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PURIFICATION OF BOVINE RHODOPSIN BY HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

Bovine rhodopsin was purified from *n*-octylglucoside-solubilized retinas by high-performance size-exclusion chromatography. In one chromatographic step, six protein fractions were separated with baseline resolution. The major fraction was identified as monomeric rhodopsin by absorption spectroscopy. Amino acid analysis of this fraction further supported the assignment. A comparison of the elution profiles of rhodopsin purified by this method with that purified by Concanavalin A–Sepharose 4B affinity chromatography suggested that rhodopsin from high-performance chromatography was slightly purer than the conventionally purified rhodopsin.

INTRODUCTION

The visual response is initiated by light absorption and energy transduction from rhodopsin, a membrane protein contained in the retina[1]. This glycoprotein (MW 41,000) consists of an 11-*cis*-retinal chromophore covalently bound to opsin by a Schiff base linkage. A number of different procedures have been reported for the isolation and purification of rhodopsin[2–11]. Each procedure typically involves the solubilization of the retina membrane proteins by a wide variety of detergents followed by the separation of rhodopsin by conventional column chromatography (size-exclusion, ion-exchange and affinity chromatography). This communication establishes the first reported purification procedure for rhodopsin using high-performance liquid chromatography (HPLC). The purity of bovine rhodopsin obtained by this procedure is slightly improved from that obtained by Concanavalin A–Sepharose 4B affinity chromatography. Furthermore, it is shown that HPLC offers a rapid method for characterizing the protein composition of solubilized rhodopsin preparations.

MATERIALS AND METHODS

Isolation of rod outer segments

The isolation and purification of rhodopsin was carried out under dim red

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light (Kodak, No. 1 Filter). Rod outer segments (ROS) were isolated by modifications of the Papermaster and Dreyer procedure[6] as described by Plantner and Kean[10]. One hundred and fifty bovine retinas (George Hormel, Austin, MN, U.S.A.) were used for the preparation. Sucrose gradients were used to purify the ROS[6,10]. Material from the 1.11–1.13 gm/ml interface was removed and placed in four Sorvall tubes. This material was then diluted with two volumes of 0.01 *M* Tris–acetate buffer, pH 7.4. Following dilution, the solutions were centrifuged at 26,200 *g* for 2 h, and the supernatant was discarded. The pellets were combined by resuspension in 20 ml of 0.01 *M* Tris–acetate buffer pH 7.4, and centrifuged at 26,200 *g* for 2 h. A 25-ml volume of 0.05 *M* Tris–HCl buffer, pH 7.0, containing 30 m*M* *n*-octylglucoside (Cal-Biochem) was added to the material in each tube. This suspension was gently homogenized with a stir bar for 5 h at 4°C. The ROS suspension was centrifuged at 26,200 *g* for 2 h. The extracted material is called crude rhodopsin throughout this communication. The absorption spectrum was measured on a Beckman Model 26 spectrophotometer (Beckman Instruments, Palo Alto, CA, U.S.A.).

Concanavalin–Sepharose affinity purification

Crude rhodopsin was made 10^{-3} *M* with respect to the following ions: magnesium chloride, calcium chloride and manganese chloride. The rhodopsin solution was then added to an equilibrated Concanavalin A–Sepharose 4B affinity column (Sigma) at a flow-rate of 0.4 ml/min. The column was eluted with 0.05 *M* Tris–HCl, pH 7.0, 30 m*M* *n*-octylglucoside and 10^{-3} *M* manganese chloride, magnesium chloride and calcium chloride at 0.4 ml/min until no absorbance at 280 nm was detected. Rhodopsin was eluted from the column by adding 0.25 *M* α-methyl glucoside. An absorption spectrum was obtained on each fraction (1.5 ml) to determine the purity of the eluted rhodopsin.

