Rapid separation of bacteriorhodopsin using a laminar-flow extraction system in a microfluidic device

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(Received 13 November 2009; accepted 4 January 2010; published online 27 January 2010)

A protein separation technology using the microfluidic device was developed for the more rapid and effective analysis of target protein. This microfluidic separation system was carried out using the aqueous two-phase system (ATPS) and the ionic liquid two-phase system (ILTPS) for purification method of the protein sample, and the three-flow desalting system was used for the removal of salts from the sucrose-rich sample. Partitioning of the protein sample was observed in ATPS or ILTPS with the various pHs. The microdialysis system was applied to remove small molecules, such as sucrose and salts in the microfluidic channel with the different flow rates of buffer phase. A complex purification method, which combines microdialysis and ATPS or ILTPS, was carried out for the effective purification of bacteriorhodopsin (BR) from the purple membrane of *Halobacterium salinarium*, which was then analyzed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight. Furthermore, we were able to make a stable three-phase flow controlling the flow rate in the microfluidic channel. Our complex purification methods were successful in purifying and recovering the BR to its required value. © *2010 American Institute of Physics.* [doi:10.1063/1.3298608]

I. INTRODUCTION

Bacteriorhodopsin (BR) is a light-sensitive protein found in the purple membrane (PM) of *Halobacterium salinarium* living in saline environments and is currently considered as a promising candidate material in the design of a molecular electron device and optical computers.[1,2] The effective biological production of BR has been investigated for such an important photophysical material.[3] A large quantity of BR is in demand for research and development, but only a small amount is available due to the difficulty in purification of the protein.[4]

The purification process of BR from cells requires multiple stages, such as harvesting, disruption of cells, protein concentration, high purity polishing of final products, and removal of salts or impurities.[5,6] Due to the number of stages required for purification, the yield of a protein present in small quantities decreases even further and so a new and effective purification process is required. For the effective purification of BR from cells, several conventional methods, such as aqueous two-phase system (ATPS), precipitation, ultracentrifugation, and gel permeation chroma-

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tography, have been reported.[5–7] Among them, purification by density gradient or differential ultracentrifugation has been the most popular method for production of high purified BR.[7] The differential ultracentrifugation was carried out at 100 000 × g and 15 °C creating a density gradient in the 40% (w/v) sucrose solution.[6] After pretreatment by ultracentrifugation, ultrafiltration and dialysis as polishing steps are carried out for the removal of excess sucrose and contaminated protein. However, since the process is time consuming and needs to be repeated several times, the purification of BR is very costly and a cumbersome procedure.[5–7]

To overcome these problems of the bulk purification system, the concept of microfluidic device was applied for fast and high purification of BR. Additionally, the microfluidic devices have been utilized for the separation of protein in various miniaturized analysis systems, also called micrototal analysis system.[8] These miniaturized analysis devices provide many advantages in chemical and analytical fields. For instance, the short molecular diffusion distance in a miniaturized device promotes diffusion chemical reactions and the large specific interface and interfacial area and small heat capacity allows for the facile and rapid control of reaction temperature.[9–12] With these benefits, ATPS and the ionic liquid two-phase system (ILTPS) using the microfluidic device[13,14] were carried out for the high purification of pretreated BR, purified by differential ultracentrifugation bulk system. Then, the microdialysis method was applied to remove excessive sucrose and salts. In the purification of the larger biomolecules, ATPS is well known for its biocompatibility, extreme partitioning of biomolecules including proteins, DNA, and others.[13,15] Among various ATPS, polyethylene glycol (PEG)-potassium phosphate system was selected due to the ease of pH control without addition of other salts. In addition, ILTPS was applied to employ ionic liquid instead of PEG phase because ionic liquid effectively removes hydrophobic protein and lipid contaminants as a biocompatible organic solution and is water immiscible.[14] Also, BR in PM could be prepared by sucrose-containing gradient step and then removed the residual sucrose from the PM.[16]

Recently, several research groups investigated the rapid analysis and separation of proteins in a microfluidic device.[17–20] For an example, Schilling *et al.*[17] reported the simple microfluidic devices that exploit transverse diffusion across two or more parallel laminar-flow streams. As another approach, the five-flow microfluidic desalting system was developed in our previous work.[20] In order to purify the target protein from the cell lysate sample containing the rich concentration of urea, our approach is based on the diffusion with the difference in the concentrations from the sample phase to the both buffer phases and the affinity capturing of urea by the metal ions.

