# Purification of the Motor Protein Prestin from Chinese Hamster Ovary Cells Stably Expressing Prestin*

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## Abstract

Prestin is regarded as the motor protein of cochlear outer hair cells (OHCs). Due to the conformational change of prestin, OHCs are believed to contract and elongate, this OHC motility realizing the high sensitivity, wide dynamic range and sharp tuning of the auditory system of mammals. Since its identification in 2000, prestin has been intensively investigated. As a result, knowledge about the structure and function of prestin has been gradually accumulated by studies using prestin-expressing cells. Purification of prestin would allow further analysis, e.g., crystal structure analysis, to obtain knowledge about prestin at the molecular level. Recently, it has been reported that recombinant prestin was purified from Sf9 insect cells and that structural analysis was carried out by electron microscopy. In the present study, an attempt was made to purify prestin from another expression system, i.e., mammalian Chinese hamster ovary (CHO) cells stably transfected with gerbil prestin. First, since it is unclear which detergents are suitable for solubilization of prestin, the best detergent for solubilization was selected from among 8 kinds of detergent commonly used for membrane protein isolation. The optimum concentration of the detergent was also determined. As a result, it was clarified that 10 mM *n*-nonyl-*β*-D-thiomaltopyranoside efficiently solubilizes prestin. Next, using this detergent, purification of prestin by anti-FLAG affinity chromatography was performed, and $84 \pm 23$ μg of purified prestin was obtained from $2 \times 10^9$ 3×FLAG-tagged prestin-expressing CHO cells.

*Key words*: Outer Hair Cell, Motor Protein Prestin, Chinese Hamster Ovary Cell, Membrane Protein, Detergent, *n*-Nonyl-*β*-D-thiomaltopyranoside, Solubilization, Anti-FLAG Affinity Chromatography, Purification

## 1. Introduction

Outer hair cells (OHCs) in the mammalian organ of Corti elongate and contract in response to changes in their membrane potential, this OHC motility being generally known as electromotility[1]-[3]. This electromotility is believed to amplify the displacement of the vibration of the organ of Corti, thus realizing the high sensitivity, wide dynamic range and sharp tuning of the auditory system of mammals[4]. The OHC electromotility had been presumed to be driven by conformational changes of a motor protein expressed in the lateral plasma membrane of the cells, and in 2000, the gene of such a motor protein was identified and termed prestin[5]. Its amino acid sequence shows that it has a calculated molecular weight of 81.4 kDa and that it is a member of the solute carrier (SLC) 26A family.
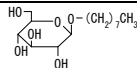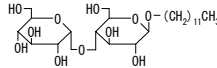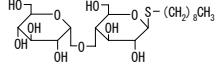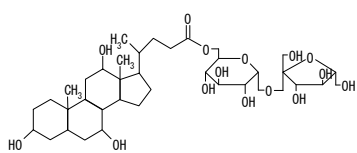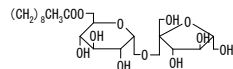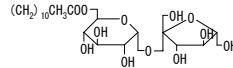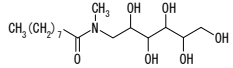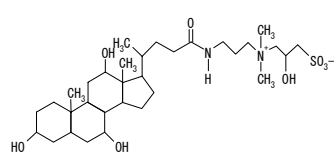
Since its identification, prestin has been intensively investigated due to its important role in mammalian hearing, and as a result, several of its features have been revealed. Prestin-transfected cells show electromotility and voltage-dependent charge movement, as do OHCs[5][6]. It has been reported that intracellular anions act as extrinsic voltage sensors, which bind to prestin and thus trigger its conformational change[7][8]. Measurements in prestin knockout mice have demonstrated that OHC electromotility is absent, resulting in significant hearing loss[9][10]. Mutational studies have suggested that prestin is a glycosylated protein[11] and is phosphorylated[12]. It has also been indicated that prestin exists as tetramers[13], which form particles with a diameter of 8-12 nm[14][15].

Although several features of prestin have been reported, further examination is required to reveal the molecular structure and mechanisms realizing its unique functions. Investigation using purified prestin, which would enable us to obtain signals from only prestin, is a promising approach to gain new information on prestin. Recently, it has been reported that recombinant prestin molecules were purified from Sf9 insect cells using a baculovirus expression system, and employing such molecules, it was revealed by electron microscopy that prestin is bullet-shaped[16]. However, although the baculovirus/Sf9 insect cell system is generally used as a high-expression system due to its well-organized and easy-to-use method, the post-translational modification pattern of prestin expressed in the Sf9 cells is considered to be different from that of native prestin. In fact, the glycosylation pattern of prestin in Sf9 cells and that in native prestin are found to differ from each other[11][16][17]. Since the glycosylation pattern is related to the protein stability, there is a possibility that the stability of prestin expressed in Sf9 cells is lower than that expressed in mammalian OHCs. Furthermore, the activity of prestin expressed in the Sf9 cells has not yet been examined. In the present study, an attempt was therefore made to purify prestin using another expression system, i.e., stably prestin-transfected mammalian Chinese hamster ovary (CHO) cells[17]. The reasons why CHO cells were used are as follows: First, the activity of prestin expressed in the transfected CHO cells has been confirmed by patch-clamp recording[17]. Second, in CHO cells, due to post-translational modification, such as glycosylation, in a manner similar to that in native protein, it is expected that prestin can be stabilized in its native conformation during the experiments. This study is the first report of purification of prestin from the mammalian cells.