High-performance size-exclusion purification of rhodopsin

A Beckman high-pressure liquid chromatograph (Model 165 variable-wavelength, series 220) was used for all preparations. Eluents were measured at two wavelengths simultaneously. Typically, these were 278 nm and 498 nm or 278 nm and 370 nm. Volumes ranging from 20 to 100 μl containing crude rhodopsin (up to 50 μ*M*) were injected into an analytical TSK-3000 (30 cm × 7.5 mm I.D.) size-exclusion column (Toyo Soda). The flow-rate was 0.5 ml/min with a pressure of 250 p.s.i. and the eluting buffer was 0.06 *M* phosphate (pH 6.5) with 0.15 *M* sodium chloride and 30 m*M* octylglucoside. All buffers and protein samples were filtered through Millex-GS 0.22-μm filters (Millipore). The entire analysis was carried out under dim red light (Kodak No. 1 filter) at 20°C. Aqueous proteins (Bio-Rad) used as standards were analyzed as described above, except that the eluting buffer did not contain *n*-octylglucoside.

Amino acid analysis

Amino acid analyses were performed using modifications of the Crestfield, Moore and Stein procedure[12]. A Waters HPLC Amino Acid system equipped with a cation-exchange column (25 cm × 4 mm I.D.) at 62°C was used. Dialysed protein preparations were hydrolysed at 108°C under vacuum for 24 h. The amino acids were detected by the fluorescence of the *o*-phthalaldehyde derivatives. The detection limit of the system is 50 pmol.

RESULTS AND DISCUSSION

Chromatography of crude rhodopsin, solubilized in *n*-octylglucoside, on a TSK-3000 size-exclusion column yields six distinct protein fractions (Fig. 1). These fractions are labeled 1 through 6 corresponding to elution volumes of 2.8, 4.2, 4.5, 5.1, 5.4 and 6.5 ml, respectively. Fraction 3, the main fraction in the chromatogram, is the only component which exhibited substantial absorption at both 498 nm and 278 nm. The absorption spectrum of this fraction displays these features more clearly (Fig. 2). The absorption band centered at 498 nm is the α-band, characteristic of native bovine rhodopsin and is attributed to the strongly allowed π–π^* transition of the retinal chromophore, covalently bound to opsin[1]. In addition to this band, the rhodopsin absorption spectrum is characterized by the β-band centered at 340 nm and the γ-band centered at 278 nm. Both bands are present in the spectrum of fraction 3. Thus, it is apparent from the absorption spectral analysis that fraction 3 consists mainly of solubilized rhodopsin.

The purity of rhodopsin preparations has generally been assessed from the ratio of the absorbances at 278 to 498 nm (A_{278}/A_{498}). Smaller ratios are associated with purer preparations since extraneous proteins associated with rhodopsin preparations would solely increase the absorbance at 278 nm. Typical values of A_{278}/A_{498} for purified rhodopsin are in the 1.65–1.85 range[11]. The data obtained from the spectra of fraction 3 yields a spectral ratio of 1.73, which is consistent with the assignment of this fraction to that of purified rhodopsin. This is further reinforced by the amino acid analysis (Table I). A direct comparison can be made of the amino acid composition of fraction 3 with that predicted for bovine rhodopsin, based on the known primary sequence[13].

The molecular weight of the chromatographic fractions can be estimated by standardizing the column with proteins of known molecular weight (Fig. 3). The use

