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TSK-TOYOPEARL GELS FOR THE PREPARATIVE SEPARATION OF STEROL CARRIER PROTEIN$_2$ FROM RAT LIVER[a]

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SUMMARY

The application of TSK-Toyopearl gels to the preparative separation of a basic and low-molecular-weight protein, sterol carrier protein$_2$ (SCP$_2$), was studied. SCP$_2$ was purified from 105 000 *g* supernatant of rat liver (S_{105}) by ion-exchange chromatography on CM-Toyopearl 650M and gel permeation on Toyopearl HW60S. Separation of S_{105} by CM-Toyopearl 650M was carried out at a high flow-rate in the presence of 10% (v/v) glycerol, a stabilizer of the protein. Toyopearl HW60S showed a significant ion-exchange effect on the elution of SCP$_2$. Using an elution buffer of ionic strength of 45 m*M*, a highly efficient purification of SCP$_2$ on HW60S was achieved. SCP$_2$ was purified approximately 5000-fold to apparent homogeneity with an overall yield of 69%.

INTRODUCTION

Sterol carrier protein$_2$ (SCP$_2$), also designated non-specific lipid transfer protein (nsL-TP), is a basic and low-molecular-weight protein[1,2] and has been shown to play an important role in intracellular transfer of both cholesterol and phospholipids and to participate in cholesterol metabolism in the liver[3,4] and various extrahepatic tissues[5–8].

Purification of SCP$_2$ has been reported by several laboratories[9–11]. However, the procedures were complicated and time-consuming, with low yields. The exact physiological role of SCP$_2$ in both the liver and extrahepatic tissues does not seem to have been fully explored as yet. In addition, the amount of tissues available is not always sufficient. Therefore, a simpler procedure for the purification of SCP$_2$ with a high yield is required.

TSK-GEL Toyopearl, a semi-rigid hydrophilic polymer, has been shown to provide high resolution of protein mixtures and good recoveries on a preparative scale[12,13]. Among Toyopearl gel derivatives, TSK-GEL ion exchanger is reported to

[a] Preliminary data were presented at the 8th International Congress of Endocrinology held in Kyoto, in July, 1988.

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be suitable for large-scale and high-speed resolution. Gel permeation on Toyopearl HW60 is said to have a small but significant ion-exchange effect on the separation of low-molecular-weight proteins[12]. This investigation was performed to study the application of Toyopearl gels to the preparative separation of SCP_2. We have established a simple and highly efficient method for the purification of SCP_2 using CM-Toyopearl 650M ion exchanger and Toyopearl HW60S for gel permeation. We describe here the application of TSK-Toyopearl gels to the chromatographic separation of SCP_2 and some properties of the protein obtained.

EXPERIMENTAL

Reagents

7-Dehydrocholesterol was purchased from Aldrich (Milwaukee, WI, U.S.A.) and NADPH from Oriental Yeast (Osaka, Japan). TSK-GEL Toyopearl CM-650M and Toyopearl HW60S were obtained from Tosoh (Tokyo, Japan). All of the reagents for amino acid analysis were obtained from E. Merck (Darmstadt, F.R.G.). Other chemicals were of analytical-reagent grade and obtained from Wako (Osaka, Japan).

Sample preparation

Adult male Sprague–Dawley rats (300–350 g) were killed by decapitation. The livers were washed with ice-cold 15 m*M* potassium phosphate buffer (pH 6.8) containing 250 m*M* sucrose, 1 m*M* EDTA and 1 m*M* dithiothreitol and homogenized in two volumes of the same buffer. The supernatant obtained after centrifugation at 20 000 *g* was centrifuged twice at 105 000 *g* at 4°C for 60 min. The resulting supernatant (S_{105}) and buffer-washed microsomes were used for the purification of SCP_2 and the assay of SCP_2 activity, respectively.

