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Study on mass production of aquaporinZ for biomimetic water purification membrane

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ABSTRACT

Aquaporin biomimetic membrane can only transfer water molecules in and out of the membrane while preventing the passage of other smaller ions and solutes. AquaporinZ (AqpZ), widely spread in *Escherichia coli* cell membrane, has shown higher water permeability than conventional membranes. Application of those exceptional properties as water purification membrane material is promising. The objective of this study was to assess protein expression conditions for AqpZ mass production. Recombinant AqpZ was successfully synthesized and identified via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. The highest level of expression of his-tagged AqpZ (12.2 mg/L) was achieved using *E. coli* BL21(DE3) host strain by the addition of 0.1 mM isopropyl-b-D-thiogalactoside and 5 h postinduction time. Subsequently, solubilization and purification of his-tagged AqpZ were carried out.

Keywords: AquaporinZ (AqpZ); AqpZ proteoliposome; Biomimetic membrane; Mass production

1. Introduction

Seawater desalination processes are the most feasible technologies to solve the water shortage problems in many of the arid countries. Among various desalination technologies, membrane-based desalination is regarded as a more energy and cost-efficient technology. However, membrane-based desalination processes such as reverse osmosis (RO) require further improvement related to energy consumption for high pressure. Energy cost for high pressure typically accounts for over 50% of the total operation cost. Another pitfall of RO membrane is that toxic

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micropollutants such as *N*-Nitrosodimethylamine and perchlorate can exist in permeate water [1]. Currently, many researchers are interested in developing high-performance membrane for water treatment. It is believed that the development of a new generation of RO membrane could reduce the operating pressure and consume less energy, which make the process much more economical and popular.

Commercial membranes are mainly synthesized by polymer materials such as polyamide and polyester. Thanks to the breakthrough in nano and biotechnology, carbon nanotube (CNT) and aquaporin (Aqp) are drawing attention as candidates for new membrane materials [2]. Especially, AquaporinZ (AqpZ), a water channel protein from *Escherichia coli*, can only convey water molecules across the cell membrane while excluding other ions and solutes [3]. Following Peter Agre, the first finder of Aqp who received novel price in chemistry 2003, industrial applications of Aqp to cosmetic, drug delivery and water filtration have been initiated by many researchers [4]. In particular, Kumar et al. investigated polymer vesicles-incorporated AqpZ, resulting in large enhancement of water permeability up to 80 times higher than that of conventional RO process [5]. It is true that this research stimulates many scientists to attempt to fabricate biomimetic membrane based on the AqpZ protein. Currently, various designs for AqpZ biomimetic membrane have been suggested and evaluated in terms of permeate flux and rejection [6,7]. Biomimetic membrane-embedded AqpZ can be composed of two layers including the active layer and the support layer. It is important that the active layer with AqpZ continuously maintains structural integrity and functionality during the membrane fabrication process. Langmuir–Blodgett technique [8], multilayer adsorption [9], vesicle fusion [6,10–13] are mainly used to prepare the active layer of biomimetic membranes. Support layers function as the support material for active layer and usually consist of porous alumina [10,11] and polymeric membrane [12,13].

Although the application of these notable properties as water purification membrane material is remarkably promising, mass production of membrane protein is extremely difficult and complicated due to the native hydrophobic properties of AqpZ. For mass production and easy purification of membrane protein, recombinant protein expression in *E. coli* system has been employed in several membrane proteins, including diacylglycerol kinase [14], glycerol facilitator [15] and CLC chloride channel homolog YadQ [16]. However, there are a few studies related to the mass production conditions of AqpZ. In this study, we established mass production conditions for AqpZ in terms of host strain, post-induction time, and inducer concentration. *E. coli* strains, which can largely produce AqpZ protein, were obtained through the recombinant DNA technology. Under the several expression conditions, purified AqpZ yields were compared via SDS-PAGE and western blot analysis.