Generally, solubilization of membrane proteins such as prestin requires the use of detergents. Although there are many kinds of detergents, it is unclear which are suitable for the solubilization of prestin. Therefore, in the first half of the present study, selection of the detergents was addressed. In this study, 8 different detergents (Table 1) (Kishida Chemical, Osaka, Japan) commonly used for membrane protein solubilization[18] were tested. In the test, the ratio of the amount of detergent-solubilized prestin to the total amount of prestin subjected to solubilization, i.e., solubilization efficiency, at various concentrations was determined by quantitative Western blotting. The most suitable detergent was selected based on considerations of the tendency of the relationship between detergent concentration and solubilization efficiency, as well as the characteristics of the

detergents. In the last half of the present paper, purification of prestin by anti-FLAG affinity chromatography is reported, including the yield at each purification step and the final amount of purified prestin, which were determined by quantitative Western blotting.

Table 1. List of the detergents tested in this work.

| Detergent | Structure | Molecular weight | cmc* (mM) | References |
|---|---|---|---|---|
| n-Octyl-β-D-glucopyranoside | | 292.37 | 25 | (18)-(20) |
| n-Dodecyl-β-D-maltopyranoside | | 510.62 | 0.17 | (18) |
| n-Nonyl-β-D-thiomaltopyranoside | | 484.61 | 2.4 | (21) |
| Sucrose monocholate | | 732.85 | 4.7 | (22) |
| Sucrose monocaprate | | 496.55 | 2.52 | (23) |
| Sucrose monolaurate | | 524.61 | 0.40 | (24) |
| n-Nonanoyl-N-methyglucamide (MEGA-9) | | 335.44 | 25 | (18)(20) (24) |
| 3-[(3-Cholamidopropyl)dimethyl-ammonio]-2-hydroxypropanesulfon-icacid (CHAPSO) | | 630.89 | 8 | (25) |

*cmc: critical micelle concentration

## 2. Materials and methods

### 2.1 Cell line

Chinese hamster ovary (CHO) cells which had been modified to stably express C-terminal 3×FLAG-tagged prestin[17] were used. The cells were cultured at 37ºC with 5% $CO_2$ in CHO-S-SFM II medium (Invitrogen, Carlsbad, CA) and then harvested and stored at -80ºC until use. The number of cells was determined by cell counting using a hemocytometer.

### 2.2 Solubilization of prestin with detergents

A schematic diagram of the preparation of solubilized and non-solubilized proteins is shown in Fig. 1. The CHO cells expressing 3×FLAG-tagged prestin were thawed and washed with 10 mM Hepes buffer (pH 7.3) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.5 mM $CaCl_2$. Then, $5 \times 10^6$ cells were incubated on ice with 100 μl of the same Hepes buffer containing detergent for 30 min. After the incubation, the samples were centrifuged at $20,360 \times g$ at 4ºC for 2 h. Proteins contained in the supernatant and those in the precipitate were defined as solubilized proteins and non-solubilized proteins, respectively. The ratio of prestin in supernatants (solubilized prestin) to that in precipitates (non-solubilized prestin) was analyzed by quantitative Western blotting.
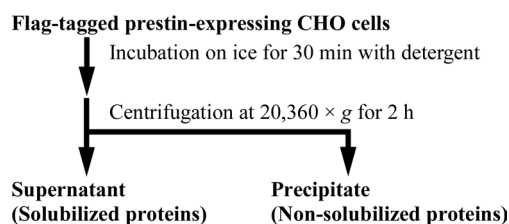
**Flag-tagged prestin-expressing CHO cells**

Incubation on ice for 30 min with detergent

Centrifugation at 20,360 × *g* for 2 h

**Supernatant**     **Precipitate**
**(Solubilized proteins)**     **(Non-solubilized proteins)**

Fig. 1  Schematic diagram of preparation of solubilized proteins and non-solubilized proteins.

## 2.3 Purification of prestin

A schematic diagram of the purification procedure is shown in Fig. 2.  Samples obtained at each step of the procedure are numbered 1 to 10 as shown in parentheses.  The CHO cells expressing 3×FLAG-tagged prestin (Fig. 2, sample 1) were thawed and washed with 10 mM Hepes buffer (pH 7.3) containing 150 mM $Na_2SO_4$.  For this experiment, $2\times10^9$ cells were used.  The cells suspended in 40 ml of the same Hepes buffer were disrupted by sonication, followed by centrifugation at 1,000 × *g* for 7 min at 4ºC to remove nuclei and undisrupted cells (Fig. 2, sample 2).  The supernatant (Fig. 2, sample 3) was centrifuged at 20,360 × *g* at 4ºC for 2 h to separate the cytoplasmic soluble fraction (Fig. 2, sample 4) and the membrane fraction (Fig. 2, sample 5).  To solubilize membrane proteins, the obtained membrane fraction (precipitate) was resuspended in 40 ml of the same Hepes buffer containing 10 mM *n*-nonyl-*β*-D-thiomaltopyranoside (Dojindo, Kumamoto, Japan), which had been found to achieve good solubilization characteristics as described below in the Results section.  This buffer was subsequently used for all procedures.  After 2-h incubation on ice, the non-solubilized proteins (Fig. 2, sample 6) were removed by centrifugation at 20,360 × *g* at 4ºC for 2 h.  The supernatant (Fig. 2, sample 7), which contained the solubilized prestin, was applied to a column (200 μl) of anti-FLAG affinity gel (Sigma) equilibrated with the buffer, prestin tagged with 3×FLAG epitope being expected to bind to the column.  The flow-through fraction (Fig. 2, sample 8), containing unbound proteins, was collected for subsequent analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.  Since some contaminating proteins were considered to be retained in the column, the column was then washed with 5 ml of the buffer to remove such contaminants, the wash fraction (Fig. 2, sample 9) also being collected.  Finally, bound proteins were competitively eluted with 1 ml of the buffer containing 250 µg/ml of 3×FLAG peptide (Sigma) (Fig. 2, sample 10).  The purification procedure described above was performed five times.  For the negative control, a sample was obtained from untransfected CHO cells with the same procedure as described above.

