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EFFICIENT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM FOR PROTEIN PURIFICATION

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SUMMARY

An efficient high-performance liquid chromatographic system, consisting of an affinity column and a high-performance size-exclusion column, was developed and applied to the purification of growth hormone receptors from rabbit livers. When a 6-ml sample of Triton X-100 extracts containing 16 mg of protein was applied to the system, 1200-fold purified receptor with a 10% recovery of binding activity from homogenates was obtained within 3–4 h. The purified receptor exhibited one main band on sodium dodecyl sulphate polyacrylamide gel electrophoresis, and the affinity constant ($K_a = 6.0 \cdot 10^9\ M^{-1}$) was found to be comparable with that of 1% Triton X-100 extract ($4.4 \cdot 10^9\ M^{-1}$). The injection of 1 ml of 3 *M* urea solution prior to receptor elution with 10 ml of 6 *M* urea solution was effective in removing non-specific binding proteins.

INTRODUCTION

A simple systematic method for protein purification is required for the production of antibodies and to determine amino acid sequences. Protein purification by tandem high-performance liquid chromatography (HPLC) should

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decrease purification times and offer an efficient method applicable even to 'intractable materials', such as membrane proteins. To this end, we previously developed an HPLC system that combined an affinity column directly with a size-exclusion column [1]. The desalting procedure was instantly carried out using the latter. Consequently, it was thought that a high receptor activity was detected.

This report verifies the high efficiency of the system, and describes the recovery of binding activity, the purification factor and the results of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the effect of column washing. The results were compared with those of the conventional open-column method in the purification of growth hormone receptor.

EXPERIMENTAL

Human growth hormone (hGH) was a gift from Sumitomo Chemicals (Osaka, Japan) and AF-formyl Toyopearl from Tosoh (Tokyo, Japan). Lactoperoxidase was obtained from Sigma (St. Louis, MO, U.S.A.) and $Na^{125}I$ from Amersham Japan (Tokyo, Japan). Sephadex G-100 and relative molecular mass (M_r) markers for SDS-PAGE were purchased from Pharmacia (Uppsala, Sweden). All other chemicals obtained from Wako Pure Chemicals (Osaka, Japan) and Nakarai Chemicals (Kyoto, Japan) were used as supplied.

Preparation of GH receptor from rabbit liver

A crude membrane fraction containing GH receptor was prepared from the liver of a female rabbit (ca. 2 kg), according to the method of Tsushima et al. [2]. Briefly, Triton X-100 (final concentration 1%, v/v) was added to a crude membrane fraction and the mixture was stirred for 1 h at room temperature, then centrifuged at 100 000 *g* for 30 min. The supernatant was collected and stored at −30 °C until use.

Radioiodination of hGH

Iodination of hGH was carried out according to the procedure reported by Tsushima et al. [2]. The specific activity was 75–110 Ci/g.

Binding studies

Binding studies of rabbit liver membrane and solubilized receptors were carried out by the method of Tsushima et al. [2], with minor modifications as follows. All dilutions were done with 50 m*M* Tris–HCl buffer containing 0.1% bovine serum albumin (BSA) and 30 m*M* $MgCl_2$ (pH 7.4).

Preparation of hGH affinity column

A 1-mg amount of hGH was coupled at pH 7.0 to 1 g (wet gel) of AF-formyl Toyopearl according to the manufacturer's instructions. The amount of hGH

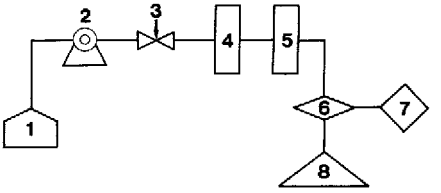

Fig. 1. HPLC system: 1 = mobile phase; 2 = pump; 3 = loop injector; 4 = affinity column; 5 = high-performance size-exclusion column; 6 = UV detector; 7 = recorder; 8 = fraction collector.

immobilized on the gel was 0.5 mg/g (wet gel), which was determined spectrophotometrically. The affinity gel bonded with hGH was packed into a 250 mm × 4.6 mm I.D. column.

High-performance liquid chromatography

The affinity column prepared was connected directly with a high-performance size-exclusion column (TSK 3000SW). The HPLC system is shown in Fig. 1. The Triton X-100 extract containing the GH receptor was diluted with an equal volume of 50 m*M* Tris–HCl buffer (pH 7.4) containing 20 m*M* $MgCl_2$ and 0.4 *M* NaCl. The fine particulate matter was removed by filtration with a membrane filter (0.45 μm). The Triton X-100 extract was injected through a large-volume loop injector (10 ml). The mobile phase was 50 m*M* Tris–HCl buffer (pH 7.4) containing 0.1% Triton X-100, 0.2 *M* NaCl, 10 m*M* $MgCl_2$ and 0.3 m*M* phenyl methyl sulphonyl fluoride. The flow-rate was 0.1 ml/min for the adsorption of protein for 60 min, then raised to 0.5 or 0.6 ml/min. A 1-ml volume of 3 *M* urea in the same buffer was injected after no protein was eluted, in order to remove non-specific proteins from the affinity column. After complete elution of these proteins, 10 ml of 6 *M* urea in the same buffer was injected. The UV absorption of the eluate was monitored at 280 nm, and the fractions were collected at 5-min intervals. The specific binding was assessed using 200 μl of each fraction and 40 000–50 000 cpm of ^{125}I-labelled hormone.