2. Materials and methods

2.1. Experimental scheme

For AqpZ mass production, AqpZ was produced in accordance with general recombinant protein technologies (Fig. 1). It is largely divided into cloning, vector construction, transformation, expression, optimization of expression condition, solubilization and purification. Due to the inherent hydrophobic properties, solubilization of AqpZ was firstly carried out using nonionic detergents and purification of solubilized AqpZ was performed via affinity chromatography. Also, the purpose of further proteoliposome characterization was to evaluate the functionality of AqpZ.

2.2. Cloning, vector construction and transformation

E. coli DH5α strain was used for cloning of the aqpZ genes. Genomic DNA of *E. coli* DH5α strain was

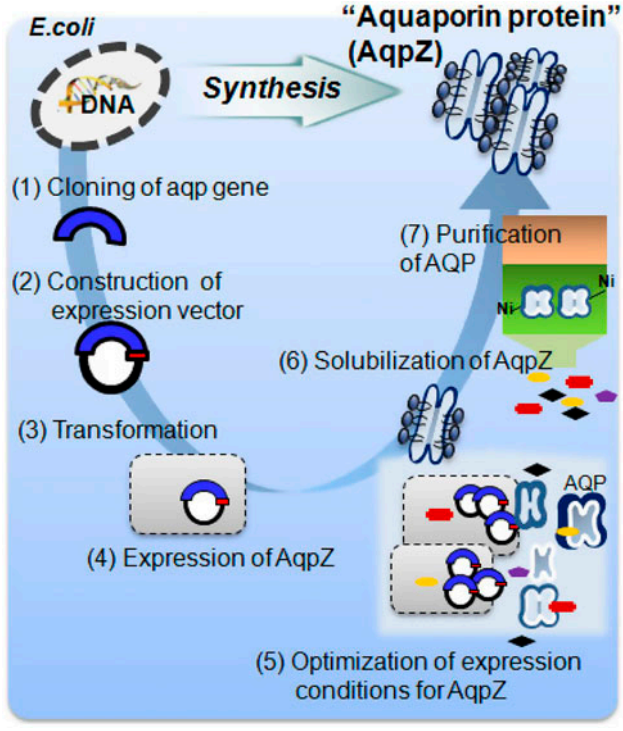

Fig. 1. Overall scheme for mass production of AqpZ.

extracted using AccuPrep Genomic DNA Extraction kit (Bioneer, Korea) and the extracted genomic DNA was amplified by polymerase chain reaction (PCR). According to Lian (2008), two primers, AqpZ-F (5′-TCCATATGTTCCGCAAATTAGCAGCTGAATGTT-3′) and AqpZ-R (5′-GCGTCGACTTATTAATCACGCTT TTCCAGC-3′), and PCR premix (Bioneer, Korea) were used for PCR. The thermal cycling conditions consisted of an initial denaturation step at 95℃ for 10 min followed by 40 cycles at 95℃ for 30 s, 55℃ for 30 s and 72℃ for 1 min with a final extension step at 72℃ for 10 min [17]. The size of the amplified DNA fragment was analyzed by means of gel electrophoresis apparatus (Advance, Japan) and the PCR product purification was performed using AccuPre™ PCR purification kit (Bioneer, Korea). For large-scale production and easy purification of AqpZ, pET-28a(+) vector (Novagen, USA) was utilized. Amplified aqpZ gene and pET-28a(+) vector were digested with two restriction enzymes, Sal I and Nde I (New England Biolabs, USA) and ligated by T4 ligase (New England Biolabs, USA) to construct recombinant AqpZ expression vector. Transformation was carried out using *E. coli* HIT Competent Cell™-21 (RBC Bioscience, Taiwan) and the recombinant clones were selected via PCR and DNA sequencing. Finally, selected recombinant clones from the two analysis methods were only used for further experiments and cultured in Luria-Bertani (LB) broth containing 50 μg/mL kanamycin.

