protein Data Bank (PDB) were expressed in *E. coli*. However, because of their high hydrophobicity, membrane proteins are typically over-expressed in *E. coli* as inclusion bodies with poor solubility and then recovered through refolding, which is time-consuming and costly [12].

For soluble expression, membrane proteins are normally expressed as fusion with other proteins to facilitate correct folding and formation of disulfide bond, such as MBP (maltose binding protein), Nus (N utilization substance), GST (gultathione S transferase) and thioredoxin, etc. [13–16]. Membrane proteins without fusion part can be obtained after digestion with specific enzymes. A drawback of this approach is that the affinity tag often needs to be cleaved off with a site-specific protease, which is costly and time consuming, and in some cases, results in difficulties in purification. Furthermore, in detergent solutions, more protease is usually required. Meanwhile, fusion tags are generally engineered at N terminus of the target proteins, after tag removal, some extra amino acid residues will be left with the target proteins, which can interfere with the biological activity of the target proteins [17,18]. Therefore, it is highly desirable to develop a new procedure to express membrane proteins with good solubility and purify them in a simple way.

Inteins are internal protein elements which can self-excise from their host proteins and catalyze ligation of exons [19]. Intein excision or self-splicing is a post-translational process. This self-splicing can be regulated and controlled by pH and temperature and does not require auxiliary enzymes or cofactors [20,21]. Most importantly, after self-splicing there is no extraordinary amino acid residues left on target

* Corresponding authors.
E-mail addresses: gebaosheng@upc.edu.cn (B. Ge), fhuang@upc.edu.cn (F. Huang).

http://dx.doi.org/10.1016/j.procbio.2017.09.024
Received 19 July 2017; Received in revised form 4 September 2017; Accepted 22 September 2017

proteins, which can keep their native N terminus and retain their biological functions [22–24]. Thus inteins have been developed as valuable tools for affinity-tag-based protein purification techniques [25,26]. However, the application of this valuable purification and self-splicing tag for membrane proteins has not been addressed [27,28].

Bacteriorhodopsin (bR) consists of 248 amino acids, a bundle of seven transmembrane α-helical, and incorporate with a retinal chromophore that is covalently bond within the helix bundle via a Schiff base linkage to Lys216 [29–32]. As a model membrane protein with similar structure as G protein coupled receptors (GPCR), bR has been extensively employed in biophysical and biochemical studies of membrane proteins, such as protein folding and relation between structure and function [33–36]. Generally, milligrams of membrane protein are needed for their structural and functional studies. However, over-expression of bR in *E. coli* could be only achieved either as inclusion bodies or fusion with protein tags, such as MBP or thioredoxin [12,37]. The following refolding or tag digestion process became challenging because of their higher hydrophobicity and low digestion efficiency.

Here, we described a protocol for a simple and efficient preparation of membrane proteins using self-cleavable ternary fusion pattern in *E. coli*. After optimization of expression conditions, soluble bR with high expression level was obtained as fusion protein, and the target protein was then purified with a relatively simple self-splicing and purification procedure. Reconstitution of bO with retinal can be completed in either DDM micelles or DOPC vesicles. The reconstituted bR shows similar characteristics as that from its native host. This work provided an important example for preparation of membrane proteins and would facilitate further studies on structural and functional studies on membrane proteins and related pharmaceutical developments.

2. Materials and methods

2.1. Materials

The pTWIN1 plasmid, DNA restriction enzymes, such as *Eco*R I, *Bam*H I, and chitin resin, were purchased from New England Biolabs (Ipswich, MA, USA). The bO gene from MPK409 strain was obtained as a donation from Prof. Janos Lanyi' (UC Irvine) lab. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and Fos Choline-14 (FC-14) were obtained from Avanti Polar lipids Inc (Alabaster, Al, USA). Sodium dodecyl sulfate (SDS) and *n*-Decyl-β-D-maltopyranoside (DDM) were from Sigma Aldrich (USA). The *E. coli* BL21 (DE3) competent cells were purchased from TIGEN Biotech Company (Beijing, China). All other reagents and chemicals were of analytical grade.

2.2. Construction of pTWIN1- Chitin Binding Domain(CBD)-intein-bO-expression plasmid

The bO gene was Polymerase chain reaction (PCR) amplified using following primers: forward primer 5′-aaaGAATTCATGTTGGAGTTA TTGCCAACAGCAGTGGA-3′ and reverse primer 5′-tttGGATCCTTAT TAGTGGTGGTGGTGATGGTGATGATGATGATGGTCGCTG GTCGCGGC CGCGCCGTC-3′. After digested with *Eco*R I and *Bam*H I, the gene was cloned into pTWIN1 vector digested with same enzymes, resulting in pTWIN1-CBD-intein-bO-expression plasmid. The construct can express bO as CBD-intein-bO-hexa-histidine fusion protein. The recombinant plasmid was then confirmed by DNA sequencing.

