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Separation of Membrane Proteins of *H. halobium* by Gel Permeation High Performance Liquid Chromatography

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High performance liquid chromatography (HPLC) separation of the SDS-solubilized membrane proteins of *Halobacterium halobium* S9 was investigated with a gel permeation column, TSK G3000 or 4000 SW. When sodium phosphate buffer (pH 7.0) containing 0.1% SDS was used for elution, both the elution volume and the elution profile of protein peaks were highly dependent on the buffer concentration. On increasing the buffer concentration, the elution volume for each increased and the resolution was also improved. Protein was recovered quantitatively from the column in the buffer concentration range between 0.01 to 0.2 M.

In contrast to the phosphate buffer system, the elution profile of the membrane protein was almost independent of the salt concentration when Tris-HCl (pH 7.0) buffer containing 0.1% SDS and various amounts of NaCl was used for elution. The best separation conditions finally determined for the membrane proteins of *H. halobium* using SW columns were as follows: 0.2 M Tris-HCl (pH 7.0) containing 0.1 M NaCl and 0.1% SDS as an elution buffer system, at a flow rate of 0.6 ml per min.

Under the above elution conditions, nine major protein peaks could be determined in the SDS-solubilized plasma membrane preparation of *H. halobium* S9 within 40 min when the absorbance was recorded at 280 nm. The resolution obtained is comparable to that of SDS-PAGE.

Keywords——*H. halobium*; membrane protein; HPLC; G 3000 SW; SDS-solubilized membrane

Introduction

Halobacterium halobium grows in high salt environments and carries out a different type of photosynthesis from that in green plants.[1,2] In order to convert the energy absorbed by the photoreceptors into chemical energy, the bacteria utilizes various ion transport systems such as an Na^+/H^+ antiporter and an amino acid synporter[3] in the plasma membrane. The electrochemical potential created by the primary photoenergy transducers such as bacteriorhodopsin[4] and halorhodopsin[5] are used by these ion transporters.

In order to characterize these transport proteins and their organization in the plasma membrane, a simple and precise analytical method for membrane protein is required. High performance liquid chromatography (HPLC) is an effective tool for the separation of biological substances, especially for the separation of soluble proteins based on their molecular weight in the presence of detergents.[6–8] The method has several advantages, such as rapid and simple operation, and easy quantitation, compared to SDS–PAGE. However, little work has been done on the separation of the membrane proteins by this method yet.

In the present work, conditions for the separation of the SDS-solubilized membrane protein of *H. halobium* were studied in detail and it was found that the method was useful for the characterization of the membrane proteins of this bacterial species.

Materials and Methods

H. halobium S9 was grown by the method described elsewhere.[9] Cells in their stationary growth phase were harvested by centrifuging 5 ml of the culture medium at $5000 \times g$ for 30 min and washed

twice with 4 M NaCl. The cell pellet was resuspended in 5 ml of distilled water, left to stand overnight after adding 300 units of DNase, then centrifuged at $100000 \times g$ for 60 min to recover the membrane fraction as a pellet. The pellet (approx. 1 mg protein) was solubilized at 60°C for 30 min after addition of 0.1 ml of 10% SDS. The solubilized membrane was diluted with 0.1 ml of the elution buffer, then subjected to a brief sonication for 1 min using a cell disrupter (Model 225 Heat System) in a water bath type probe. The solubilized membrane preparation was centrifuged at $5000 \times g$ for 10 min to remove insoluble contaminants before use.

HPLC separation was carried out using a Toyo Soda model 803 liquid chromatograph equipped with a precolumn (7.5×50 mm) and a TSK G3000 or 4000 SW column (7.5×600 mm), which were extensively washed with the elution buffer [0.2 M Tris-HCl (pH 7.0) containing 0.1 M NaCl and 0.1% SDS] before use. Vo and Vt of the column were determined using Blue dextran and distilled water, respectively.

One to 20 μl of the solubilized membrane preparation was injected from the bottom of the column and eluted at a speed of 0.6 ml/min with an appropriate buffer. The elution peaks were monitored by measurement of the absorbance at 280 nm using a ultraviolet (UV) monitor and recorded on a strip chart recorder. Approximately 30 μg of protein gave an appropriate peak height at 0.05 A full scale. SDS-solubilized standard protein mixture was also treated under the conditions used for membrane sample to calibrate the molecular weight of membrane components.