2.3. Expression of AqpZ

In order to confirm the expression of AqpZ, a single colony of recombinant *E. coli* including AqpZ expression vector was cultured in LB broth containing 50 μg/mL kanamycin. Induction conditions of AqpZ were addition of 1 mM isopropyl-b-D-thiogalactoside (IPTG) and 2 h postinduction time at 37℃ [3]. Cells were harvested by centrifugation at 9,000 g for 20 min and then resuspended in 1/100 culture volume of lysis buffer (100 mM K_2HPO_4, 1 mM MgSO4, 1 mM Phenylmethylsulfonyl fluoride and 10 μg/mL Deoxyribonuclease I (pH 7.0)). Probe type sonicator was used for complete lysis of harvested cell and then the lysates were divided into soluble and insoluble fraction by centrifugation at 9,000 g for 5 min. SDS-PAGE and western blot were carried out to verify the expression of AqpZ. NuPAGE® Novex 10% Bis-Tris Gel (Invitrogen, USA) was used for SDS-PAGE. Transfer of protein bands for western blot was performed using iBlot transfer stack (Invitrogen, USA). Two antibodies, mouse penta-his monoclonal antibody (Novagen, USA) and HRP conjugated goat anti-mouse IgG (Santa Cruz, USA), were sequentially used to induce the reaction with his-tagged AqpZ for 12 h and 1 h, respectively. In the end, ECL western blot detection reagents (Amersham, USA) were used for detection of his-tagged AqpZ on the X-ray film.

2.4. Expression assay of AqpZ

For evaluating the various expression conditions for AqpZ, small-scale expression screens were carried out by means of altering the expression conditions such as host strain, inducer concentration, and post-induction time. In order to identify the effect of host strains, C41 (DE3) and C43(DE3) strains (Lucigen Corporation, USA), mutant strains of *E. coli* BL21(DE3) were additionally employed. YT medium (5 g yeast extract, 8 g tryptone, 5 g NaCl/L) was used as growth media for C41(DE3) and C43(DE3) strains. For optimum inducer concentration, no induced, 0.05, 0.1, 0.5, 1.0, 2.0 mM IPTG were investigated, respectively. 1, 2, 3, 4, 5, 7 h of post-induction time were also investigated, respectively. Other expression conditions including induction temperature and induction timing were identical with small-scale expression experiments and expression of AqpZ was also confirmed by western blot.

2.5. Solubilization and purification of AqpZ

Solubilization of expressed AqpZ was carried out using detergent. Differential centrifugation was primarily conducted to separate membrane fractions into the cell lysate. Supernatant recovered by low-speed centrifugation at 10,000*g* for 20 min was sequentially used for ultracentrifugation at 160,000*g* for 1 h. Pellet separated from the ultracentrifuge (Hitachi, Japan) was resuspended with solubilization buffer (1.5% dodecylmaltoside (DDM), 100 mM K_2HPO_4, 10% glycerol, 5 mM *β*-mercaptoethanol, 200 mM NaCl and pH 8.0) and was incubated on ice for 1 h. Finally, solubilized membrane fractions were achieved from the supernatant by centrifugation at 160,000 g for 45 min.

Ni-NTA agarose (Qiagen, Germany) was used for affinity chromatography to obtain solubilized AqpZ from solubilized membrane fractions. Ni-NTA agarose and solubilized membrane fractions were mixed in the ratio of 1:4 and poured into the separation column (Qiagen, Germany). Following 1 h incubation at 4℃, separation column was washed twice by 4-fold volume of washing buffer (1.5% DDM, 100 mM K_2HPO_4, 10% glycerol, 5 mM *β*-mercaptoethanol, 200 mM NaCl, 100 mM imidazole, pH 7.0). Purified AqpZ was recovered from the elution step using elution buffer (containing 1 M imidazole and other components were identical with washing buffer). Entire purification

steps were investigated by SDS-PAGE and western blot and purified AqpZ was quantified through Qubit fluorometer (Invitrogen, USA).

2.6. Preparation of AqpZ proteoliposome

AqpZ proteoliposome, a particular liposome containing AqpZ between the lipid bilayer, was fabricated for functional characterization of purified AqpZ. *E. coli* total lipid extract (Avanti Polar Lipids, USA) was used for preparation of liposome and 100-mM Mops-Na (pH 7.5), 1.25% (w/v) octylglucoside; purified AqpZ (final concentration 50–100 mg/mL) were sequentially added into the liposome solution to construct AqpZ proteoliposome. The final mixture was rapidly blended with 25 volume of assay buffer (50 mM MOPS, 150 mM *N*-methyl-D-glucamine, pH 7.5) to remove remaining detergents. In conclusion, AqpZ proteoliposome was recovered by centrifugation at 160,000 g for 45 min and resuspended into assay buffer. Transmission Electron Microscopy (TEM) (Joel, Japan) and electrophoretic light scattering apparatus (Ozuka Electric, Japan) were used to examine the morphology and dimension of AqpZ proteoliposome.