Protein assays

Protein concentrations of the crude membrane and Triton X-100 extract were estimated by a modification of the method of Lowry et al. [3]. The protein concentration of purified receptors was estimated by the method of Bohlen et al. [4], using fluorescamine.

SDS-PAGE

SDS-PAGE was carried out on 7.5% acrylamide gels (1 mm thick) according to the method of Laemmli [5]. SDS gels were stained with silver, using a Wako silver stain kit.

RESULTS

Purification of GH receptor by HPLC

Triton X-100 extract containing GH receptors was applied to the HPLC system consisting of a high-performance affinity column and a size-exclusion column. Fig. 2 shows the elution profile of GH receptor following the injection of 6 *M* urea solution (10 ml). After elution of unretained proteins between 70 and 120 min, 10 ml of 6 *M* urea solution were injected at 190.5 min. A major active peak (marked with an asterisk in Fig. 2), representing hGH binding activity, which emerged 23 min later (213.5 min), had weak absorption at 280 nm. The sharp peak detected at 280 nm at 222 min is due mainly to the blank peak containing Triton X-100.

Scatchard analysis of purified receptor

The eluent containing GH receptor was fractionated and submitted to the [^{125}I]hGH binding assay for Scatchard analysis [6]. Fig. 3 shows the Scatchard plots for the initial homogenates, Triton X-100 extracts and purified receptor. The affinity constant (K_a) calculated from the slopes in Fig. 3 was $1.5 \cdot 10^9\ M^{-1}$ for the initial homogenates, $4.4 \cdot 10^9\ M^{-1}$ for the Triton X-100 extract (before chromatography) and $6.0 \cdot 10^9\ M^{-1}$ for the purified receptor (after chromatography). This means that the affinity constant changed during the Triton X-100 extraction, but hardly changed during the HPLC purification.

Table I gives the purification factor and recovery obtained by these procedures. The actual purification obtained by a single application of the present

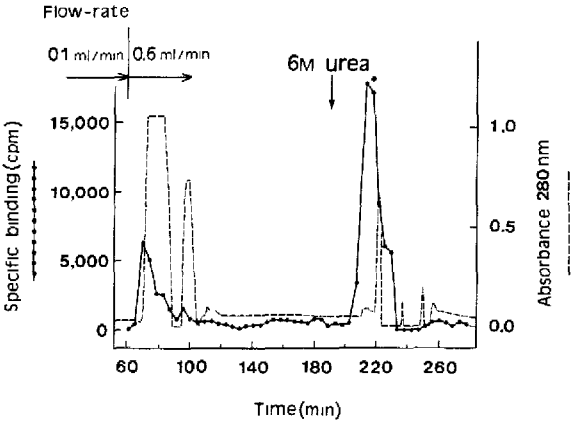

Fig. 2. Elution profile of GH receptor by affinity chromatography and size-exclusion HPLC. Flow-rate, 0.1 ml/min (0–60 min) then 0.6 ml/min, sample, 16 mg of protein per 6 ml of Triton X-100 extract; affinity column, 150 mm × 4.6 mm I.D. (hGH-Formyl Toyopearl); high-performance size-exclusion column, 600 mm × 7.6 mm I.D. (TSK 3000SW).

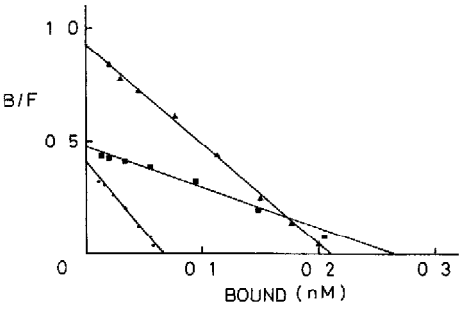

Fig. 3. Scatchard analysis of the binding activity. The ratio of bound to free [^{125}I]hGH was plotted against the concentration of bound hGH in each preparation: (▲) purified GH receptors; (●) Triton X-100 extract; (■) initial homogenates.

TABLE I

PURIFICATION FACTORS AND RECOVERIES

All data based on Fig. 3. For protein estimates of individual fractions, see Experimental.

