*Journal of Chromatography*, 616 (1993) 327–332
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6852

# Short Communication

# High-recovery protein purification by high-performance gel-permeation chromatography: application to human serum biotinidase

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(First received December 7th, 1992; revised manuscript received March 12th, 1993)

ABSTRACT

A reproducible, high-yield high-performance gel-permeation chromatographic method for proteins was developed and applied to the final step of purification of human serum biotinidase. One diol-type silica gel column, Tosoh TSK-gel 3000 SW (300 mm × 7.9 mm I.D.; average pore size 30 nm), and two Jasco Bio-Fine GFC SI 150-K columns (300 mm × 7.9 mm I.D.; average pore size 15 nm) were connected in series. A 0.1 *M* sodium phosphate buffer (pH 6.0) solution containing 0.3 *M* sodium chloride, glycerol (2.5%, v/v) and the non-ionic detergent Nonidet P-40 (NP-40, 0.15%, v/v) was utilized as an eluent. Recovery of the protein bovine serum albumin from the separating columns was measured by a spectrophotometric method and found te be 77.0 ± 3.74% (mean ± S.D.). The recovery of the total activity of human serum biotinidase was 72.6 ± 13.0%. Since biotinidase activity was not recovered from the column in the absence of NP-40, the introduction of this non-ionic detergent to the mobile phase was shown to be essential for the final purification step of human serum biotinidase.

## INTRODUCTION

Human serum biotinidase (EC 3.5.1.12; BIN), a thiol-type enzyme, has been recently isolated and found to be a 76-kDa glycoprotein [1–3].

High-performance gel-permeation chromatography (HPGPC) was shown to be a suitable and easy to perform protein separation method compared with the reversed-phase high-performance liquid chromatography system (RP-HPLC) [4]. However, HPGPC gel (diol-type silica gel) sometimes shows relatively strong hydrophobic binding ability for some apparently hydrophilic proteins (unpublished personal observations). Hydrophilicity of human serum biotinidase, as calculated from the amino acid composition [2] according to Capaldi and Vanderkooi [5], is relatively high (46.5%). However, since enzyme

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0378-4347/93/$06.00 

activity could not be recovered from the HPGPC column, hydrophobic interactions between biotinidase and diol gel cannot be excluded. High-recovery purification of human serum biotinidase was achieved when the non-ionic detergent Nonidet P-40 (NP-40) was included in the eluent of the HPGPC system. A protein recovery test from the column using bovine serum albumin (BSA) as the reference protein was also performed.

## EXPERIMENTAL

### *Chemicals and reagents*

BSA, γ-immunoglobulin (IgG), ovalbumin from hen egg white, carbonic anhydrase from bovine erythrocytes, α-chymotrypsinogen A from bovine pancreas, soy bean trypsin inhibitor, α-lactalbumin from bovine milk, ribonuclease A and biotinyl-4-amino-benzoate (BPAB) were purchased from Sigma (St. Luis, MO, USA). Nonidet P-40 (NP-40) was purchased from Nacalai Tesque (Kyoto, Japan). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) molecular mass standards (broad range) kit was purchased from Bio-Rad Labs. (Richmond, CA, USA). Gel filtration calibration kit was purchased from Pharmacia (Uppsala, Sweden). Aprotinin was from Takara Shuzo (Kyoto, Japan).

### *Specimens*

Outdated human blood was supplied by the National Children's Hospital (Setagaya-ku, Tokyo, Japan). Serum was obtained by centrifuging the blood at 600 *g* for 15 min at 4°C. Transparent serum was obtained by centrifuging the serum at 40 000 *g* for 12 min at 4°C and was stored at −80°C.

### *HPGPC*

An HPLC pump (Model 2150 HPLC pump with 2152 LC controller; Pharmacia LKB, Uppsala, Sweden) was utilized. Sample injection was carried out with a Model U6K universal liquid chromatography injector (Millipore, Milford, MA, USA, USA) with a 2-ml loop. A line filter (GL Sciences, Tokyo, Japan) was inserted between the injector and the column. One HPGPC diol-type silica gel column, Tosoh TSK-gel 3000 SW (300 mm × 7.9 mm I.D.; average pore size 30 nm), and two Jasco Bio-Fine GFC SI 150-K columns (300 mm × 7.9 mm I.D.; average pore size 15 nm) were connected in series. A 0.1 *M* sodium phosphate buffer (pH 6.0) solution containing 0.3 *M* sodium chloride, glycerol (2.5%, v/v) and the non-ionic detergent Nonidet P-40 (NP-40; 0.15%, v/v) was utilized as an eluent. The flow-rate was 0.8 ml/min. Eluting protein was detected at a wavelength of 290 or 280 nm with a Model 655A variable-wavelength UV monitor (Hitachi, Tokyo, Japan).