2.3. Optimization of CBD-intein-bO-pTWIN1/BL21 expression in E. coli

Optimization of culture conditions was performed for maximum expression of soluble CBD-intein-bO in BL21 (DE3) cells. Freshly transformed cells were picked up and grown in 5 ml LB broth and cultured overnight with shaking, then cultures were inoculated into 200 ml fresh Terrific Broth (TB) medium containing 100 μg/ml Ampicillin. When cultures grown to OD_{600} as 0.2–0.4, 0.6–0.8, 1.2–1.4 and 1.8–2.0 at 37 °C in TB media, protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) with final concentration of 0.1 mM, 0.3 mM, 0.5 mM, 0.7 mM and 1.0 mM respectively. The culture was further continued at 18 °C after induction for another 20 h. Different culture temperatures, such as 37 °C, 18 °C and 10 °C after induction were also optimized using similar method. Samples were taken at regular time and analyzed using dot-blot analysis.

2.4. Protein purification and self-cleavage

Three liters of freshly transformed *E. coli* cells were cultured at optimized conditions. After 20 h of induction at 18 °C, cells were harvested by centrifugation at 8000 × *g* for 20 min at 4 °C. Cell pellets were suspended in 100 ml lysis buffer (50 mM Tris-HCl pH 8.5, 300 mM NaCl, 0.05% (m/v) lysozyme, 0.04 mg/ml DNA enzyme, 4 M urea, 1 mM Phenyl methane sulfonyl fluoride (PMSF)), and then passed through a high pressure cell disruptor for about 3 times at 4 °C. The cell lysates were centrifuged at 8000 × g for 30 min at 4 °C, and then supernatant was collected and membrane components were extracted by centrifugation at 100000 × *g* for 1 h at 4 °C. This membrane components sedimentation was suspended in solubilization buffer (50 mM Tris-HCl, pH 8.5, 300 mM NaCl, 1% FC-14 (w/v)) with gently mixing for 16 h at 4 °C. After solubilization, the sample was centrifuged at 8000 × *g* at 4 °C for 20 min and then filtered with 0.45 μm filters. The supernant was then applied onto Ni^{2+} affinity chromatography column pre-equilibrated with 3–5 column volume of equilibration buffer (50 mM Tris-HCl, pH 8.5, 300 mM NaCl, 0.02% FC-14(w/v), 25 mM imidazole). After washed with washing buffer (50 mM Tris-HCl, pH 8.5, 300 mM NaCl, 0.02% FC-14(w/v), 100 mM imidazole), the aimed fusion protein was eluted with elution buffer (50 mM Tris-HCl, pH 8.5, 300 mM NaCl, 0.02% FC-14(w/v), containing 500 mM imidazole). The eluent was then buffer exchanged into 1 × PBS (pH 7.4) containing 0.02% (w/v) DDM for self-cleavage.

After that, the solution was loaded onto the chitin affinity chromatography column pre- equilibrated with 1 × PBS (pH7.4), 0.02% (w/v) FC-14. the column was firstly washed with 10 column volume 1 × PBS (pH7.4) containing 0.02% (w/v) FC-14, and incubated at 25 °C three days for on-column self-cleavage. The bO was finally recovered as flow-through. The collected fraction was then buffer exchanged into 10 mM sodium phosphate (pH 6.2) containing 0.02% (w/v) DDM and kept for further analysis.

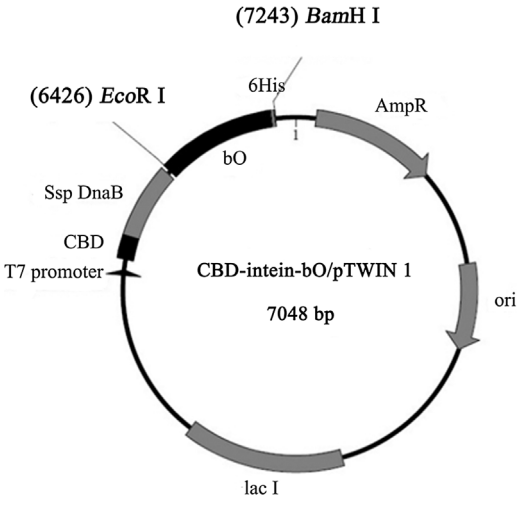

Fig. 1. The diagram of constructed pTWIN1-CBD-intein-bO expression plasmid. The target gene of bO can be cloned using *Eco*R I and *Bam*H I cloning sites. The construct resulted in a hexa-histidine tag fused at the C-terminus of bO, and a CBD-intein tag fused at the N-terminus.