## 2.4 Western blotting

Samples obtained from 3×FLAG-tagged prestin-expressing CHO cells and known amounts of 3×FLAG-tagged bacterial alkaline phosphatase (3×FLAG-BAP) control protein (Sigma) were subjected to SDS-PAGE.  After electrophoresis, the proteins were transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ), followed by blocking with 5% skimmed milk in PBS containing 0.05% Tween 20 (PBS-T).  The membranes were then incubated with mouse anti-FLAG primary antibody (Sigma, St. Louis, MO) at a dilution of 1:10,000 in PBS-T, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverley, MA) at a dilution of 1:3,000 in PBS-T.  Immunoreactive bands were visualized with ECL Western blotting detection reagents (GE Healthcare).  Images were captured using the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA).

**Flag-tagged prestin-expressing CHO cells (1)**
　　Sonication

　　Centrifugation at 1,000 × *g* for 7 min

**Precipitate (2)**
**(Nuclei and undisrupted cells)**

**Supernatant (3)**
　　Centrifugation at 20,360 × *g* for 2 h

**Supernatant (4)**
**(Cytoplasmic soluble fraction)**

**Precipitate (5)**
**(Membrane fraction)**
　　Incubation on ice for 2 h with 10 mM
　　*n*-nonyl-*β*-D-thiomaltopyranoside

　　Centrifugation at 20,360 × *g* for 2 h

**Precipitate (6)**
**(Non-solubilized proteins)**

**Supernatant (7)**
**(Solubilized proteins)**
　　Application on an anti-FLAG affinity column

**Flow-through fraction (8)**

**Proteins bound to the column**
　　Washing the column with the buffer

**Wash fraction (9)**

　　Elution with 250 μg/ml of 3×FLAG peptide
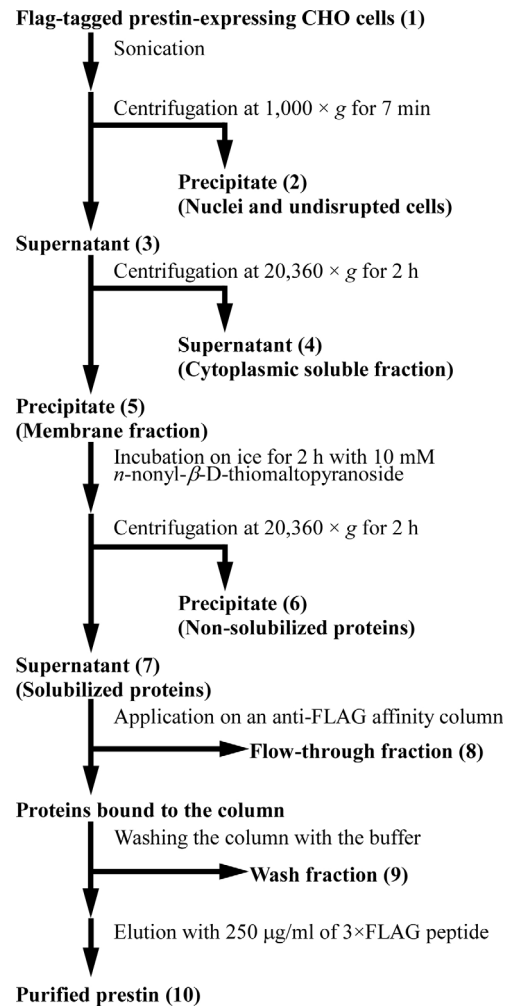
**Purified prestin (10)**

Fig. 2　Schematic diagram of purification procedure.　Numbers in parentheses represent sample numbers. As starting material for purification of prestin, $2\times10^9$ 3×FLAG-tagged prestin-expressing CHO cells were used.

## 2.5 Quantitative analysis using Western blotting

First, the linearity of the relationship between known amounts of 3×FLAG-BAP, which has the same epitope as 3×FLAG-tagged prestin, and the signal intensities obtained by Western blotting were examined five times.　The reason why 3×FLAG-BAP was used is that equal amounts of proteins show the same signal intensities in Western blotting if such proteins have the same 3×FLAG epitope[26].

When the relationship between the known amounts of 3×FLAG tagged protein and the signal intensities of their bands is linear, the ratio of the amount of solubilized prestin to the total amount of prestin subjected to solubilization, i.e., the solubilization efficiency *SE*, is given by

$$SE = \frac{I_{\text{solubilized}}}{I_{\text{solubilized}} + I_{\text{non-solubilized}}}, \tag{1}$$

where $I_{\text{solubilized}}$ and $I_{\text{non-solubilized}}$ are signal intensities of the bands showing solubilized and non-solubilized prestin, respectively.　Also, the amount of obtained purified prestin can be determined by comparing the signal intensity of the band showing prestin with those of the bands showing known amounts of 3×FLAG-BAP.