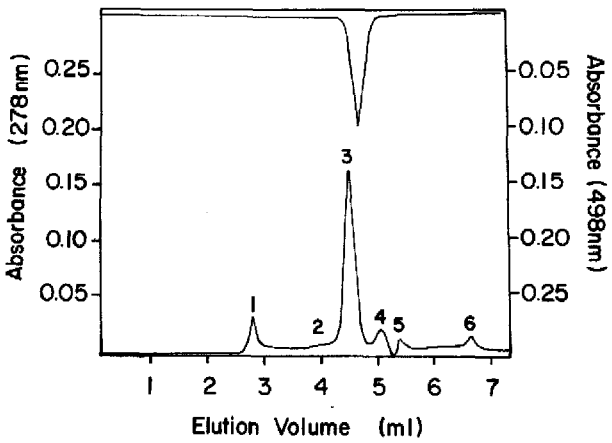

Fig. 1. High-performance size-exclusion chromatograph of crude bovine rhodopsin. Chromatographic conditions: column, TSK-3000 SW (30 cm × 7.5 mm I.D.); eluent 60 m*M* phosphate buffer, pH 6.5; 150 m*M* NaCl and 30 m*M* octylglucoside; flow-rate 0.5 ml/min. Top curve corresponds to 498 nm absorbance. Bottom curve corresponds to 278 nm absorbance. The top elution profile is offset to the right for clarity. Fraction numbers 1, 2, 3, 4, 5 and 6 correspond to peaks which are eluted with 2.8, 4.2, 4.5, 5.1, 5.4 and 6.5 ml, respectively. Fraction 2 is more apparent on an expanded scale.

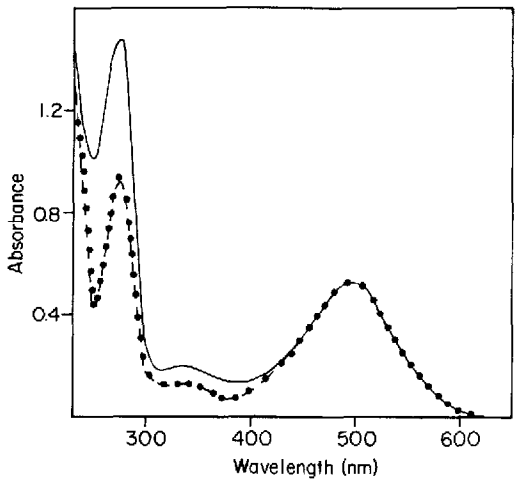

Fig. 2. Comparative absorption spectra of bovine rhodopsin. Absorption spectrum of crude rhodopsin (———) in 30 m*M* *n*-octylglucoside, 50 mM Tris · HCl buffer, pH 7.0. Absorption spectrum of post-Concanavalin A peak fraction (. . .) in 30 m*M* *n*-octylglucoside, 50 m*M* Tris-HCl buffer, pH 7.0. Absorption spectrum of fraction 3 from HPLC (– – –) in 30 m*M* *n*-octylglucoside, 50 m*M* phosphate buffer, pH 6.5 (see Fig. 1). Optical pathlength was 1 cm. Measurements were made at room temperature.

TABLE I

AMINO ACID ANALYSIS OF PROTEIN FRACTIONS FROM HPLC PURIFICATION OF CRUDE BOVINE RHODOPSIN

Bovine rhodopsin data from ref. 12.

Amino acids	*Bovine rhodopsin*	*Fraction*				
		1	*2*	*3*	*4 + 5*	*6*
Asx	20	26	29	20	19	23
Thr	28	16	20	23	12	13
Ser	15	23	31	13	60	39
Glx	29	31	33	30	36	40
Pro	20	14	12	19	9	10
Gly	23	31	35	25	42	46
Ala	29	25	22	31	21	27
Cys	10	3	—	4	1	3
Val	31	19	17	27	12	14
Met	16	5	1	10	1	1
Ile	22	14	11	18	8	9
Leu	29	25	18	26	15	15
Tyr	18	9	4	15	7	7
Phe	31	15	14	29	9	8
His	6	5	5	5	5	6
Lys	11	13	12	11	10	11
Arg	7	13	1	8	9	9

of standards in the absence of the *n*-octylglucoside detergent or a denaturing detergent (SDS) precludes accurate molecular weight determinations. However, a comparison of rhodopsin's elution volumes (4.5 ml) with that of the standards reveals that the rhodopsin–detergent micelle is eluted together with the native form of bovine serum albumin (MW 67,000) (Fig. 3). This suggests that the detergent micelle of fraction 3 contains monomeric rhodopsin (MW 41,000).