3. Results and discussion

3.1. Expression of his-tagged AqpZ

Fig. 2 demonstrates western blot result of AqpZ expression. AqpZ, expressed by recombinant technology, includes 6 histidine residues at the end of the *N*-terminal of AqpZ due to primer sequence. Histidine residues provide binding sites with specific antibodies used for western blot, resulting in showing of protein band on the X-ray film. Lines 1, 2–4 (Fig. 2) indicate no IPTG-induced *E. coli* cell and 1-mM IPTG-induced *E. coli* cell for 2 h, respectively. Lines 2, 3 and 4 (Fig. 2) represent total protein, soluble protein and insoluble protein, correspondingly. Through the comparison between lines 1 and 2 (Fig. 2), it is confirmed that IPTG is confirmed as inducer of AqpZ expression. Concerning the structure of AqpZ, it mainly exists in tetrameric structures (approximately 80 kDa) at native condition and is divided into monomers (approximately 23 kDa) under the denaturing conditions such as SDS solution [3].

However, apparent molecular weight of AqpZ are analyzed by 19, 51 kDa, respectively. Electrophoretic behaviors of hydrophobic proteins such as AqpZ are considerably affected by acrylamide concentration [3]. Apparent the molecular weight measured in this study correctly corresponds with the results from Borgnia et al. From the lines 3 and 4 in Fig. 2, AqpZ has been revealed as integral membrane protein (IMP),

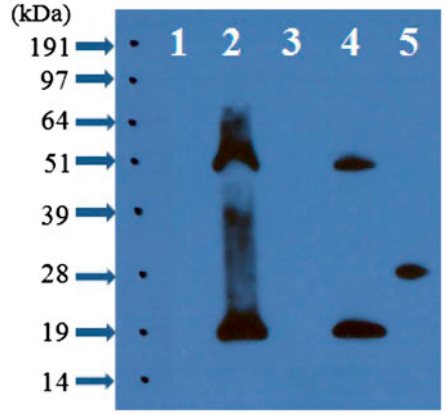

Fig. 2. Confirmation of AqpZ expression using western blot. Transformant *E. coli* BL21(DE3) carrying AqpZ expression vectors was lysed and its extract was loaded. (Line 1, no induced; Line 2–4, 1 mM IPTG induced for 2 h (Line 2: total protein, Line 3: soluble protein, Line 4: insoluble protein); Line 5: positive control (27 kDa)).

permanently attached to the cell membrane. In most cases, IMPs robustly combine with cell membranes due to strong hydrophobic interactions. Therefore, solubilization of AqpZ should be performed in advance for the purification of AqpZ.

3.2. Effects of expression conditions on AqpZ production

Currently, researchers have summarized several factors affecting the production of membrane protein including host strains, promoters, detergent, position and length of fusion protein [18]. In order to acquire optimum expression conditions for AqpZ, we perform several small-scale expression experiments at different conditions including host strain, inducer concentration and post-induction time.

Overexpression of membrane protein is typically toxic to the cell growth and causes plasmid instability. Consequently, many researchers mainly employ commercial mutant *E. coli* strains, C41(DE3) and C43 (DE3), to overcome detrimental effects of membrane protein expression [19]. However, BL21(DE3) strain is certainly estimated as the proper strain for expression of AqpZ as shown in Fig. 3. Considerable expression level variances among those strains are observed. In fact, accurate mechanism regarding toxicological change of protein overexpression in mutant *E. coli* strains is not even fully understood. In addition, molecular biological experiments should be required to elucidate the result clearly. The effects on inducer concentration and postinduction time are also evaluated by quantitative western blot analysis. Among several inducer concentrations (no induced, 0.05, 0.1,