In this study, we present a new microfluidic protein separation technology for obtaining highly purified protein that combines the selective purification of two-phase extraction and the rapid separation of three-flow desalting system. This microfluidic separation system was carried out using ATPS and ILTPS as purification methods of the protein sample, and the three-flow desalting system for the removal of salts from a sucrose-rich sample. Partitioning of the protein sample was observed in ATPS or ILTPS with various pHs and flow rate of the buffer phase. The microdialysis system was applied to remove small molecules in the microfluidic channel. The integrated purification method, which combined ATPS or ILTPS and microdialysis, was carried out for the effective purification of BR from the PM of *H. salinarium*. In addition, we were able to maintain a stable three-phase flow by controlling the flow rate in the microfluidic channel. Our complex-integrated purification methods successfully achieved the purification of BR of high purity and established the basis on the purification of the BR to its required value in the microfluidic system.

II. MATERIALS AND METHODS

A. Strain and chemicals

A saline bacterium *H. salinarium* R1 (ATCC 29341) was grown at 37 °C in Hb medium,[4,21] which contains the following (per liter): 250 g NaCl, 30 g $MgCl_2 \cdot 6H_2O$, 35 g $MgSO_4 \cdot 7H_2O$, 7 g KCl, and 0.2 g $CaCl_2 \cdot 2H_2O$, (Junsei Chemical., Tokyo, Japan). Hexafluorophosphate (1-n-hexyl-3-methylimidazolium) ionic liquid was supplied by Fluka. Si (100) wafer was obtained from LG

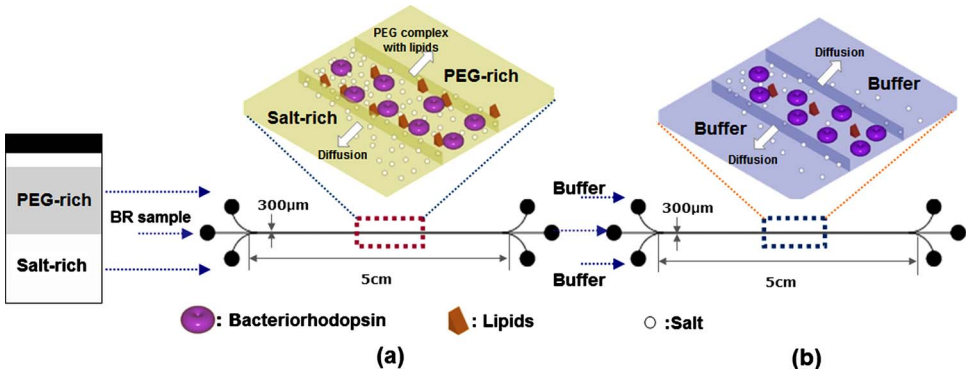

FIG. 1. Schematic of a complex microfluidic purification system by combining two different types of the separation process (two-phase extraction process and three-flow desalting system). (a) Two-phase extraction process (ATPS and ILTPS) was performed to purify BR from the cell lysate sample. PEG-rich (or ionic liquid phase) and water-rich phases were injected through both sides inlet ports by pressure-driven flow. Subsequently, sample solution was introduced into the middle inlet channel. Under the formation of the laminar-flow extraction system, impurities diffuse away from the cell lysate stream depending on the difference in hydrophobicity and biomolecular interactions. (b) Three-flow desalting dialysis was applied for the removal of contaminated proteins and excessive sucrose after fractionation of the sample stream from the laminar-flow extraction process.

Siltron (Korea) and negative photoresist, SU-8, was obtained from MicroChem. Polydimethylsiloxane (PDMS) (Sylgard 184) was purchased from Dow Corning. Other chemicals and reagents were used of an analytical grade.