Protein was determined by the method of Lowry.[10] Protein standard Kit was purchased from Boehringer Mannheim., DNase from Sigma, and special-grade SDS for protein solubilization from Nakarai. All other reagents used were from Wako and were of special reagent grade.

Results

The elution behavior of the protein standards on a G 3000 SW column by Na phosphate buffer was essentially the same as reported previously.[7] When the buffer concentration was increased, the calibration curves shifted to higher volume and the exclusion limits slightly higher. Higher resolution was also attained at higher buffer concentration. When the solubilized membrane preparation was applied to the column under the same elution conditions as standard proteins, the elution profile was markedly dependent on the buffer concentration. Typical elution profiles at 0.02 and 0.1 M Na phosphate concentration are given in Fig. 2. Protein was recovered quantitatively in the buffer concentration range between 0.02 to 0.2 M.

On the other hand, when Tris-HCl buffer was used as the elution solvent, the elution profile was rather independent of the salt concentration in the buffer. In the presence of only 0.02M NaCl, a much higher exclusion limit was obtained with Tris buffer than with 0.2 M Na phosphate buffer. As expected from the elution behavior of the standard proteins in the Tris buffer system, no significant difference in the elution profile of the membrane proteins was observed between 0.02 to 0.2 M NaCl concentration. Typical elution profiles at 0.02 and 0.1 M NaCl are shown in Fig. 4. The best separation was attained when 0.1 M NaCl concentration was used. Under this condition, nine major protein peaks could be determined in the membrane preparation. The last peak eluted after peak IX appeared in the Vt of the column. This peak may be attributed to small molecules other than protein.

As shown in Fig. 4, the highest molecular weight protein (peak I) was almost in the exclusion limit of the G 3000 SW column, but the use of a G 4000 SW column, which has a

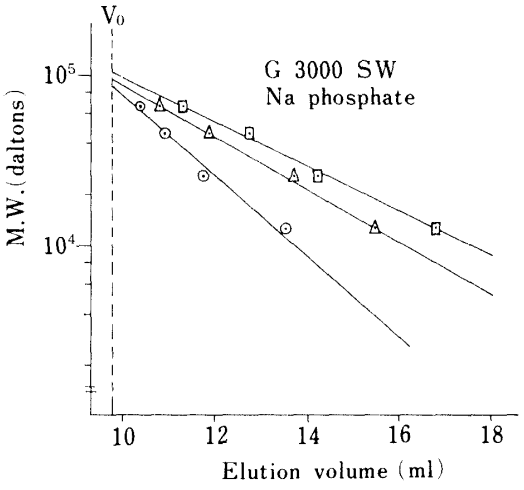

Fig. 1. Molecular Weight Calibration Curves for Na-phosphate Buffer System

Standard proteins[cyt. C (12.5 K), α-A-chymotrypsinogen (25 K), albumin (45 K) and BSA (68 K)] were solubilized with SDS and eluted with 0.02 M (⊙) 0.05 M (△) and 0.1 M (□) Na phosphate (pH 7.0) containing 0.1% SDS. The flow rate of the buffer was 0.6 ml/ min and the chart speed was 0.5 cm/min. Elution peaks were recorded at 280 nm.

higher exclusion limit, made it possible to further resolve the components in the molecular weight range above 100 K daltons (Fig. 5). On the other hand, G 3000 SW could resolve the molecular weight range around 40 to 80 K daltons much better than the G 4000 SW column.

The molecular weights of the protein peaks determined from the calibration curves for G 3000 SW and 4000 SW columns at different buffer concentrations were summarized in Table I.

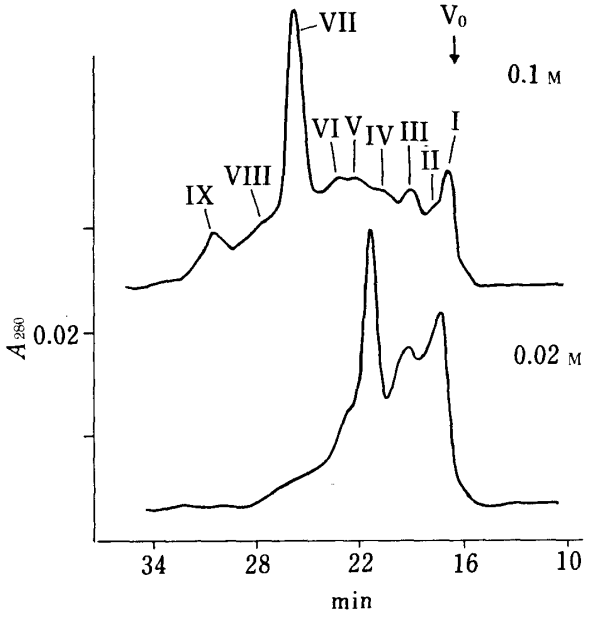

Fig. 2. Typical Elution Profile of the Membrane of *H. halobium* S9 from a G 3000 SW Column with Na Phosphate Buffer

SDS-solubilized plasma membrane preparation of *H. halobium* S9 (approx. 30 μg as protein) was applied. HPLC conditions were the same as in Fig. 1.