Assay of SCP_2 activity

SCP_2 activity was determined by measuring the activation of 7-dehydrocholesterol reductase [E.C. 1.3.1.21] according to the method of Noland *et al.*[9] with minor modifications. Each assay mixture contained 50 μM 7-dehydrocholesterol, microsomes (2 mg of protein), 0.5 m*M* NADPH, SCP_2 in various concentrations, 15 m*M* potassium phosphate buffer (pH 6.8) containing 5 m*M* 2-mercaptoethanol and 10% (v/v) glycerol in a total volume of 1 ml. After a preincubation for 6 min, the reaction was started by the addition of 7-dehydrocholesterol and maintained under a nitrogen atmosphere at 37°C for 1 h. After the reaction had been terminated by the addition of 1 ml of 30% (w/v) potassium hydroxide solution and 2 ml of ethanol, sterols were extracted twice with 4 ml of light petroleum. The solvent was evaporated and the residue was dissolved in 750 μl of cyclohexane. The concent of 7-dehydrocholesterol was determined by three-wave quantitative calculation at 276, 282 and 290 nm, using a Hitachi Model 220A double-beam spectrophotometer.

Chromatography

All procedures were carried out at 0–4°C. The S_{105} from rat liver (460 ml) was diluted with the same volume of 15 m*M* potassium phosphate buffer (pH 6.8) containing 5 m*M* 2-mercaptoethanol and 10% (v/v) glycerol and then applied to a CM-

Toyopearl 650M column (20 × 8 cm I.D.) equilibrated with the same buffer. The flow-rate was 100 ml/min. The column was washed with 2 l of the same buffer and the protein was eluted with 3 l of 60 m*M* potassium phosphate buffer (pH 6.8) containing 5 m*M* 2-mercaptoethanol and 10% (v/v) glycerol. The eluate was concentrated to 250 ml using a Lab Cassette system equipped with eight cassettes of PT-10 000 membrane (Millipore, Bedford, MA, U.S.A.), and further concentrated to 50 ml using an Amicon ultrafiltration cell with a YM-5 membrane (Amicon, Danvers, MA, U.S.A.).

The concentrated fraction from the CM column was separated by gel permeation chromatography using Toyopearl HW60S. The effect of the ionic strength of the buffer on the separation and the recovery of SCP_2 was examined using 15, 30, 45 and 60 m*M* potassium phosphate buffer (pH 6.8) containing 5 m*M* 2-mercaptoethanol and 10% (v/v) glycerol. The concentrated fraction from the CM column (3 ml) was applied to a Toyopearl HW60S column (40 × 2 cm I.D.) equilibrated with eluting buffer as mentioned above. The flow-rate was 0.45 ml/min and 1.8-ml fractions were collected and the SCP_2 activity of each fraction was assayed. The UV absorption of column effluent was monitored at 280 nm.

For the preparative scale, the concentrated fraction (50 ml) was applied to a Toyopearl HW60S column (100 × 4 cm I.D.) equilibrated with 45 m*M* potassium phosphate buffer (pH 6.8) containing 5 m*M* 2-mercaptoethanol and 10% (v/v) glycerol. The flow-rate was 2.8 ml/min. The column was eluted with the same buffer and 15-ml fractions were collected. The UV absorption of the collected fractions was measured at 220 nm. The fractions containing SCP_2 activity were pooled and concentrated to 2 ml using an Amicon ultrafiltration cell with a YM-5 membrane.

Protein analysis

Size-exclusion high-performance liquid chromatography was performed on a Shodex Protein WS-802.5F column (600 × 8 mm I.D.) with a pre-column (50 × 6 mm I.D.) (Showa Denko, Tokyo, Japan) using 50 m*M* potassium phosphate buffer (pH 6.5) containing 150 m*M* potassium sulphate, 5 m*M* 2-mercaptoethanol and 10% (v/v) glycerol as the mobile phase. The flow-rate was 0.5 ml/min. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[14] using a slab gel consisting of 10–20% gradient separating gel. Molecular weight marker proteins, a mixture of monomer and polymers of cytochrome C (Oriental Yeast), were used. Gels were stained using Coomassie Brilliant Blue R-250. Analytical isoelectric focusing was performed using a LKB Ampholine polyacrylamide gel plate (pH 3.5–9.5). p*I* determinations were made with an isoelectric focusing p*I* calibration kit, pH 3–10 (Pharmacia, Piscataway, NJ, U.S.A.). For amino acid analysis, the sample was injected into a reversed-phase HPLC system equipped with a Nucleosil $5C_{18}$ octadecylsilica (250 × 4.6 mm I.D.) (Ishizu, Osaka, Japan) for removing 2-mercaptoethanol, glycerol and potassium phosphate buffer. The sample was hydrolyzed with 3 *N* mercaptoethanesulphonic acid at 110°C for 44 h. The hydrolysate was analysed on a Hitachi Model 835 amino acid analyser. Protein concentrations were determined by the method of Bradford[15] with bovine γ-globulin as a standard.