### *Biotinidase preparation*

Partially purified biotinidase preparations were obtained essentially as peviously described [1–3]. Serum was sequentially purified with ammonium sulphate fractionation, DEAE-Cellulofine (Chisso, Tokyo, Japan) ion-exchange chromatography, Sephadex G-200 (Pharmacia) GPC and hydroxyapatite HPLC (Koken, Tokyo, Japan) (Fig. 1).

### *Determination of biotinidase activity*

Biotinidase activity was determined by the HPLC–fluorimetric method as previously described [3]. Synthetic substrate of BPAB was used.

### *Protein content*

Protein concentrations were assayed by using a BCA protein assay kit from Pierce (Rockford, IL, USA). BSA was used as a standard protein.

### *Polyacrylamide gel electrophoretic analysis of biotinidase*

SDS-PAGE analysis was performed according to the procedure of Laemmli [6]. Acrylamide concentrations of 7.5 and 12% were used.

### *Recovery tests*

The protein recovery test from the column was performed as described previously [4]. The recovery test for total enzyme activity was similarly carried out.

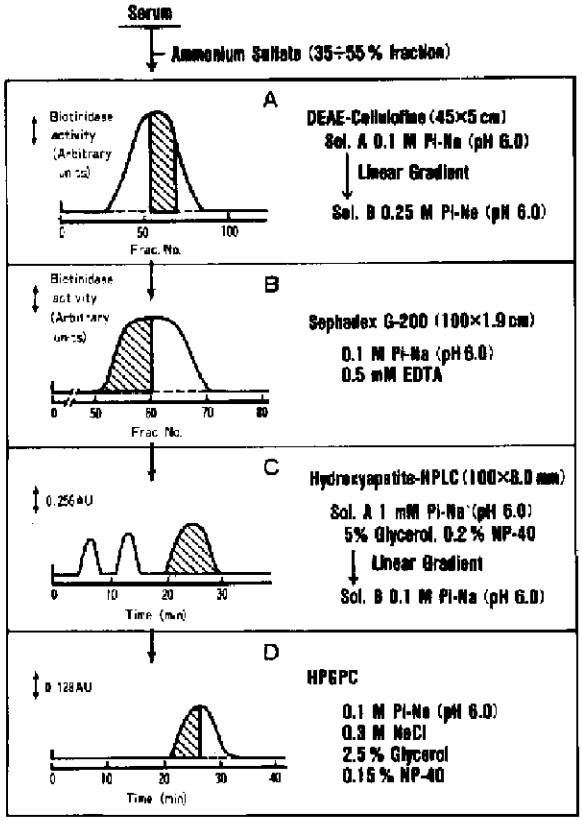


Fig. 1. Purification protocol for human serum biotinidase. Steps A and B: biotinidase activity was measured. Steps C and D: UV absorbance at 290 nm was recorded. The fractions shown as hatched areas were collected for the next step of purification.

## RESULTS AND DISCUSSION

Although the 76-kDa human serum biotinidase has been reportedly purified using a diol-type silica gel HPGPC column [1], our preliminary biotinidase purification trial using a Tosoh TSK 3000-SW column was not reproducible. On the other hand, it has recently been found that recovery of biotinidase activity from the polyacrylamide gel (4.8% acrylamide concentration) of isoelectric focusing is satisfactory only in the presence of non-ionic detergent (Nonidet P-40; 2%, v/v) [3], hence suggesting that the reduction in recovery and/or reproducibility of HPGPC column was due to hydrophobic interactions between biotinidase and the column. Therefore, a mobile phase system including the non-ionic detergent NP-40 was developed. Since Triton X-100 showed relatively stronger inhibitory effect on enzyme activity than NP-40 on a similar enzyme (lipoamidase) [7], we chose NP-40 in this study. Because of high UV absorbance of NP-40 at 280 nm, a low concentration of this detergent (0.15%, v/v) was chosen to optimize detection of proteins. Extremely acidic buffer conditions (pH 2.1) have been shown to increase the separation efficiency of silica gel [4]. However, because of the likely damage of the eluting biotinidase under