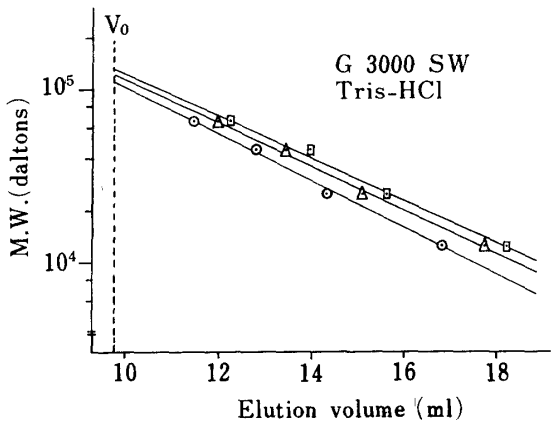

Fig. 3. Molecular Weight Calibration Curves for Tris-HCl System

Protein standard mixture was eluted with Tris-HCl (pH 7.0) containing various concentrations of NaCl [0.02 M (⊙), 0.1 M (△) and 0.2 M (⊡)] and 0.1% SDS. Other HPLC conditions were the same as in Fig. 1.

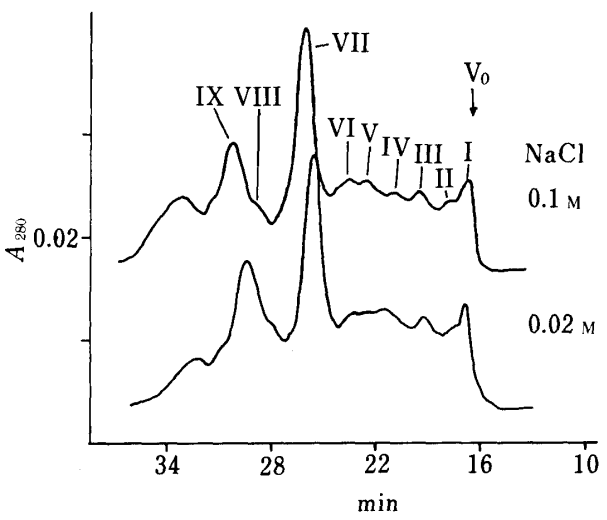

Fig. 4. Typical Elution Profile of the Membrane Proteins of *H. halobium* S9 from a G 3000 SW Column with Tris-HCl Buffer

Samples were the same as in Fig 2 and other HPLC conditions are the same as in Fig. 3.

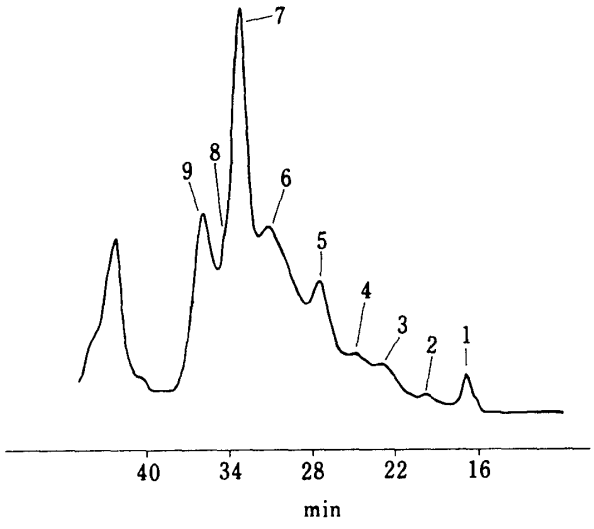

Fig. 5. Typical Elution Profile of the Membrane Proteins of *H. halobium* S9 from a G 4000 SW Column

The sample was the same as in Fig. 3. Elution was carried out with 0.2 M Tris-HCl (pH 7.0) containing 0.1 M NaCl and 0.1 % SDS. Other HPLC conditions were the same as in Fig. 3.