B. Preparation of purple membrane

The recovery of PM was performed through two steps: The first step being cell lysis and the second step being the breakdown of DNA. Cells were harvested by centrifugation (8000 rpm, 30 min), washed twice in basal medium, the pellet resuspended in water purified with deoxyribonuclease (1 mg/100 ml, Sigma-Aldrich, St. Louis, MO), and stirred for 6 h at room temperature. The concentrated sample was then placed on a sucrose gradient from 20% to 46% sucrose (w/v), and ultracentrifuged using a swinging bucket rotor for 18 h at 20 000 rpm at 4 °C. The PM residing in the 40% (w/v) sucrose layer was collected and was used for further purification of BR in the microfluidic system.

C. Purification of BR using the microfluidic device

Microfluidic device was fabricated using the usual soft lithography and replica molding methods, and the material for the replica molding was PDMS. PDMS is highly transparent and thus suitable for detection.[22] Figure 1 shows the layout and dimensions of the microchannel for a three-phase flow and schematic of purification of BR in the microfluidic channel. The microchannel is approximately 5 cm in length, 300 μm in width, and 50 μm in depth for the three-phase flow microfluidic device. ATPS was made by mixing PEG 8000 (Sigma-Aldrich) and potassium phosphate solution (8:11, w/w). Stock solution of 24 wt % PEG 8000 and 24 wt % potassium phosphate solutions were prepared in de-ionized water. Potassium phosphate solutions were prepared with different molar ratios of dibasic:monobasic, resulting in pHs of 6.0–8.0. For chip experiment, the bottom and top phases were carefully separated using a syringe after the partition in aqueous two phases. As can be seen in Fig. 1(a), both sides of the inlet channel were injected as PEG-rich phase and water-rich phase after the settlement of ATPS, respectively. Controlling molar ratio of KH_2PO_4 and K_2HPO_4 could make changes from pH 6.0 to 8.0. The middle phase of inlet channel was introduced to the pretreated BR sample by differential ultracentrifugation. In the case of ILTPS, one side phase of inlet channel was applied to the ionic liquid instead of PEG-rich phase. After employing ATPS and ILTPS, the sample stream of BR was fractionated, and then delivered to the microdesalting channel for the removal of sucrose and contaminated proteins [see

Fig. 1(b)]. Both sides of inlet channel were injected as 20 mM Tri-HCl buffer solution (pH 6.0–8.0). The flow rate of the sample phase kept 1.5 ml/h, and that of both side phases was varied to 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 ml/h using syringe pumps (KDS200, KD Scientific). Flow rates were controlled independently. Formation of the three-phase flow was observed with an optical microscope (Olympus SZX12, Tokyo, Japan).

D. Analytical method

The concentration of BR was determined by the following equation: $\mathrm{BR(g/l)}=26\,500\times(A^{0}_{568}-A^{24}_{568})/63\,000$.[4,7] The molecular weight of BR is approximately 27 kDa and the molar extinction coefficient is 63 000/M cm. The absorbance at 568 nm (A_{568}) was first measured in the dark (A^{0}_{568}). The mixture was exposed to light for 24 h to remove retinal from PM, and again the A_{568} was measured (A^{24}_{568}). In native membrane the retinal protein complex has an absorbance maximum at 568 nm. Therefore, the purity of BR was expressed as follows: $\mathrm{purity(P)}=1/(A_{280}/A_{568})$. A_{280} and A_{568} are the absorbances at 280 and 568 nm, respectively.[4,7]

The concentration of sucrose was determined by high performance liquid chromatography with an ion exchange column (Aminex HPX-87H, 300×7.8 mm^2, Bio-Rad) at 50 °C using 5 mM H_2SO_4 aqueous solution as a mobile phase. The flow rate of the mobile phase was 0.6 ml/min and the absorbance was measured by an UV/visible detector (Waters 2487). The analysis of protein was carried out by a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Axima CFR V2.1.0, Shimadzu, Kyoto, Japan). 2,5-dihydroxybenzoic acid (DHB) was used to record a positive ion spectrum. The purified proteins were analyzed by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated in 15% acrylamide gel and visualized by Coomassie brilliant blue staining. Molecular sizes of the model proteins were analyzed by comparison with an SDS-PAGE standard marker (Intron, Seongnam, Korea).