To obtain the linearity of the relationship between the known amounts of 3×FLAG-BAP and the signal intensities of their bands, Western blotting was carried out for 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35 pmol of 3×FLAG-BAP.　Images were analyzed using the Volume Tools in Quantity One image analysis software (Bio-Rad Laboratories) as follows:

Bands showing 3×FLAG-BAP were surrounded by rectangles, the width of which was determined to be 5 mm, i.e., the width of each lane of the Western blots.   As the molecular weight of 3×FLAG-BAP is 49 kDa, the top and bottom edges of the rectangles were determined at the positions of 62 kDa and 47.5 kDa, respectively, such positions being decided based on those of molecular weight markers (P7708, New England Biolabs, Beverly, MA).   Band intensity was obtained by integrating the intensities of the pixels within the rectangles.   Background intensity was also obtained from an empty lane.   Net intensity was calculated by subtracting the background intensity from the band intensity. The obtained net intensities were plotted against the amount of 3×FLAG-BAP.

### 2.6 Determination of solubilization efficiency of prestin

To determine the solubilization efficiency, 1/100 volume of solubilized proteins and such volume of non-solubilized proteins were Western blotted on the same membrane. The reason why 1/100 volume of the solubilized proteins and the same volume of non-solubilized proteins were subjected to Western blotting is that the net intensities of the bands showing prestin were expected to be within the linear range of the relationship between the net intensities and the known amounts of protein, i.e., 0.05-0.35 pmol of 3×FLAG-BAP.

Images were analyzed using Quantity One image analysis software.   Regions of interest were determined as follows: The width of the rectangles which surround bands showing 3×FLAG-tagged prestin was determined to be 5 mm.   Since bands showing prestin were detected as broad bands with the highest intensity around 100 kDa, the top and bottom edges of the rectangles were determined at the positions of 175 kDa and 83 kDa, respectively, such positions being decided based on those of molecular weight markers. Solubilization efficiency of prestin was calculated using the obtained net intensity and equation (1).

### 2.7 Determination of the amount of purified prestin

To determine the solubilization efficiencies, the linearity of the relationship between the known amounts of 3×FLAG-BAP and the net intensities of their bands obtained by Western blotting was first confirmed.   One set of solubilized and non-solubilized prestin using the 8 different detergents at various concentrations was then Western blotted on the same membrane, and the intensity of the band showing solubilized prestin ($I_{solubilized}$) was compared with that of the band showing non-solubilized prestin ($I_{non-solubilized}$), thus determining the solubilization efficiencies.

By contrast, to determine the amount of the purified prestin, the net intensity of the band showing purified prestin is necessary for simultaneous comparison with those of bands showing known amounts of 3×FLAG-BAP.   Therefore, 1/10,000 volume of obtained purified prestin and 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 and 0.35 pmol of 3×FLAG-BAP were Western blotted on the same membrane.   The reason why 1/10,000 volume of the obtained purified prestin was subjected to Western blotting is that the amount of prestin was expected to be between 0.05 and 0.35 pmol.   In this experiment, to reduce experimental error, the acrylamide concentration of the separation gel was set at 15% so that all bands were narrow. The net intensity of the band showing 1/10,000 volume of the obtained purified prestin was substituted into the linear regression equation describing the relationship between the known amounts of 3×FLAG-BAP and the net intensities of their bands.   Thus, the amount of purified prestin in the 1/10,000 volume of the obtained sample was determined.

## 3. Results

### 3.1 Solubilization of prestin

An example of the results of Western blotting of 3×FLAG-BAP and that of the

relationship between the known amounts of 3×FLAG-BAP and the net intensities of their bands are shown in Fig. 3(a) and (b), respectively. Good linearity was observed ($r^2 = 0.95$). From five experiments, $r^2$ was found to be $0.96 \pm 0.02$ (mean ± SD). The values of detected intensity differed from membrane to membrane. However, the reproducible good linearity of the relationship between the amounts of protein and the net intensities of their bands ensured that the amounts of 3×FLAG-tagged protein could be determined from the net intensities of the bands obtained by Western blotting on the same membrane.

The solubilizaiton efficiencies of the 8 kinds of detergents listed in Table 1 at various concentrations were examined by Western blotting. Figure 4 shows an example of Western blotting when 10 mM $n$-nonyl-$\beta$-D-thiomaltopyranoside was used. Bands showing prestin were detected as broad bands having the highest intensity around 100 kDa, which is greater than the predicted molecular weight of 3×FLAG-tagged prestin of 84.4 kDa. This is probably because that prestin was glycosylated[11]. Bands indicating prestin were surrounded by rectangles as shown in Fig. 4, and net intensities of solubilized and non-solubilized prestin, i.e., $I_{solubilized}$ and $I_{non-solubilized}$, were obtained by image analysis. The solubilization efficiency was determined to be 74% using equation (1).
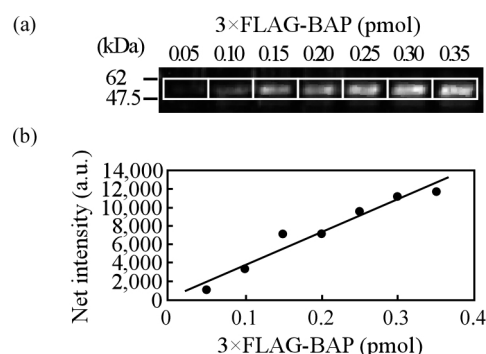