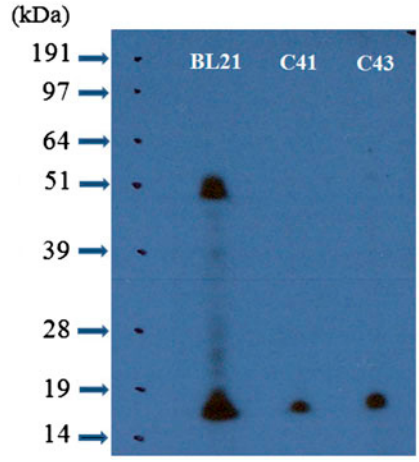

Fig. 3. Host-strain effect on expression of His-AqpZ. (Line 1, *E. coli* BL21(DE3); Line 2, *E. coli* C41(DE3); Line 3, *E. coli* C43(DE3)).

0.5, 1.0, 2.0 mM) and post-induction time (1, 2, 3, 4, 5, 7 h), it has been shown that induction with 0.1 mM IPTG and 5 h of post-induction time have been decided as the maximum expression levels, respectively (Fig. 4). Lysogenic strain, such as BL21(DE3), typically requires low inducer concentration ranging from 0.1 to 0.2 mM, since the activities of T7 RNA polymerase are partially maintained without inducer. Post-induction time would be the most variable factor among several induction conditions in this study. Even though post-induction time for AqpZ was formerly 2–3 h [3,20], current research results for post-induction time of AqpZ are 7–8 h [5] and 10 h [21], respectively. It was reported that several expression conditions including induction timing, tag protein length and position and induction temperature should be considered for the scale-up of membrane protein.

3.3. Production of solubilized AqpZ

Induced by optimum conditions and harvested *E. coli* cell includes AqpZ as well as protein, carbohydrate, DNA, lipid, etc. In order to purify AqpZ molecules separately, we adopted commercial Ni-NTA resin for affinity chromatography. Since histidine residues in recombinant protein molecules have high affinity with Ni-NTA resin, purification of recombinant protein is typically performed by single affinity chromatography. Nevertheless, purification of hydrophobic protein including AqpZ requires solubilization steps due to robust hydrophobic interactions. It was reported that detergent, kaotropic ions, ultrasonic and hydrolysis enzyme can be used to solubilize membrane protein from cell membrane [22]. In this study, 1.5% *n*-dodecyl *β*-D-maltoside (DDM) is used for solubilization of AqpZ. Comparing the lines 2 and 4 in Fig. 5, DDM is the critical component for solubilization of membrane protein. It is believed that DDM, possessing large micelle size, is the most suitable non-ionic detergent and mainly used for solubilization of lac permease and potassium channel protein, respectively [23,24].

Final purification of solubilized membrane fraction is carried out by affinity chromatography. Fig. 6 shows SDS-PAGE and western blot results of affinity chromatography for AqpZ. To monitor overall purification processes, every fraction sample including total solubilized membrane, flow through, washing and