III. RESULTS AND DISCUSSION

A. Purification of BR using ATPE in the microfluidic device

To purify BR from the pretreated sample containing impurities, such as lipids and excess sucrose, experiments were conducted using ATPS, ILTPS, and dialysis (see Fig. 1). The pretreated sample of PM is made up of 25% lipids, of which 60% are phospholipids and 30% are glycolipids, with a lipid to BR ratio of about 10:1. BR is the only protein in the PM of *H. halobium* (also called *H. salinarium*) and the lipid and hydrophobic proteins exist as the residue of components.[6,23,24] In order to apply ATPS in the microfluidic device, top and bottom phases after settlement of PEG and potassium phosphate solution were separated and then injected into the inlet channel. Protein extraction in ATPS has been shown that impurities (mainly consisted of several lipids) are transported by a difference in hydrophobicity and interactions of biomolecules, such as van der Waals interaction, hydrogen bond, and ionic interactions with the surrounding phase.[25]

In a sequential desalting approach [see Fig. 1(b)], small molecules—such as sucrose and salts—were removed more rapidly than the large proteins from the sample phase to both adjacent phases. Figure 2 shows the three-phase (PEG-rich phase/BR sample/salt-rich phase) flow in a 5 cm long microchannel. The three-phase flow and the clear liquid-liquid interface were formed over the entire microchannel. The three-phase flow formed a laminar flow, where the Reynolds number of the three-phase flow was less than 1. It is related to the fact that molecular diffusion governs the mass transfer in the microfluidic device, where the turbulence flow hardly occurred.

To characterize the two-phase extraction technique, experiments were conducted using ATPS. Figure 3 shows the recovery rate and purity of BR with increasing pH. The pH of ATPS was adjusted to controlling the molar ratio of KH_2PO_4 and K_2HPO_4 based on the ratio of 8:11 (PEG and potassium phosphate, w/w). The recovery rate of BR gradually increased as pH increased (pH 6.0–7.0), and reached a constant rate at pH values larger than 7.0. In general, BR is stable in the range of pH 6.5–7.5, but begins to aggregate at about pH 8.0. It is supposed that the loss of BR decreased with increasing pH due to the formation of aggregated BR because it is difficult for the

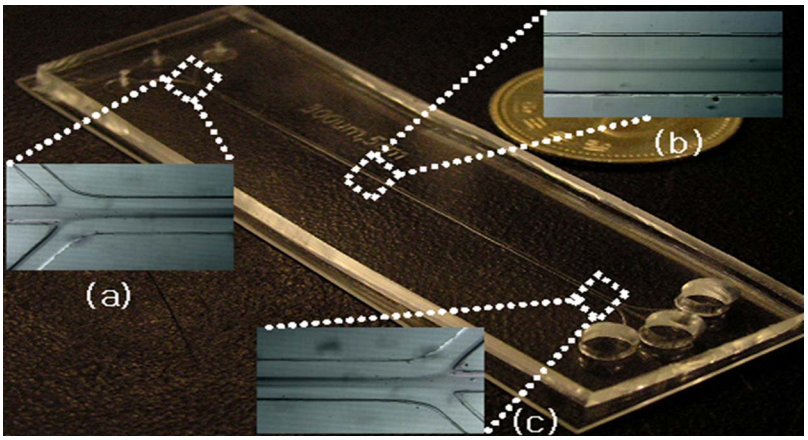

FIG. 2. Photograph of microfluidic device and the formation of the stable three-phase stream in the microchannel. (a) Center region near the inlets of the microchannel, (b) middle area of the microchannel, and (c) center region near the outlets of the microchannel. The flow rates of the PEG-rich and the salt-rich phases were 0.2 and 3.0 ml/h, respectively, and that of the sample phase kept at 0.7 ml/h.

larger molecule to diffuse from the middle phase to adjacent phases. As expected, the purity of BR, based on the reference purity of BR (0.476), increased with the decreasing of pH corresponding to the recovery rate results described above.