RESULTS AND DISCUSSION

Ion-exchange chromatography on CM-Toyopearl 650M

A 460-ml volume of S_{105} was processed on a CM-Toyopearl 650M column. SCP_2 was eluted from the column with 60 m*M* phosphate buffer. In contrast to soft gel ion exchangers such as CM-cellulose, CM-Toyopearl 650M is applicable to the high-speed separation of viscous samples such as S_{105} containing 10% (v/v) glycerol, and thereby seems to be suitable for the first step of the purification. In this study, a high flow-rate (100 ml/min) was achieved even in the presence of 10% (v/v) glycerol and the original supernatant was processed successfully within 1 h. The eluate from the CM column was concentrated to 50 ml. Thus, SCP_2 was purified 16-fold with 129% recovery in the initial step.

Gel permeation chromatography on Toyopearl HW60S

Fig. 1 shows the effect of the ionic strength of eluting buffer on the elution profile and the recovery of SCP_2 from the Toyopearl HW60S column. There was no significant effect of ionic strength on the retention of the protein peak at 280 nm. In contrast, the retention time of SCP_2 activity was increased as the molarity of the buffer was reduced from 60 to 15 m*M*. Baseline separation of SCP_2 activity from the protein peak at 280 nm was achieved at less than 30 m*M*. On the other hand, the recovery of SCP_2 activity was decreased on reducing the ionic strength of the buffer. The recovery of SCP_2 activity at 60, 45, 30 and 15 m*M* were 94.7, 68, 51.4 and 30.6%, respectively. A concentration of 45 m*M* was chosen for the eluting buffer in order to optimize the separation and recovery of the protein.

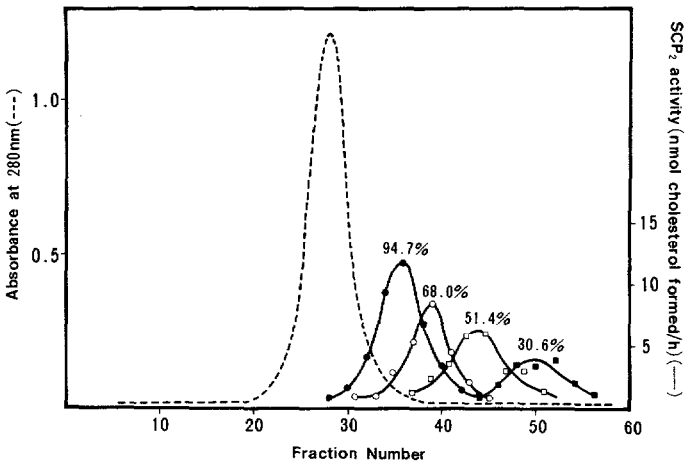

Fig. 1. Effect of buffer ionic strength on elution profile and recovery of SCP_2. A 3-ml volume (33.6 mg of protein) of the concentrated fraction from CM-Toyopearl 650M was applied onto a Toyopearl HW60S column (40 × 2 cm I.D.) equilibrated with (●) 60, (○) 45, (□) 30 and (■) 15 m*M* potassium phosphate buffer (pH 6.8) containing 5 m*M* 2-mercaptoethanol and 10% (v/v) glycerol. The column was eluted with the same buffer at a flow-rate of 0.45 ml/min and 1.8-ml fractions were collected. Dashed line, absorbance at 280 nm; solid lines, SCP_2 activity. Percentage values indicate the recovery of SCP_2 activity using each ionic strength of elution buffer.