Table I. Apparent Molecular Weights of Membrane Proteins of *H. halobium* S 9 obtained from the Calibration Curves under Various HPLC Conditions

Peak No.	G 3000 SW column				Peak No.	G 4000 SW column
	Na phosphate (pH 7.0)		0.2 M Tris-HCl (pH. 7.0) NaCl			0.2 M Tris-HCl (pH 7.0) 0.1 M
	0.02 M	0.1 M	0.01 M	0.1 M		
I		98	125	120	1	1400
					2	598
					3	275
II	72	82	93	101	4	165
III		64	72	76		
IV		47	55	57	5	85.5
V	38.5	33.8	44	47		
VI		28.5	34.5	36.5	6	32.8
VII	20.5	17.9	21.8	23.0	7	20.0
VIII	13.1	12.0	14.3	15.0	8	14.2
IX	7.2	7.2	10.8	11.1	9	9.9
						(× 1000 daltons)

It is clear that the apparent molecular weights of membrane components varied depending on the buffer composition. Phosphate buffer gave a smaller molecular weight value than Tris buffer.

When the same membrane preparation was subjected to polyacrylamide slab gel electrophoresis in 0.1% SDS (12.5% gel), eight bands were detected as follows: 106, 90, 78, 54, 44, 37, 19 and 18 K daltons (not shown). These molecular weights are similar to the values obtained on the G 3000 SW column using Tris-HCl buffer. However, the background was still high in the higher molecular weight region after extended destaining treatment for several days, so that the peaks could only be detected by dual wavelength scanning (500—565 nm) of the gel. As a result, loss of the low molecular weight components such as bacteriorhodopsin occurred and the relative peak intensity in the low molecular component region decreased.

Discussion

SDS–PAGE has so far been used for the analysis of membrane proteins. The method is rather time-consuming and requires considerable skill, though it can produce quite a high resolution of protein components.

On the other hand, the present gel permeation HPLC procedure allowed us to separate soluble proteins at high speed and in the presence of detergent such as guanidine hydrochloride[8] or SDS.[6,7] The elution volumes of standard proteins were proved to correlate linearly to their known molecular weights. The method was applied to the membrane proteins of *H. halobium* and was found to work successfully with an SDS-containing buffer system.

Two different elution buffer systems, Na phosphate and Tris-HCl were tested for HPLC, and Tris buffer was found to give much better resolution than phosphate buffer. In the Tris-HCl system, the exclusion limit was comparatively higher in the presence of 0.02 M NaCl than that obtained with 0.1 M Na phosphate buffer. Changes in the flow rate of the buffer did not alter the resolution significantly.

The elution profile of the SDS-solubilized membrane preparation obtained with Tris-HCl buffer containing 0.1 M NaCl was almost identical to that obtained with 0.1 M Na phosphate

buffer, but slight differences were observed in the molecular weight range below 20 K daltons.

Salt concentration in the buffer could be increased to 0.6 M without any precipitation of SDS at room temperature when the SDS concentration was maintained at 0.1%.

Purified purple membrane, which contains bacteriorhodopsin as a single protein component, gave a single peak at an elution volume of around 15.5 ml in the Tris-HCl buffer system (*cf.* peak VII in Fig. 4 and peak 7 in Fig. 5) on the G 3000 SW column and the apparent molecular weight from the calibration plot was 22000, which is rather smaller than that obtained by another method.[11] The same molecular weight for bacteriorthodopsin has been obtained occasionally by SDS–PAGE.[12] Further, the molecular weight of the membrane components obtained from the calibration plots for different buffer systems and different salt concentrations were not always identical (Table I). This suggests that the method is quite convenient to separate and characterize the membrane components, but for the precise determination of the molecular weights of the components, another method is required. Similar uncertainty in the molecular weight calibration of a membrane protein has also been reported for Sepharose column chromatography,[13] where the calibration curve obtained for water soluble proteins is not strictly applicable as an indication of the size of detergent-solubilized membrane proteins.

In spite of the uncertainty in molecular weight determination, the HPLC method was proved to have a resolution of each membrane component comparable to that of SDS–PAGE, with a markedly shorter analysis time. It also offered quantitative recovery of each component, especially the lower molecular weight components. Thus this method should be useful to follow changes of a certain membrane component under various conditions, *e.g.*, during the growth stages of cells.[14]

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