To examine the effectiveness of the ATPS, the continuous cycles of separation were carried out described in Table I, which shows the results of recovery rate and purity with successive batch processes of ATPS in the microfluidic device. The recovery rate of BR decreased to 90.23%, 84.54%, and 80.73% after each batch, respectively. In addition, the purity of BR increased sharply to 0.485 after one batch ($P=0.435$), but it reached a constant level at the third successive batch processes using ATPS. Based on these results, we determined that the optimum condition for the effective purification using ATPS is at pH 7.0 and two batch processes. To improve the reproducibility of BR purification from several devices, all devices used in this study were verified by the formation of laminar flow once introducing the sample solutions with syringe pumps at the same flow rate condition. In addition, the recovery and purity of BR were analyzed through the results obtained by using three different devices.

B. Purification of BR using ILTPS in the microfluidic device

As another approach, ionic liquid was introduced to the inlet channel instead of a PEG-rich phase, as done using ATPS. As can be seen in Fig. 3, the recovery rate and purity profiles for BR were very similar to those of ATPS. However, the value of recovery rate decreased sharply to less than 85% with decreasing pH in comparison with ATPS. As pH increased, the formation of more aggregated BR was inclined to reduce the loss of protein. The purity of BR achieved a value of above 0.476 over the value of pH 7.0.

It is supposed that ILTPS is a more effective purification method for higher purity of BR than ATPS because pretreated BR was composed of most hydrophobic proteins and lipids. Hydrophobic contaminated biomolecules were effectively removed from the sample phase to the ILTPS due to the larger hydrophobicity of the ionic liquid phase in comparison with PEG-rich phase. However, the value of recovery for BR using ILTPS was less than that of ATPS because some of the BR-lipid complex was removed together with the lipid from the sample phase to the ionic phase. Thus, it is concluded that ILTPS carried out at pH 7.0 gives the optimum recovery and purity for BR.