Fraction	Specific activity (pmol/mg)	Purification factor	Recovery (%)
Whole homogenate	0.120	1	100.0
Triton X-100 extract	0.786	6.55	28.4
HPLC system 1	152	1280	10.1
HPLC system 2	125	1040	9.8

method was ca. 200-fold, and the recovery was ca. 35%. The results of the two independent experiments indicate satisfactory reproducibility.

Effect of column washing by 3 M urea

Fig. 4 shows the elution profiles of GH receptor obtained by washing with 3 *M* urea (1 ml) followed by injection of 6 *M* urea (10 ml). Compared with the major active peak at 213.5 min in Fig. 2, the peak at 425 min (marked with an asterisk in Fig. 4) eluted after injection of 6 *M* urea indicated the weaker 280-nm UV absorption. This suggested that the washing with 1 ml of 3 *M* urea solution effectively removed non-specifically retained protein.

The purity of the isolated GH receptor was examined by SDS-PAGE under reducing conditions. The results are shown in Fig. 5. In lanes B and C, the single main band was observed at M_r 66 000, indicating that the GH receptor was highly purified. Comparison of the results shown in lane B and lane C indicates that the washing with 3 *M* urea solution effectively removed non-specific binding proteins. When 4 ml of the Triton X-100 extract containing 11 mg of protein were applied, the purified receptor was obtained containing as much as 50 μg of protein, which is shown in lane C in Fig. 5.

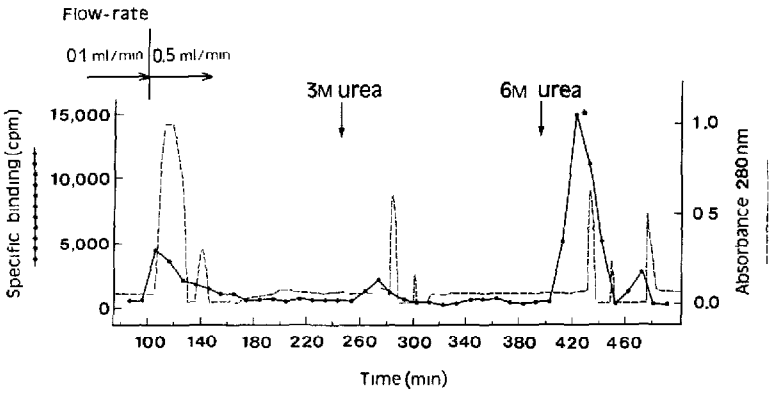

Fig. 4. Elution profile of GH receptors by affinity chromatography and size-exclusion HPLC following 3 *M* urea column washing. Flow-rate, 0.1 ml/min (0–100 min) then 0.5 ml/min; sample, 11 mg of protein per 4 ml of Triton X-100 extract; other conditions as in Fig. 2.

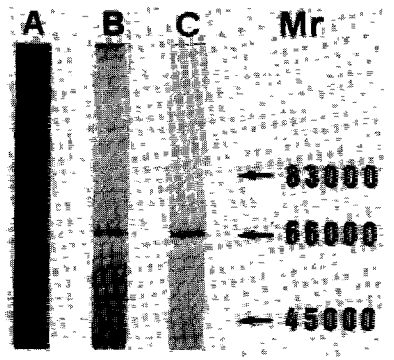

Fig. 5. 7.5% SDS-PAGE of GH receptors: lane A, Triton X-100 extract (80 μg); lane B, purified GH receptors (2 μg); lane C, purified GH receptors after 3 *M* urea column washing (9 μg).

DISCUSSION

In the previous reports [7,8] dealing with the purification of GH receptor by the conventional multiple open-column method, the final recovery of binding activity was found to be less than 1% when Triton X-100 was used for solubilization. A similar degree of purification (1200-fold) could be achieved with a 10% recovery by this HPLC system within 3–4 h. It was reported previously that ca. 50% of binding activity of GH receptor was inactivated during storage in 6 *M* urea solution for 30 min [1]. On account of the rapid desalting on the size-exclusion column in the present method, the denaturation seemed to be minimized. Continuous purification may help to a great extent to decrease the loss of receptor protein content. Conversely, in the conventional

open-column method, considerable amounts of receptor may be inactivated by 6 *M* urea solution and also may be lost during affinity chromatography, desalting procedure, isoelectric focusing chromatography and size-exclusion chromatography. The high reproducibility obtained in two independent experiments results from simple procedures and the strict control of flow-rate and pressure in the HPLC apparatus. As shown in lane C in Fig. 5, the purified receptor containing 50 μg of protein was obtained in a single analysis. This indicates that the scarce protein can be sufficiently purified, although the affinity column and the size-exclusion column were used on an analytical scale in the present system.

We believe that the present HPLC system is helpful for rapid purification of proteins, owing to its simple procedure and its applicability to other types of biointeraction, such as immunoaffinity.

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