Fig. 3 Evaluation of the linearity of the quantitative Western blotting. (a) Western blot of 3×FLAG-BAP. SDS-PAGE was performed on a 15% polyacrylamide gel. Net intensities were obtained by subtracting the background intensity from the band intensities acquired by integrating the intensities of the pixels within the white rectangles. (b) Relationship between the known amounts of 3×FLAG-BAP and the net intensities of their bands. The filled circles indicate the data obtained by quantitative Western blotting. The solid line shows the linear regression: $I = 88 + 36,100M$; $r^2 = 0.95$, where $I$, $M$ and $r$ are the net intensity, the known amount of 3×FLAG-BAP and the correlation coefficient, respectively.
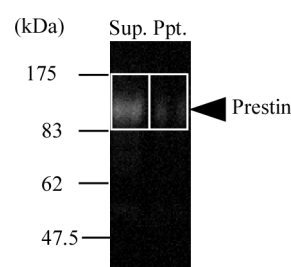


Fig. 4 Representative data of Western blotting. SDS-PAGE was performed on a 10% polyacrylamide gel. Cells were incubated on ice with 10 mM $n$-nonyl-$\beta$-D-thiomaltopyranoside for 30 min followed by centrifugation at $20,360 \times g$ for 2 h. Sup: supernatant (solubilized proteins), ppt: precipitate (non-solubilized proteins). The arrowhead shows prestin. Net intensities, $I_{solubilized}$ and $I_{non-solubilized}$, were obtained by subtracting the background intensity from the band intensities acquired by integrating the intensities of the pixels within the white rectangles. Solubilization efficiency was determined from $I_{solubilized}$ and $I_{non-solubilized}$.

The relationships between the detergent concentration and the solubilization efficiency using the 8 kinds of detergents are shown in Fig. 5. When the concentrations of detergent are higher than their critical micelle concentrations (cmc), the detergents form micelles. In such cases, membrane proteins are reported to be solubilized by detergents[27]. In the present study, when *n*-octyl-*β*-D-glucopyranoside, *n*-nonyl-*β*-D-thiomaltopyranoside, sucrose monocholate or *n*-nonanoyl-*N*-methyglucamide (MEGA-9) was used, solubilization efficiency sharply increased around each cmc. Since this tendency agrees with the above-mentioned knowledge about membrane protein solubilization and with those in the previous reports in which target membrane proteins were solubilized in active form[20][21][28], these 4 kinds of detergents may uniformly solubilize prestin without denaturation. On the other hand, when *n*-dodecyl-*β*-D-maltopyranoside, sucrose monocaprate, sucrose monolaurate or 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonicacid (CHAPSO) was used, solubilization efficiency gradually increased with increasing concentrations of detergents. This tendency is contrary to the above-mentioned knowledge about membrane protein solubilization. Furthermore, the results showing a gradual increase of solubilization efficiency indicate that a part of prestin was solubilized at low detergent concentrations and that another part of prestin was solubilized at high detergent concentrations, suggesting that the condition of prestin solubilized at low detergent concentrations was different from that of prestin solubilized at high detergent concentrations. Hence, there is a possibility that these 4 kinds of detergents nonuniformly solubilized prestin, perhaps with denaturation. Therefore, in the present study, the candidates for use in prestin solubilization were limited to the former four detergents.
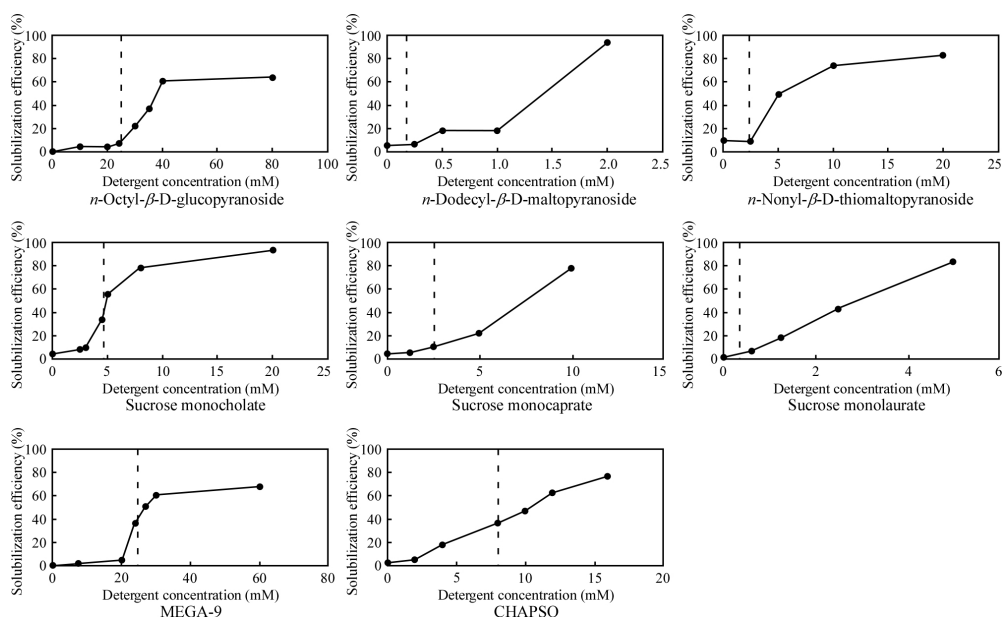