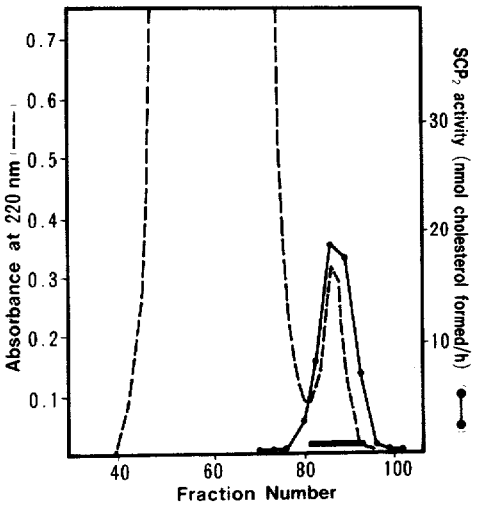

Fig. 2. Elution profile on Toyopearl HW60S. A 50-ml volume (1340 mg of protein) of the concentrated fraction from CM-Toyopearl 650M was applied to a Toyopearl HW60S column (100 × 4 cm I.D.) equilibrated with 45 m*M* potassium phosphate buffer (pH 6.8) containing 5 m*M* 2-mercaptoethanol and 10% (v/v) glycerol. The column was eluted with the same buffer at a flow-rate of 2.8 ml/min and 15-ml fractions were collected. Dashed line, absorbance at 220 nm; solid line, SCP_2 activity. The marker bar indicates the fractions which were pooled for the following step.

For the preparative separation of SCP_2, the concentrated fraction (50 ml) from the CM column was injected on to the preparative Toyopearl HW60S column (100 × 4 cm I.D.). As shown in Fig. 2, SCP_2 was eluted as a small and symmetrical peak and was separated from a large protein peak. The pooled fractions were concentrated to 2 ml with an Amicon ultrafiltration cell with a YM-5 membrane. There was a 303-fold purification and 54% recovery in this step. Table I shows the results for the specific activities and recoveries during the purification of the protein. The protein was purified 4969-fold with a 69% yield.

Properties of the purified protein

The final preparation of the protein eluted as a single and symmetrical peak on high-performance gel permeation, which coincided exactly with the SCP_2 activity.

TABLE I

PURIFICATION OF STEROL CARRIER PROTEIN$_2$ FROM RAT LIVER

Sample	*Volume (ml)*	*Total activity (units)*[a]	*Total protein (mg)*	*Specific activity (nmol cholesterol formed/h/mg protein)*	*Yield (%)*	*Purification factor (fold)*
S_{105}	460	27 123	22 310	1.216	100	1
CM-Toyopearl 650M	50	34 951	1755	19.9	129	16
Toyopearl HW60S	2.1	18 714	3.1	6037	69	4969

[a] One unit of SCP_2 activity is defined as the activity that causes an increment of 1 nmol/h in the conversion of 7-dehydrocholesterol to cholesterol.

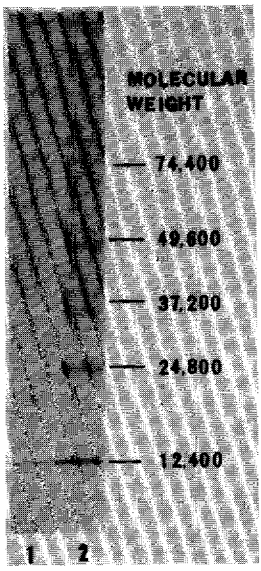

Fig. 3. SDS-PAGE of the purified protein (lane 1) and standard proteins (lane 2).

The protein also gave a single protein band on SDS-PAGE (Fig. 3). These results indicate that the purified protein was homogeneous. The molecular weight of the purified protein was 12 300 as determined by SDS-PAGE. The protein had a p*I* of 9.1 as determined by analytical isoelectric focusing. The most abundant amino acid was lysine which accounted for 16.5 mol-%. The protein contained no arginine or tyrosine and only small amounts of histidine and tryptophan. The correlation coefficient of the amino acid composition was 0.996 between the purified protein and SCP_2 reported by Noland *et al.*[9]. Hence, the purified protein is thought to be identical with SCP_2 as purified by Noland *et al.*[9]. The UV spectrum of the protein showed a low absorbance at 280 nm, as expected from the low tyrosine and tryptophan contents. The maximum absorbance occurred at 216 nm. Therefore, monitoring effluent at *ca.* 220 nm, as opposed to 280 nm, appears to give a more exact approximation during gel permeation chromatography.

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