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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM FOR THE RAPID PURIFICATION OF GROWTH-HORMONE RECEPTOR IN RABBIT LIVERS

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SUMMARY

A system consisting of high-performance affinity chromatography and size-exclusion chromatography has been developed for the rapid purification and isolation of relatively labile membrane proteins, such as growth-hormone receptor. The crude membrane sample containing growth-hormone receptor was obtained from rabbit livers by ultracentrifugation, followed by solubilization with Triton X-100. The sample was introduced into the high-performance affinity gel column through a large-volume loop injector. After removal of unretained proteins, the fraction containing the growth-hormone receptor was eluted with 6 *M* urea solution. The eluate from the affinity column passed directly into the size-exclusion gel column, where rapid desalting and separation from contaminants were achieved. The eluate was monitored by UV absorption at 280 nm and fractionated for the subsequent binding assay with ^{125}I-labelled human growth hormone.

INTRODUCTION

Affinity chromatography has frequently been used as an effective method for the purification of membrane receptors from a variety of contaminant proteins. However, a difficulty is often encountered in cases of strong affinity, such as between receptor and hormone[1,2] and between avidin and biotin[3], where the adsorbed species are not effectively eluted by a specific elution method, but by a non-specific method with the use of urea or guanidinium salt solutions as denaturing agents. The use of denaturants at high concentration tends to deform the tertiary structure of proteins, resulting in low recoveries of activity. In order to avoid such difficulties, it is desirable for the active substances to be desalted as quickly as possible.

A site of the receptor specific for human growth hormone was identified in a

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cultured human lymphocytes[4], and Tsushima and Friesen[5] developed a sensitive radiolabelling assay method for growth hormone, using the particulate membrane fraction from the liver of pregnant rabbits. The specific binding sites for growth hormone have also been demonstrated in human livers[6] and pregnant mice[7]. Recently, the receptors for a variety of polypeptide hormones have been solubilized and partially purified for the production of their antibodies[8–13]. The partial purification and characterization of growth-hormone receptor was reported by Tsushima and Friesen[14].

The aim of this work was to develop a high-performance liquid chromatographic (HPLC) system that permits the rapid purification of relatively labile membrane proteins, such as growth-hormone receptor. The system, consisting of a high-performance affinity column and a size-exclusion column connected in series, minimizes the contact of the receptor with the denaturing agent, so that the lowering of the binding activity is markedly restricted and separation is achieved in a short period of time.

EXPERIMENTAL

Hormone and chemicals

Human growth hormone was a gift from Sumitomo Chemicals (Osaka, Japan). Lactoperoxidase was obtained from Sigma (St. Louis, MO, U.S.A.), bovine serum albumin from Armour (Chicago, IL, U.S.A.) and $Na^{125}I$ from Amersham Japan (Tokyo, Japan). Molecular-weight markers used for the calibration in size-exclusion chromatography were the products of Oriental Yeast (Tokyo, Japan). All other chemicals were purchased from Wako (Osaka, Japan) and Nakarai Chemicals (Kyoto, Japan) and were used as supplied.

Preparation of crude growth-hormone receptor

A crude membrane fraction containing growth hormone receptor was prepared from the liver of a rabbit (female, *ca.* 2 kg) according to the method reported by Tsushima and Friesen[14]. The protein concentration of the suspension was adjusted to 2–5 mg/ml and Triton X-100 was added to a final concentration of 1% (v/v). The fraction was stored at −20°C until used.

Binding studies

Human growth hormone was iodinated with $Na^{125}I$ according to the method of Thorell and Johansson[15]. The specific activity was 20–80 Ci/g. Binding studies were carried out according to the method of Tsushima and Friesen[14].

Preparation of the affinity column

The affinity gel was prepared by coupling 1 mg of human growth hormone to 1 g (wet gel) of AF-formyl Toyopearl (Toyo Soda, Tokyo, Japan) at pH 7 according to the manufacturer's instructions. The amount of human growth hormone immobilized on the gel was 0.5 mg/g (wet gel), as determined spectrometrically. The affinity gel thus prepared was packed into a 25 cm × 4.5 mm I.D. column.