Fig. 5 Relationships between the detergent concentration and the solubilization efficiency. The filled circles, connected by solid lines, indicate the solubilization efficiency obtained by quantitative Western blotting. Dashed lines show each critical micelle concentration (cmc). In the case of *n*-octyl-*β*-D-glucopyranoside, *n*-nonyl-*β*-D-thiomaltopyranoside, sucrose monocholate and MEGA-9, the solubilization efficiency of prestin sharply increased around the cmc, while when the other detergents were used, solubilization efficiency gradually increased with increasing detergent concentration.

It has been reported that the use of nonionic detergents containing a sugar group, including *n*-octyl-*β*-D-glucopyranoside and *n*-nonyl-*β*-D-thiomaltopyranoside, are expected to increase the possibility of solubilization of membrane proteins without denaturation[29] and that *n*-nonyl-*β*-D-thiomaltopyranoside is more stable than *n*-octyl-*β*-D-

glucopyranoside[30].   In the present study, therefore, *n*-nonyl-*β*-D-thiomaltopyranoside was used for solubilization of prestin.   The detergent concentration was decided based on a report that it should be close to the saturation point of solubilization efficiency[29].   Since such point for *n*-nonyl-*β*-D-thiomaltopyranoside in the present study was 10 mM, it was concluded that 10 mM *n*-nonyl-*β*-D-thiomaltopyranoside should be applied for prestin purification.

### 3.2 Purification of prestin

Purification of prestin was performed.   To visualize all proteins in the sample and to detect prestin, 1/10,000 volume of each sample obtained at each step of the purification procedure was subjected to SDS-PAGE with Coomassie brilliant blue staining, and Western blotting with probing by anti-FLAG antibody.   Representative data are shown in Fig. 6. From the cells (Fig. 2, sample 1; Fig. 6, lanes 1), nuclei and undisrupted cells (Fig. 2, sample 2; Fig. 6, lanes 2) were removed by sonication followed by centrifugation at 1,000 × *g*.   The obtained supernatant (Fig. 2, sample 3; Fig. 6, lanes 3) containing prestin was separated into a cytoplasmic soluble fraction (Fig. 2, sample 4; Fig. 6, lanes 4) and a membrane fraction (Fig. 2, sample 5; Fig. 6, lanes 5) by centrifugation at 20,360 × *g*. Prestin was detected in the isolated membrane fraction (Fig. 6B, lane 5).   Membrane proteins were then solubilized from the membrane fraction with 10 mM *n*-nonyl-*β*-D-thiomaltopyranoside.   Non-solubilized proteins (Fig. 2, sample 6; Fig. 6, lanes 6) and solubilized proteins (Fig. 2, sample 7; Fig. 6, lanes 7) were separated by centrifugation at 20,360 × *g*.   From the net intensity of non-solubilized prestin ($I_{\text{non-solbilized}}$; Fig. 6B, lane 6) and that of solubilized prestin ($I_{\text{solubilized}}$; Fig. 6B, lane 7), in this case, the solubilization efficiency was determined to be 71%.   The solubilized proteins were then applied to an anti-FLAG affinity column.   Some of the prestin did not bind to the column and was found in the flow-through fraction (Fig. 2, sample 8; Fig. 6, lanes 8).   The ratio of net intensity in Fig. 6B, lane 7 to that in lane 8 was 100:44, indicating that 56% of the prestin was bound to the column and that 44% of the prestin passed through.   To remove nonspecifically retained contaminants, the column was washed with the buffer (Fig. 2, sample 9; Fig. 6, lanes 9).   The prestin was then competitively eluted from the column using 3×FLAG peptide (Fig. 2, sample 10; Fig. 6, lanes 10).   The overall recovery rate of prestin for the entire purification procedure was calculated to be 34% based on the net intensity of the band in Fig. 6B, lane 1 and that in lane 10.

Since purified prestin was not detected by Coomassie brilliant blue staining due to its low concentration (Fig. 6(a), lanes 10), 1/200 volume of the obtained purified prestin, the amount of which was 50 times as much as that loaded on the gel shown in Fig. 6(a), and the same volume of the sample obtained from the untransfected cells (negative control) were loaded onto another SDS-PAGE gel.   The gel was stained with Silver Stain Kit (Bio-Rad Laboratories), which has high sensitivity.   The result is shown in Fig. 7.   In lane 1, which shows the obtained purified prestin, a major band around 100 kDa (arrowhead) indicating prestin and a weak band around 40 kDa were detected.   In lane 2, which shows the sample obtained from the untransfected cells, a band around 40 kDa was also detected.   Since 40-kDa protein was detected in lane 2, this protein was probably derived from CHO cells. In lane 1, the band intensity of prestin was much higher than that of the 40-kDa protein, indicating that prestin was the major protein in the sample.