Fraction 3 may still contain small amounts of opsin which could have been present in non-dark adapted retinas. In order to establish the elution pattern for opsin, purified rhodopsin (fraction 3) was partially photobleached with white light at 4°C. The absorbance of the α-band decreased with a concomitant increase in a band centered at 370 nm (data not shown). This band is attributed to the chromophore which is no longer bound to the protein. The elution pattern of this preparation shows a corresponding decrease of the intensity of fraction 3 and the appearance of a new band with multiple peaks (eluting volume range 2.8–3.9 ml). Extensive photobleaching of the rhodopsin results in the complete conversion of fraction 3 to give new bands in the chromatogram. Thus, it is not expected that fraction 3 contains measurable amounts of opsin since baseline separation occurs between photobleached rhodopsin and native rhodopsin. It is interesting to note that the opsin elution profile on HPLC is similar to that observed in agarose column chromatography[14].

The remaining fractions from the chromatograph, *viz.* fractions 1, 2, 4, 5 and 6 are proteins or protein mixtures which act as contaminants in the rhodopsin purification. As can be seen from Fig. 2, a substantial decrease in the intensity of the γ-band occurs after chromatography. The yield of extraneous protein from the column is essentially quantitative. The sum of the absorbances at 278 nm from fractions 1, 2, 4, 5 and 6 equals the absorption difference between the γ-bands in the crude and purified rhodopsin preparations. These fractions have substantially different amino

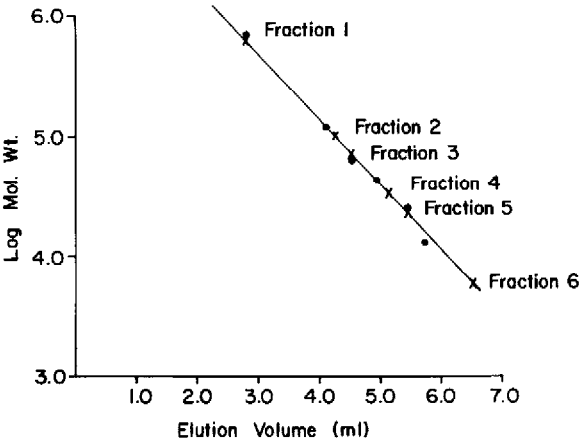

Fig. 3. Calibration curve of the TSK-3000 size-exclusion column and elution volumes of the protein fractions isolated via HPLC purification of crude rhodopsin. Thyroglobulin, aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen and ribonuclease were used to standardize the column (●). Protein fraction 1 is at the exclusion limit of the column (greater than 300,000). Fractions 2 through 6 (×) correspond to effective molecular weights for the protein–detergent micelle of 110,000, 67,000, 32,000, 25,000 and 2000, respectively.

acid compositions from that of purified rhodopsin (Table I). Other separation methods which have been employed have been unable to resolve these protein components. Thus, HPLC offers a new method for further characterization of other membrane proteins contained in the retina.

It should be noted that small absorbances occur at 498 nm for fractions 1 and 2. Since these fractions are eluted faster than fraction 3, it is expected that the proteins are of higher molecular weight (Fig. 3). Fraction 1 may contain aggregated rhodopsin since it is eluted together with high-molecular-weight proteins at the exclusion limit, while fraction 2 may contain rhodopsin dimers. In order to test this hypothesis, crude rhodopsin was concentrated prior to chromatography by Amicon ultrafiltration, a process known to cause rhodopsin aggregation. The chromatograph displayed larger absorbances at 278 nm and 498 nm for these fractions. Thus, these fractions probably contain small amounts of aggregated rhodopsin in addition to some other protein contaminants.

A comparison can be made of the purity of rhodopsin obtained by this method with that of rhodopsin purified by conventional methods. In order to make the comparison accurate, crude rhodopsin was taken from the same ROS preparation used for the HPLC purification. When crude rhodopsin was purified by Concanavalin A affinity chromatography, both the yield (nearly 90%) and the elution profile were nearly identical with those which were previously reported[11]. The absorption spectrum of the peak fraction from Concanavalin A which contained the lowest spectral ratio and hence the purest rhodopsin, yielded a ratio of 1.79 which is slightly higher than the ratio obtained by HPLC-purified rhodopsin (1.73). All other spectral bands were identical with those from the HPLC preparation (Fig. 2), indicating that the HPLC-purified rhodopsin is as pure as conventionally purified rhodopsin. Each preparation was further subjected to HPLC analysis (Figs. 4 and 5). Fraction 3, containing purified rhodopsin, was the main band present in these chromatographs. Minor fractions consistent with fractions 4 and 6 (Fig. 1) were also present in much lower