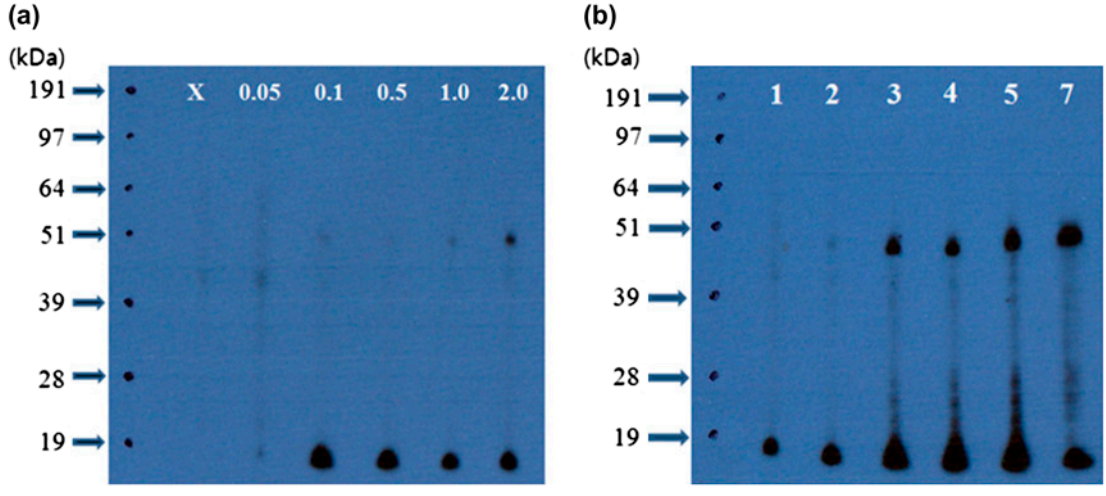

Fig. 4. Optimization of expression conditions for AqpZ. (a) Effect of IPTG concentration (Line 1, no induced; Line 2, 0.05 mM; Line 3, 0.1 mM; Line 4, 0.5 mM; Line 5, 1.0 mM; Line 6, 2.0 mM) (b) Effect of post-induction time (Line 1, 1 h induced; Line 2, 2 h; Line 3, 3 h; Line 4, 4 h; Line 5, 5 h; Line 6, 7 h).

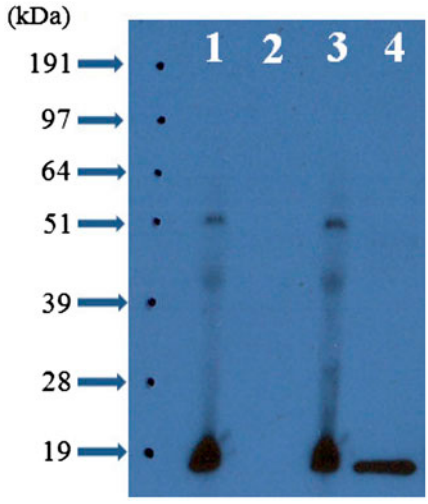

Fig. 5. Solubilization of AqpZ (Line 1 and 3, total protein; Line 2, solubilized without DDM; Line 4, solubilized with DDM).

elution fraction were loaded into SDS-PAGE and western blot analysis. To be specific, line 1 represents solubilized membrane fraction containing AqpZ and line 2 is the flow through sample, with no affinity with Ni-NTA resin. Lines 3 and 4 represent flow-through samples from washing and lines 5 and 6 represent flow-through samples from elution, respectively. Through SDS-PAGE and western blot analysis, it was observed that solubilized AqpZ containing histidine residues completely combines with Ni-NTA resin and is recovered from only elution fraction not washing fraction. Since a few recovery losses of solubilized AqpZ fairly occur during purification, supplementary researches related to enhance the efficiency of purification are required. In this study, final yield of solubilized AqpZ was 12.2 mg/L, relatively larger than 2.5 [3] and 9.05 mg/L [19]. It was reported that approximately 10 mg of AqpZ, is enough to utilize for further researches such as reconstitution into other structures [25]. Until now, the highest yield for AqpZ was approximately 200 mg/L when maltose binding protein (MBP), 42.5 kDa of monomer, is applied to the fusion protein [21]. However, MBP is excluded from this study since MBP can adversely affect the three-dimensional structure of AqpZ.

3.4. Characterization of AqpZ proteoliposome

Oocyte, mammalian cell swelling assay and proteoliposome assay are usually utilized in many research areas for functional assay of membrane protein. Proteoliposome assay, mainly used in channel gating mechanism research, can provide higher time resolution due to no interaction with other proteins [26]. Fig. 7 indicates TEM image of AqpZ proteoliposome deposited onto a 200-mesh carbon-coated copper grid. AqpZ proteoliposome represents a relatively spherical structure and has 194.0 ± 58.9 nm of dimension.