The amount of purified prestin was determined by quantitative Western blotting.   An example of the results of Western blotting is shown in Fig. 8(a).   The linear regression line was obtained by plotting the relationship between the known amounts of 3×FLAG-BAP and the net intensities of their bands, as shown in Fig. 8(b).   The linear regression equation was $I = 372 + 24,300M$, where $M$ is the amount of 3×FLAG-BAP and $I$ is the net intensity of the band.   The net intensity of the band showing 1/10,000 volume of the obtained purified

prestin was 3,200, which is displayed by the dashed line in Fig. 8(b). By substitution of 3,200 into the linear regression equation, i.e., by finding the intersection of the linear regression line with the dashed line, the amount of purified prestin in 1/10,000 volume of the obtained sample was determined to be 0.12 pmol. This result indicates that the total amount of the obtained purified prestin was 1.2 nmol, corresponding to about 120 μg since the molecular weight of prestin was estimated to be about 100 kDa based on Fig. 7. This yield is the best value in our experiments. In five experiments, the amount of purified prestin obtained from $2 \times 10^9$ prestin-expressing CHO cells ranged from 60 to 120 μg, the mean $\pm$ SD being $84 \pm 23$ μg.
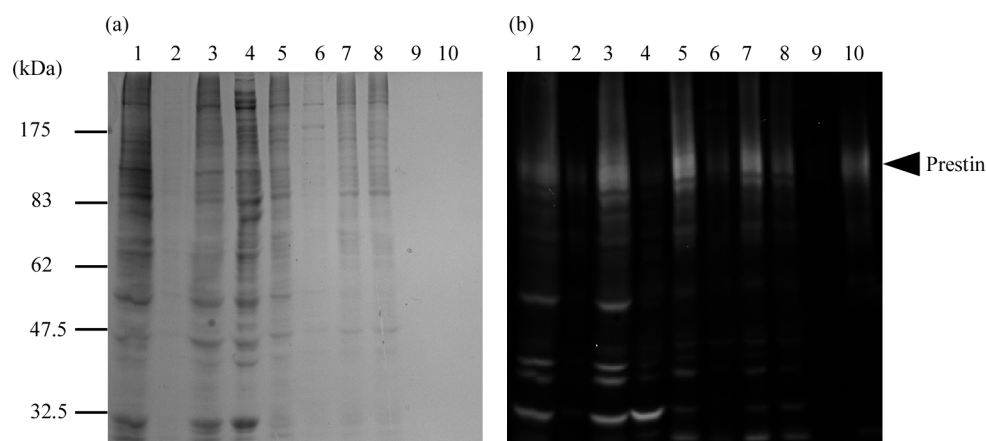


Fig. 6  SDS-PAGE and Western blot analysis of prestin purification. Analysis was carried out on 1/10,000 volume of the samples obtained at each step. (a) Coomassie brilliant blue-stained gel and (b) corresponding immunoblot. SDS-PAGE was performed on a 10% polyacrylamide gel. The arrowhead shows prestin. Lanes 1: total proteins of FLAG-tagged prestin-expressing CHO cells (Fig. 2, sample 1). Lanes 2: nuclei and undisrupted cells (Fig. 2, sample 2). Lanes 3: supernatant after sonication and centrifugation at $1,000 \times g$ (Fig. 2, sample 3). Lanes 4: cytoplasmic soluble fraction (Fig. 2, sample 4). Lanes 5: membrane fraction (Fig. 2, sample 5). Lanes 6: non-solubilized membrane proteins (Fig. 2, sample 6). Lanes 7: solubilized membrane proteins (Fig. 2, sample 7). Lanes 8: flow-through fraction (Fig. 2, sample 8). Lanes 9: wash fraction (Fig. 2, sample 9). Lanes 10: purified prestin eluted from the column using 3×FLAG peptide (Fig. 2, sample 10).
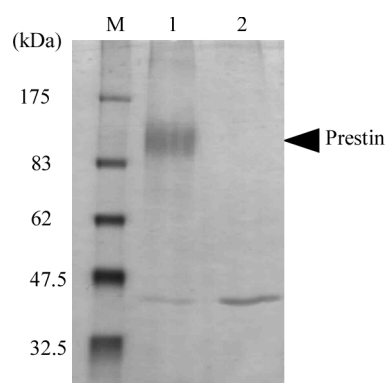


Fig. 7  Silver-stained SDS-PAGE gel loaded with purified prestin and with the sample obtained from the untransfected CHO cells. SDS-PAGE was performed on a 10% polyacrylamide gel. Analysis was carried out on 1/200 volume of purified prestin and the same volume of the sample obtained from the untransfected CHO cells. The arrowhead shows prestin. Lane M: molecular weight marker. Lane 1: purified prestin (Fig. 2, sample 10). Lane 2: sample obtained from untransfected cells (negative control).
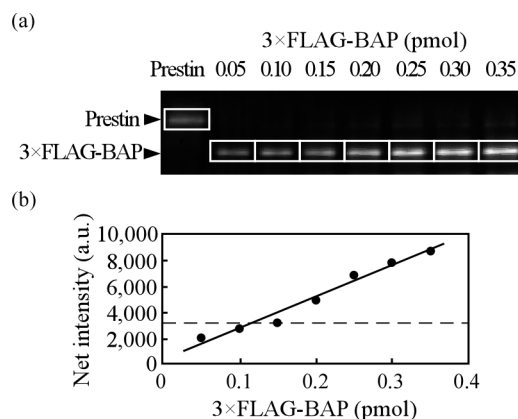
(a)



(b)



Fig. 8   Quantitative Western blotting.   (a) Western blot of 1/10,000 volume of obtained purified prestin and 0.05-0.35 pmol of 3×FLAG-BAP.   SDS-PAGE was performed on a 15% polyacrylamide gel.   (b) Relationship between the known amounts of 3×FLAG-BAP and the net intensities of their bands.   The filled circles indicate the data obtained by quantitative Western blotting.   The solid line shows the linear regression: $I = 372 + 24,300M$; $r^2 = 0.97$, where $I$, $M$ and $r$ are the net intensity, the known amount of 3×FLAG-BAP and the correlation coefficient, respectively.   The dashed line indicates the net intensity of the band showing 1/10,000 volume of the obtained purified prestin.   By finding the intersection of the regression line with the dashed line, the amount of 1/10,000 volume of the obtained purified prestin was determined to be 0.12 pmol.

