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One-step purification of delipidated Bacteriorhodopsin by aqueous-three-phase system from purple membrane of *Halobacterium*

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ABSTRACT

Bacteriorhodopsin (BR), the only protein in the purple membrane (PM) of certain extreme halophilic microorganisms, functions as a light-driven proton pump using light energy to generate transmembrane proton gradient for ATP synthesis. BR naturally aggregates in a highly ordered two-dimensional hexagonal array of trimers in the PM of *Halobacterium*. The BR in the isolated PM can be employed to generate a photocurrent in a photocell. However, delipidated BR (deBR) has been reported be more efficient than BR for photocurrent generation. In the present work, detergent CHAPS was included in anaqueous three-phase system (A3PS) to remove the lipids in the out layer of the BR trimer during the preparation of deBR. A3PS that consisted of polypropyleneglycol (PPG), polyethyleneglycol (PEG), and phosphate buffer purified deBR directly from the cell lysate of *Halobacterium salinarum* with a recovery yield of 89.7%. CHAPS along with the contaminant bacterioruberin pigment were partitioned into the top PPG-rich phase while deBR was mainly located at the interface between PEG-rich phase and the lower phosphate phase. After further purification by using ultrafiltration to remove PEG, the purified deBR when immobilized on indium tin oxide (ITO) glass was able to generate 60% higher photocurrent density.

Keywords: Aqueous three-phase system; Purple membrane; Bacteriorhodopsin; Delipidation; Membrane protein; Photocurrent

1. Introduction

Bacteriorhodopsin (BR) is the only protein in the purple membrane (PM) patch of extreme halophilic bacteria *Halobacterium salinarum*. It is arranged in a highly ordered two-dimensional hexagonal array of trimers and each monomer functions as an independent light-driven proton pump to transfer protons unidirectionally from the cytoplasmic side to the extracellular side of the membrane. The established proton gradient is then used by the bacteria to drive the synthesis of ATP. Each monomer of BR consists of a membrane integral protein, bacteriopsin (BO) with seven trans-membrane helices and a central region occupied by a retinal chromophore covalently bound via a Schiff base to lysine 216. In the PM structure, lipids amount to about 25% by weight, fill the spaces between the BR molecules in the lattice and are in close contact with BO (Blaurock and Stoeckenius, 1971; Henderson and Unwin, 1975; Jost et al., 1978; Glaeser et al., 1985).

As a two-dimensional crystal, BR exhibits exceptional thermal and photostability that can lead to a considerable interest in the incorporation of BR film into electronic circuitry for the generation of a photocurrent. The suggested applications include artificial retinas, photochromic data storage, holographic cameras, and information processing (Xu et al., 2004; Hampp, 2000; Miyasaka et al., 1992; Birge et al., 1999).

In order to enhance the generated photocurrent, various methods have also been investigated for the immobilization of BR on different substrates including drop drying (Saga et al., 1999), Langmuir–Blodgett film formation (Miyasaka and Koyama, 1992), electrophoretic sedimentation (Min et al., 1998), antibody mediated oriented immobilization (Koyama et al., 1994), host guest mediated immobilization (Chen et al.,

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2003) and encapsulation in polymeric or sol–gel based supports (Chen et al., 1991; Weetall, 1996).

Traditionally, BR is isolated and purified by a tedious and time-consuming sucrose density gradient ultracentrifugation. Its scale-up, however, is a big engineering challenge. The lack of easily available purified BR limits its commercial applications. Aqueous two-phase system (ATPS) consists of two immiscible aqueous phases that are a non-toxic, environmentally friendly, with an easily scalable extraction process (Zijlstra et al., 1998). The purifications of various biomolecules, cells, and membrane proteins have been achieved by using ATPS (Albertsson, 1973; Yoshida et al., 1983; Minuth et al., 1995; Morre and Morre, 2000). The purification mechanism of ATPS is mainly based on surface property differences rather than on size and density of the biomolecules (Zijlstra et al., 1998). Recently, an extremely effective isolation and purification of BR, from the lysate of *Halobacterium* by ATPS, has been reported (Huh et al., 2010; Shiu et al., 2013). In actual fact, the high purity BR obtained either by using density gradient ultracentrifugation or ATPS is still associated with various lipids. By stripping the lipids that reside outside the BR trimers in PM, one can obtain delipidated BR (deBR). When deposited as a monolayer on a gold electrode surface, deBR generates a considerably greater photocurrent density than that of BR as reported by Patil et al. (2011). BR as a membrane protein has a strong affinity for lipids. And when incubated in certain detergents, the lipids that are associated with the BR can be stripped off to produce delipidated BR. Previously, ionic detergent 3-[(cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS) is a very effective delipidation agent. BR delipidation using CHAPS has little effect on both the maximal absorption wavelength of the BR molecules (with a shift of only ~10 nm (Heyes and El-Sayed, 2003)) and its activity to generate a photocurrent (Patil et al., 2011). The removal of CHAPS from deBR, however, is a tedious process, that requires chromatography or diafiltration.