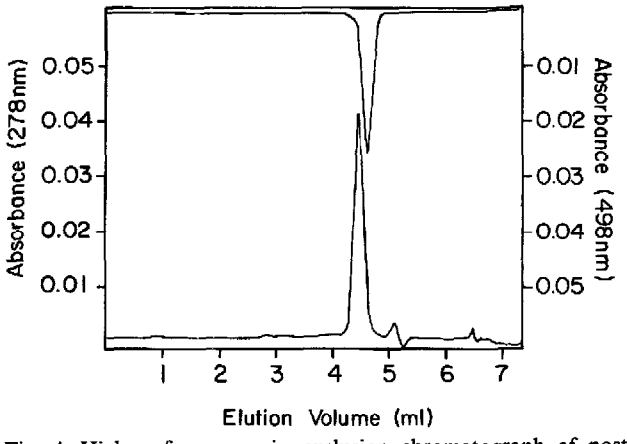

Fig. 4. High-performance size-exclusion chromatograph of post-Concanavalin A-purified rhodopsin (peak fraction). Chromatographic conditions: column, TSK-3000-SW (30 cm × 7.5 mm I.D.); eluent 60 m*M* phosphate buffer, pH 6.5, 150 m*M* NaCl and 30 m*M* octylglucoside; flow-rate 0.5 ml/min. The A_{278} and A_{498} legends are the same as Fig. 1. Elution volumes for the A_{278} peaks (bottom profile) are 4.5, 5.1 and 6.5 ml.

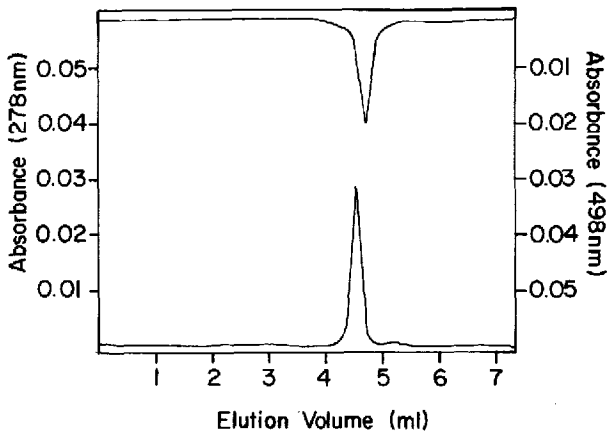

Fig. 5. High-performance size-exclusion chromatograph of fraction 3 (see Fig. 1). Chromatographic conditions: column TSK-3000-SW (30 cm × 7.5 mm I.D.); eluent 60 m*M* phosphate buffer, pH 6.5, 150 m*M* NaCl and 30 m*M* octylglucoside; flow-rate 0.5 ml/min. Elution volumes for the A_{278} peaks (bottom profile) are 4.5 and 5.1 ml.

amounts. However, these bands are more apparent in the Concanavalin A-purified rhodopsin (Fig. 4) suggesting that the HPLC-purified rhodopsin is less contaminated.

CONCLUSION

The main objective of this study was to determine the effectiveness of high-performance size-exclusion liquid chromatography as an alternative purification method for bovine rhodopsin. The results demonstrated that HPLC is an easy and efficient technique for rhodopsin purification. Spectroscopic and chromatographic analyses indicate that this method of purification may yield rhodopsin of slightly higher purity than that achieved by using Concanavalin A affinity chromatography. In addition, this method allows separation of rhodopsin aggregates from monomers, and bleached forms of rhodopsin from the native molecule. It is likely that this method can be used to purify rhodopsin isolated from other species as well. Because of its higher yields and greater resolution capabilities, HPLC provides several advantages over present rhodopsin purification techniques. With the use of preparative columns, it is expected that large-scale rhodopsin preparations can be routinely employed.

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