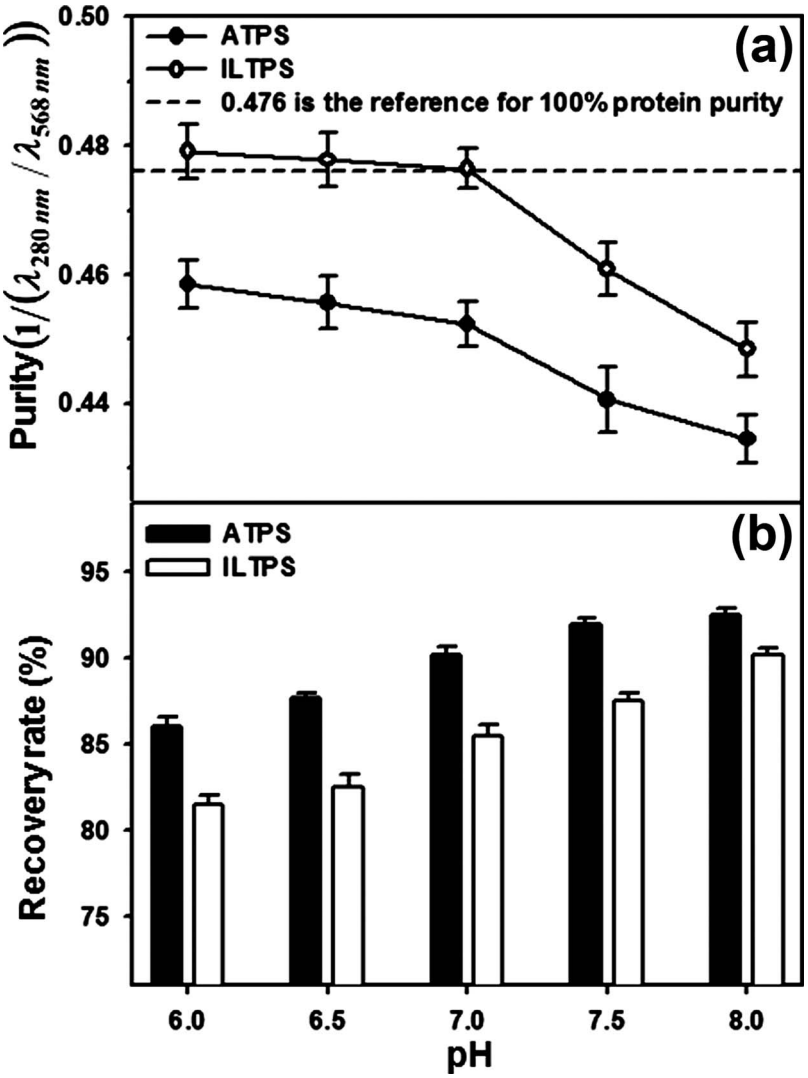

FIG. 3. Effect of pH on (a) the recovery rate and (b) the purity of BR using two kinds of purification techniques (ATPS and ILTPS) in the microfluidic device.

C. Dialysis for removal of sucrose in the microfluidic device

For removing of excessive sucrose from a pretreated sample containing 40% sucrose by differential ultracentrifugation, the concept of dialysis was applied to the microfluidic channel. Figure 1(b) shows the schematic of dialysis for the removal of small molecules, such as sucrose and salts in the microfluidic channel. The three-phase flow was formed as the stable laminar flow. Neither the both boundary flow nor the sample flow invaded the adjacent flow throughout the

TABLE I. Recovery and purity of BR, and the removal of sucrose with different pHs in the microdialysis system.

No. of successive ATPS	$1/(\lambda_{280\ nm}/\lambda_{568\ nm})$	Purity (%)	Yield (%)	Removal of sucrose (%)
1	0.435	94.76	90.23	17.42
2	0.485	100	84.54	31.08
3	0.488	100	80.73	41.26

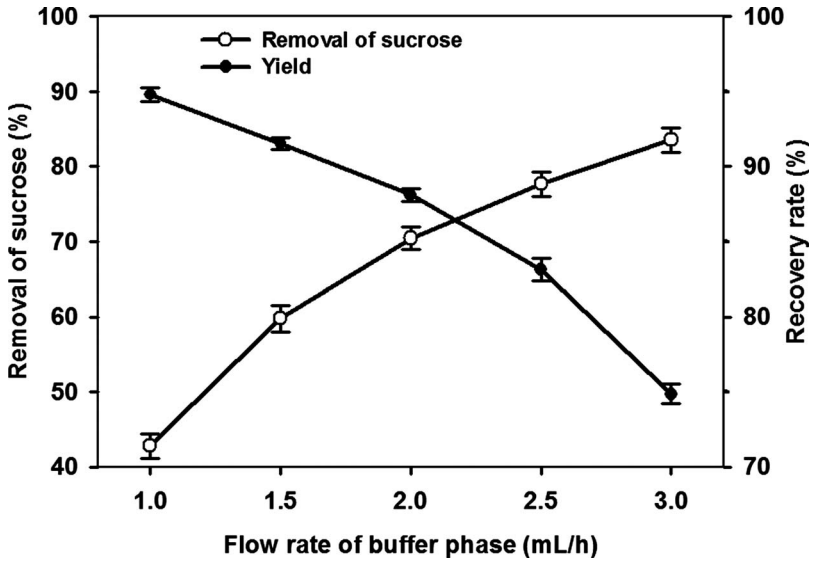

FIG. 4. Effect of flow rate of both buffer phases on the recovery rate and purity of BR in the microdialysis system.