It has been demonstrated by Albertsson (1973) that the PPG phase formed in an aqueous multiphase partition system can effectively extract Triton X-100 and lipids in the purification of hydrophobic membrane protein phospholipase A1 from *Escherichia coli*. In order to develop a one-step scalable deBR purification process for the preparation of a high photocurrent density electrode, in the present study, PEG-phosphate ATPS, developed for BR purification (Shiu et al., 2013) will be modified by adding polypropylene glycol (PPG) to form a three-phase system (A3PS). The PPG-rich phase is expected to remove most of the lipids and delipidation agent CHAPS added together with lysed *H. salinarum* cells. The deBR recovered from the interface between phosphate and PEG-rich phase will be used in a photocell to study its effect on enhancing photocurrent density.

2. Materials and methods

2.1. Chemicals

Polypropylene glycol 2024 (PPG) and the ionic detergent 3-[(cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS) were obtained from Sigma (St. Louis, USA). Polyethylene glycol 8000 (PEG) and lethicin were purchased from Amersco (Solon, OH, USA) and Nacalai Tesque Inc. (Tokyo, Japan), respectively. Other chemicals used were of analytical grade.

2.2. Cell cultivation

H. salinarum, provided by Professor Dieter Oesterhelt (Max Planck Institute of Biochemistry, Martinsried, Germany) was grown in100 mL culture medium containing 25 g NaCl, 2 g $MgSO_4{\cdot}H_2O$, 0.3 g sodium citrate, 0.2 g KCl, 1 g bacterogical peptone (Oxoid, L37) with pH adjusted to 7.2 in a 250 mL flask. The culture was shaken at 150 rpm, 37 °C under continuous illumination (40 W) for 48 h. The cells were collected by centrifugation at 8000 rpm (Beckman Avant J25, rotor JA10) at 4 °C for 15 min. The collected cell pellet was then resuspended in 400 mL fresh culture medium and cultured for another 48 h under the same conditions. The final optical density (OD_{660}) of the cell culture was 2.3.

2.3. Purification and delipidation of BR

For BR purification, a 75 mL *H. salinarum* culture was harvested by centrifugation (Beckman Avant J25, rotor JA10) at 8000 rpm at 15 °C for 15 min. The obtained cell pellet was suspended in 5 mL deionized water containing 68.7 μL DNase (329.6 U, Sigma–Aldrich). After mixing thoroughly for 30 min at room temperature, 10 mL 24% (w/w) potassium phosphate solution (22 g monobasic potassium phosphate, 2 g dibasic potassium phosphate, pH 8) and 10 mL 24% (w/w) PEG solution were added and mixed thoroughly for 1 h. To enhance the rate of phase separation, the mixture was centrifuged (Beckman Optimal L90K, swing bucket rotor SW28) at 8000 rpm for 15 min at 4 °C. A purple band, which formed at the interface between the upper PEG-rich phase and the lower phosphate-rich phase, was collected with a pipette and then subjected to a low speed (4000 rpm) centrifugation to pellet the cell debris within the collected purple band. A purple precipitate can be obtained by centrifuging the supernatant at a higher centrifugation speed (24,000 rpm) at 4 °C for 30 min. After washing twice with 30 mL deionized water, the purple precipitate was resuspended in 10 mL deionized water as the purified BR solution. BR delipidation was then carried out by using CHAPS detergent to remove lipids in the outer layer of the purified BR according to Szundi's method (Szundi and Stoeckenius, 1987). Briefly, 0.6 mL BR solution (~80 μg BR) was gently mixed with 41.4 μL, 20 mM CHAPS solution at room temperature overnight. Delipidated BR was recovered by centrifugation (Eppendorf centrifuge, rotor FA-45-24-11) at 15,000 rpm, 4 °C for 30 min. To ensure complete delipidation, the process was repeated several times with 3-fold higher concentration CHAPS (60 mM) and for 1 h at a time. The deBR was recovered by centrifugation (Eppendorf centrifuge, rotor FA-45-24-11) at 15,000 rpm, 4 °C for 30 min and washed three times with deionized water.

2.4. One-step purification of delipidated BR

Cell pellets, harvested from 75 mL *H.salinarum* culture by centrifugation (Beckman Optimal L90 K, swing bucket rotor SW28) at 8000 rpm for 15 min was suspended in 1.5 mL deionized water containing 68.7 μL DNase solution (329.6 U) for 30 min at room temperature to disrupt the cells. The cell lysate suspension was directly subjected to the BR delipidation process. Approximately 2 mL cell lysate was mixed with 3.6 mL 40 mM CHAPS for 1 h followed by10 mL 24% (w/w) potassium phosphate (22 g monobasic potassium phosphate, 2 g dibasic potassium phosphate, pH 8), 10 mL 24% (w/w) PEG 8000 solution and 5 mL polypropylene glycol. After mixing thoroughly for 30 min at room temperature, it was centrifuged