## 4. Discussion

There are many kinds of detergents which can be used to solubilize membrane proteins. The choice of detergent to solubilize a target protein in its functional state is one of the important aspects in studies of membrane proteins.   In the present study, 8 kinds of detergents (Table 1) commonly used for membrane protein solubilization[18] were tested. As a result, it was determined that 10 mM *n*-nonyl-*β*-D-thiomaltopyranoside was suitable for solubilization of prestin.

Purification of prestin was attempted using 10 mM *n*-nonyl-*β*-D-thiomaltopyranoside. To improve the purity, unnecessary proteins should be removed as much as possible before applying the sample to the affinity column.   Therefore, in the present study, nuclei (Fig. 2, sample 2), cytoplasmic soluble proteins (Fig. 2, sample 4) and non-solubilized membrane proteins (Fig. 2, sample 6) were removed before chromatography.   Especially, removal of cytoplamic soluble proteins, which account for more than half of all proteins contained in the cells (Fig. 6(a), lanes 4), is thought to be effective.

As shown in Fig. 7, lane 1, prestin was purified from the crude fraction of the solubilized membrane proteins (Fig. 6(a), lane 7).   In lane 1 of Fig. 7, two bands around 100 kDa and 40 kDa were detected.   Since the 40-kDa protein was also detected in the sample obtained from the untransfected cells (negative control), as shown in lane 2, this 40-kDa protein was considered to be derived from CHO cells.   Since the band intensity of prestin detected around 100 kDa was much higher than that of the 40-kDa protein in lane 1, prestin was considered to be the major protein in the obtained sample.   The reason why the intensity of the band detected around 40 kDa in lane 2 is higher than that in lane 1 is considered to be as follows.   The 40-kDa protein may be relatively weakly bound to the anti-FLAG affinity column.   In the case of purification of prestin from transfected cells, prestin was strongly adsorbed onto the affinity column, resulting in lowered capacity of the column to absorb the 40-kDa protein.   By contrast, in the case of the preparation of the sample from the untransfected cells, since there was no prestin, the capacity of the column to absorb the 40-kDa protein was probably higher than that in the case of the purification of prestin from the transfected cells, thus resulting in the amount of 40-kDa protein obtained in the case of the sample from untransfected cells being larger than that in the case of prestin

purification.

In SDS-PAGE and Western blotting in the present study, bands showing prestin were detected as broad bands having the highest intensity around 100 kDa, which is greater than the predicted molecular weight of 3×FLAG-tagged prestin of 84.4 kDa.   The reason why the molecular weight of prestin detected in SDS-PAGE and Western blotting was found to be higher than the predicted molecular weight and why the detected bands were characterized by broadness should be that prestin expressed in CHO cells is probably modified by different lengths of carbohydrate chains[11][17], resulting in modified prestin being heavier than the predicted molecular weight and varying in molecular weight.   The position of the bands agrees with a previously reported finding[11].

Prestin has recently been reported to form homo-oligomers[13][16].   On the other hand, it has been reported that there are interactions between prestin/prestin and prestin/GLUT5 in transiently transfected HEK293T cells[31].   In the present study, since prestin was purified from CHO cells, which do not express GLUT5[32], and since no band was detected in the purified samples other than those showing prestin and the 40-kDa protein (Fig. 7, lane 1), prestin probably forms homo-oligomers in the transfected CHO cells.

In the present study, it was shown that prestin could be purified from 3×FLAG-tagged prestin-expressing CHO cells using 10 mM $n$-nonyl-$β$-D-thiomaltopyranoside and the anti-FLAG affinity column (Figs. 6 and 7) and that the amount of purified prestin acquired from $2×10^9$ cells was $84 \pm 23$ μg (mean $\pm$ SD; $n$=5).   Causes of the variation of yield are considered to be differences in the expression level of prestin in the cultured cells and the efficiency of prestin binding to and elution from the affinity column.   To increase the yield with little variation, further refinement of the cell culture conditions, such as cell density and culture time, and that of the operation of the affinity chromatography, such as flow rate of the solution, will be necessary.   In the next step, the activity of purified prestin should be confirmed.   Nevertheless, it is considered that the success of prestin purification as demonstrated in this study is important and meaningful.   This result is expected to expand the possibilities for further study of prestin.   For example, molecular structure, pure electrophysiological properties, interaction characteristics with other biomolecules and so on are expected to be revealed.

## 5. Conclusion

The present study demonstrated that $84 \pm 23$ μg of purified prestin could be obtained from $2×10^9$ 3×FLAG-tagged prestin-expressing CHO cells using 10 mM $n$-nonyl-$β$-D-thiomaltopyranoside and an anti-FLAG affinity column.

## Acknowledgements

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