microchannel and each phase was collected from each outlet channel. It is related to the fact that the mass transfer in the microfluidic device, where the turbulence flow cannot occur, is under the control of diffusion, not convection. This means that the interfacial area and the thickness of the sample phase play a significant role for the more rapid and effective removal of small molecules in the dialysis system in the microfluidic channel. Figure 4 shows the recovery rate and the purity profile of BR with different flow rates of the buffer phase in the microdialysis system. It is supposed that the microfluidic channel, which is able to form a thickness of the sample phase with controlling the flow rate of the buffer phase, would enlarge the contact area and contact time. Therefore, the flow rate for the buffer phase was increased and the purity of BR was increased. This could be explained by the fact that the smaller the molecules are, the more rapid the molecular diffusion is from the sample phase to both buffer phases. However, the recovery rate of BR decreased slightly with increasing the flow rate of the buffer phase. In addition, as the flow rate of the buffer phase was increased, the diffusion force in the sample phase was increased. It was interpreted that the larger difference in the concentration gradient had, the more small molecules were transported from the sample phase to both buffer phases. Therefore, for applying efficient microdialysis, the flow rate of 2.5 ml/h in the buffer phase was carried out.

Figure 5 shows the results of removal efficiency of sucrose and recovery rate of BR with different pHs in the microdialysis system. The recovery rate of BR increased with the increase in pH, but the removal efficiency of sucrose slightly decreased. It was previously mentioned that small molecules, such as sucrose and salts, were thought to be drawn with the proteinlike clusters of BR with sucrose surrounding it, while the more amount of BR protein diffused from sample phase to buffer phase at the lower range of pH. However, the reference purity of BR was not achieved using just the microdialysis system.

For a more efficient and higher purification of BR, microdialysis was carried out to remove excess sucrose after hydrophobic contaminating proteins were removed by using either ATPS or ILTPS. Table II shows the purification table using this method. In the case of ATPS and ILTPS, a higher purity of BR than the reference purity was achieved but was only able to remove 17.42% and 35.6% of the sucrose, respectively. Comparing ATPS and ILTPS, the purity and removal of sucrose using ILTPS obtained more excellent results than using ATPS, but the value of recovery rate using ILTPS was much lower than using ATPS. For the removal of sucrose and contaminated proteins, the integrated purification method, such as combined ATPS or ILTPS and microdialysis was applied in the microfluidic device. The purity of BR and removal rate of sucrose using the

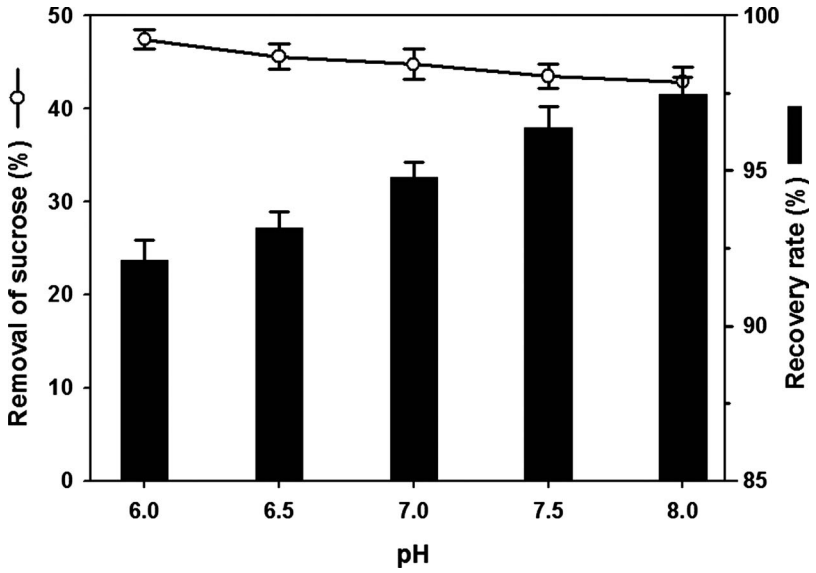

FIG. 5. Effect of pH on the recovery rate of BR and the removal efficiency of sucrose using the three-flow dialysis system in the microfluidic device.

integrated purification method was higher than that in the single purification method, but the recovery of BR was lower. Additionally, the value of specific purity and purification fold increased through employing the integrated purification method.