High-performance liquid chromatographic system

The affinity column and the size-exclusion column were directly connected in

series. The crude membrane sample containing growth-hormone receptor was diluted with an equal volume of 50 m*M* Tris–HCl buffer solution (pH 7.4) containing 0.2 *M* sodium chloride and 10 m*M* magnesium chloride and injected through a large-volume loop injector. The mobile phase was 50 m*M* Tris–HCl buffer solution (pH 7.4) containing 0.1% Triton X-100. The flow-rate of the mobile phase was 0.1 ml/min for the first 60 min, then increased to 0.5 ml/min. A 10-ml portion of 6 *M* urea solution was injected after complete elution of unretained proteins. The eluate from the size-exclusion column was monitored by UV absorption at 280 nm and fractionated every 3 min. The specific binding of ^{125}I-labelled human growth hormone was determined, using 200 μl of each fraction and 50 000 cpm of the labelled hormone.

RESULTS AND DISCUSSION

Separation characteristics of size-exclusion chromatography

The separation characteristics of the size-exclusion column with the mobile phase containing 0.1% Triton X-100 was determined by using the following proteins as molecular-weight markers: cytochrome *c* (mol. wt. 12 400), adenylate kinase (32 000), enolase (67 000), lactate dehydrogenase (142 000) and glutamate dehydrogenase (290 000). The plot of log (molecular weight) *versus* elution volume was reasonably linear, as shown in Fig. 1. A crude membrane fraction containing the growth-hormone receptor was applied to the column. The mobile phase used was 50 m*M* Tris–HCl buffer solution (pH 7.4) containing 0.1% Triton X-100 and 0.2 *M* sodium chloride. The elution profile is shown in Fig. 2, where the active fraction was eluted between 10 and 18 ml. The shoulder on the peak may be due to the aggregated growth-hormone receptor. From these results, the apparent molecular weight of growth-hormone receptor from rabbit livers was estimated to be *ca.* 240 000 (elution volume 15.3 ml).

Effect of urea solution on binding activity

The stability of the growth-hormone receptor in 6 *M* urea solution, which was used for protein desorption in affinity chromatography, was investigated in a batch experiment. A crude membrane fraction was exposed in 6 *M* urea–50 m*M* Tris–

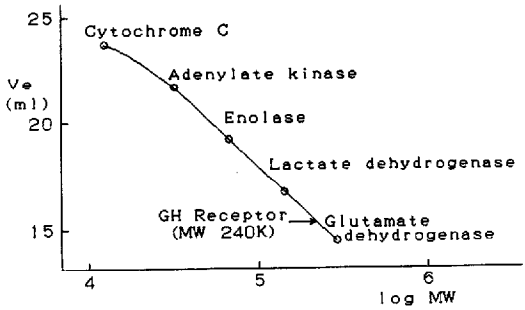

Fig. 1. Relationship between elution volume and log MW of standard proteins on the size-exclusion column.

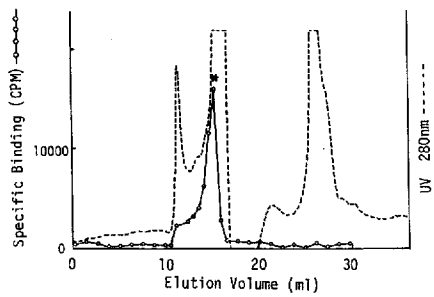

Fig. 2. Elution profile of growth-hormone receptor (marked with an asterisk) on a single size-exclusion column.

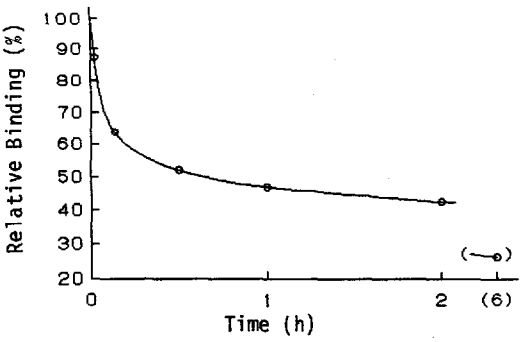

Fig. 3. Effect of 6 *M* urea solution on the binding activity of growth-hormone receptor.