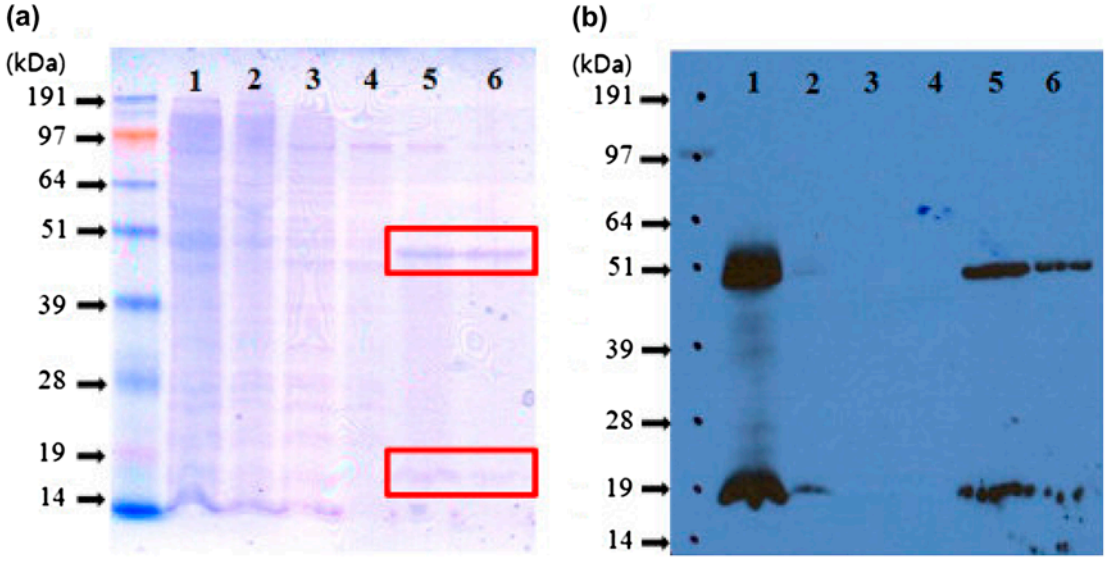

Fig. 6. Purification of AqpZ in *E. coli* cell homogenate using Ni-NTA agarose (a) SDS-PAGE (b) western blot (Line 1, total AqpZ solubilized with DDM; Line 2, Ni-NTA agarose column flow-through sample after applying *E. coli* cell homogenate; Line 3–4, flow-through sample from washing; Line 5–6, flow-through sample from elution). Square boxes indicate the position of purified AqpZ.

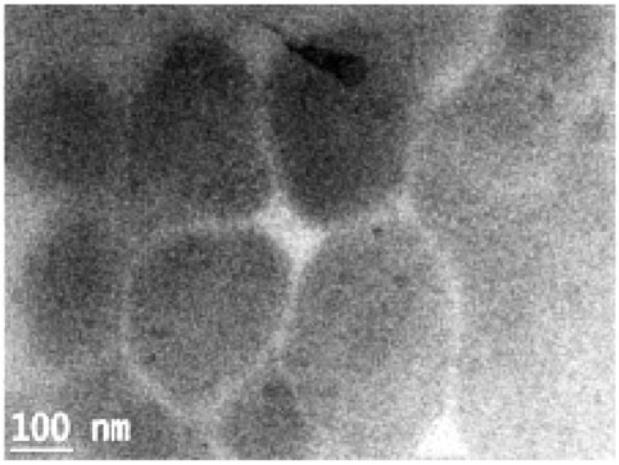

Fig. 7. TEM image of AqpZ proteoliposome.

4. Conclusion

In this study, mass production of solubilized AqpZ has been investigated for developing new membrane with higher flux and lower energy consumption. It is expected that various designed biomimetic membranes would be developed by using mass-produced AqpZ protein under optimized conditions. Robust AqpZ biomimetic membrane is expected to overcome the drawback of conventional membrane used in water treatment. Some conclusions made from this study are as follows.

(1) Recombinant AqpZ expression and purification systems have been established. In this study, final yield of solubilized AqpZ is 12.2 mg/L. In addition, further studies for obtaining higher yield of solubilized AqpZ are required.
(2) Optimum expression conditions are determined as BL21(DE3), addition of 0.1 mM IPTG and 5 h post-induction time. Other expression conditions such as fusion protein, expression temperature and induction timing are needed to be established for optimum expression of AqpZ.

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