To verify the characteristics of the purified BR proteins in the integrated microfluidic device, SDS-PAGE and MALDI-TOF analyses were performed. As shown in Fig. 6(a), the band intensity of each lane represents the twofold increase in BR protein. A purity of approximately 97% of BR was successfully obtained using this integrated purification method. Furthermore, the sample collected from the outlet port was analyzed by means of a MALDI-TOF mass spectrometry in a positive mode. Figure 6(b) shows the MALDI-TOF mass spectra of 50 μg/ml BR protein sample after purification from the integrated microfluidic device. MALDI-MS spectroscopy of the desalted sample shows a major signal at $m/z=26\,370-27\,100$. Thus, this integrated purification method successfully achieved the purity and recovery of BR to its required purity value. It is suggested that the microfluidic device is an appropriate purification tool for the rapid and high yield purification of protein and by combining various separation technologies, a higher purity, and recovery rate of protein is successfully achieved.

IV. CONCLUSIONS

This investigation showed the creation of a microfluidic device that utilizes protein purification technology and was successfully carried out for the high purity and recovery rate of BR. The

TABLE II. Purification profile related to each step.

Purification step	Recovery rate (%)	Removal of sucrose (%)	Total purity (P)	Specific purity (P/mg)	Purification fold
Pretreated sample	100	···	0.412	2.02	1.00
ATPS	90.23	17.4	0.435	2.35	1.16
ILTPS	84.32	35.6	0.493	2.85	1.41
ATPE+dialysis	78.92	65.3	0.503	3.14	1.55
ILTPS+dialysis	72.06	75.5	0.508	3.45	1.71

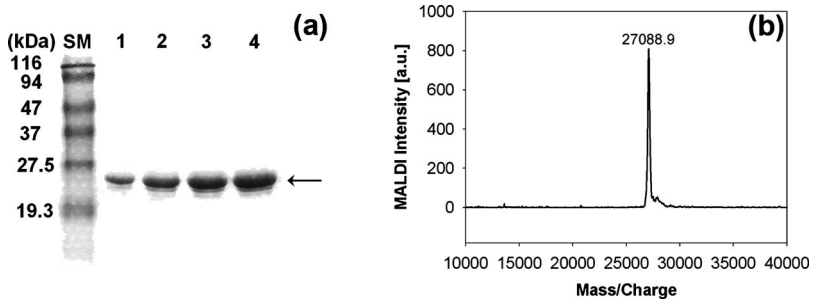

FIG. 6. Characterization of purified BR proteins by (a) SDS-PAGE. SM, molecular size marker; lane 1, 0.25 μg BR; lane 2, 0.50 μg BR; lane 3, 0.75 μg BR; lane 4, 1.00 μg BR; black arrow, target BR. (b) MALDI-TOF analysis recorded with the DHB matrix from the 50 μg/ml BR sample purified through the microfluidic device. The purified target BR was analyzed as a protein sample having a molecular weight of 27 088.9 Da.

microfluidic channel from pretreated PM was effectively achieved to form a stable flow phase and to control the thickness of sample phase with adjusting the flow rate. Furthermore, ATPS and ILTPS using microfluidic channel from pretreated PM were adopted as removal of hydrophobic contaminated protein and lipids. The purity of BR was successfully achieved to above the value of reference purity (P=0.476) employing ATPS and ILTPS from pretreated samples (P=0.412). For the more efficient purification of BR in the microfluidic device, a dialysis step was added for the removal of sucrose and salts after ATPS or ILTPS. By applying the on-chip purification system, we obtained higher purity and recovery rate of BR using two integrated purification systems (0.503% and 78.92% of ATPS+microdialysis; 0.508% and 72.06% of ILTPS+microdialysis). In general, the advantages of microfluidic separation system compared to conventional techniques are that they provide a means to obtain target purification samples with a small amount of sample, rapid purification times, and improved yield. In bulk separation studies, the purity of BR was obtained in the range of 60%–99% purity.[26–29] Therefore, we achieved similar or higher purity and yield compared to conventional BR separation techniques. This result demonstrates that the microfluidic device designed to implement protein purification methods can be successfully applied for the production of the high purified BR.

ACKNOWLEDGMENTS

This work was supported in part by the IT Leading R&D Support Project from the Ministry of Knowledge Economy through KEIT and WCU (World Class University) program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (Grant No. R32-2008-000-10142-0).

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