HCl–0.1% Triton X-100 for various time periods at 25°C, and the incubation mixture (200 μl) was chromatographed on the size-exclusion column. The eluate was fractionated and subjected to binding activity measurement with ^{125}I-labelled human growth hormone. The control experiment was conducted with the use of membrane fraction that had not been incubated in 6 *M* urea solution. The results in Fig. 3 indicate that the binding activity was decreased to about half of the control within the first 30 min. The activity was irreversibly lost, because it was not recovered even in the urea-free solution.

High-performance affinity–size-exclusion chromatographic system

The chromatogram obtained by the above-mentioned method is shown in Fig. 4, the amount of sample applied being about 10 mg of protein per injection. The flow-rate of the mobile phase was maintained at 0.1 ml/min for the first 60 min in order to complete the affinity adsorption, then increased to 0.5 ml/min to eliminate

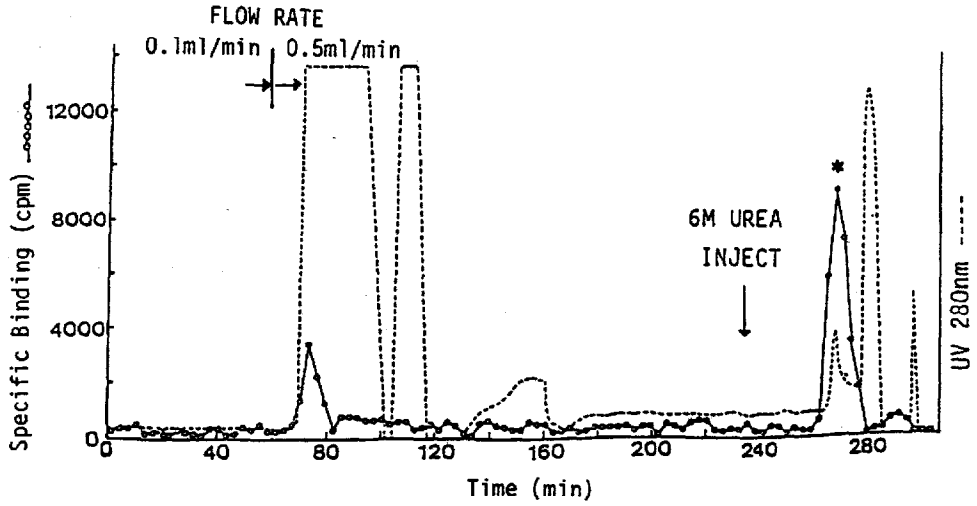

Fig. 4. Elution profile of growth-hormone receptor (marked with an asterisk) in high-performance affinity–size-exclusion chromatography.

the unretained proteins, which were eluted between 70 and 160 min. After elution of unretained proteins, 10 ml of 6 *M* urea solution were injected through a large-volume loop injector. A major active peak, apparently representing growth-hormone receptor, emerged 32 min later. Evidently, the active fraction, which was eluted from the affinity column, entered the size-exclusion column on average within 1.4 min, because the elution volume of growth-hormone receptor on the single size-exclusion column was 15.3 ml (retention time 30.6 min at a flow-rate of the mobile phase of 0.5 ml/min), as shown in Fig. 2. Thus, the denaturation caused by the urea solution was minimized, and rapid separation from contaminants was achieved. The peak that eluted at about 70 min, exhibiting binding activity, may be ascribed to either overloading of the affinity column or the presence of other active proteins. As no peaks were eluted between 160 and 250 min, the total elution time can be reduced by at least 1 h.

In conclusion, the proposed method is suitable for the rapid purification of relatively labile proteins, and the antibody-bound affinity gel column can also be applied to the present system.

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