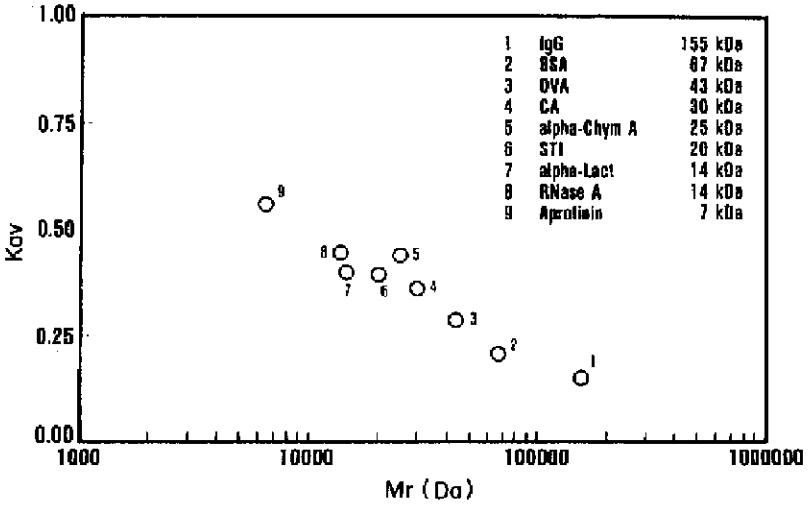


Fig. 2. Calibration curve. $K_{av}$ values were used for the calibration curve. The $K_{av}$ value for each protein was defined and calculated using the following equation: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where $V_e$ = elution volume for the protein, $V_0$ = column void volume = elution volume for Blue Dextran 2000 (exclusion limit) and $V_t$ = total bed volume = inclusion limit for tyrosine.

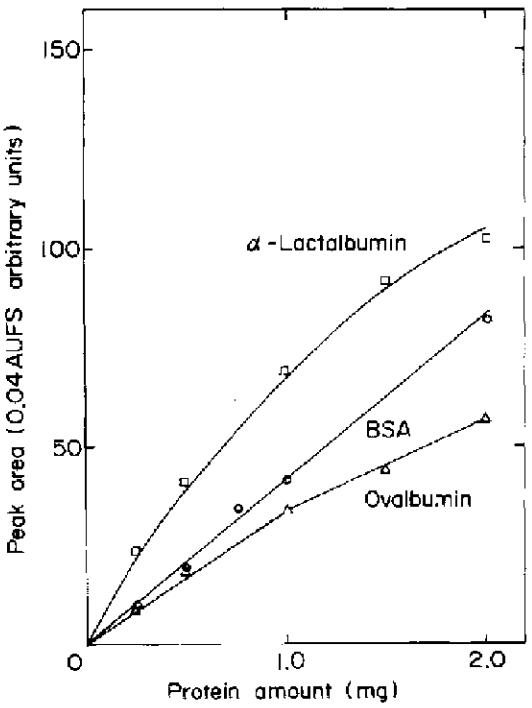


Fig. 3. Relationship between injected protein amounts and area under the peaks for three representative proteins.

such extremely non-physiological conditions, a pH 6.0 phosphate buffer was chosen instead. The addition of 0.3 *M* sodium chloride improved the linearity of the calibration curve. In order to prevent a phase separation phenomenon, 2.5% (v/v) glycerol was finally added.

Under such eluent conditions, adequate peak symmetry and protein separation were obtained (Fig. 2). The total area of the peaks was found to be correlated with the amount of three injected proteins (Fig. 3). From a linear regression analysis, $r$ values of 0.997 ($P = 0.00016$), 0.995 ($P = 0.00049$) and 0.985 ($P = 0.0021$) were obtained for BSA, ovalbumin and α-lactalbumin, respectively, thereby suggesting high recovery from the column. In order to verify this, three recovery tests using BSA as a reference protein were carried out using the spectrophotometric protein assay method. The results obtained are shown in Table I. The protein recovery of BSA was satisfactory with little inter-analysis variation (coefficient of variation 4.86%) using a 900-mm-long column.

TABLE I

PROTEIN RECOVERY OF BSA FROM THE HPGPC SYSTEM

Recovery test and protein determination were performed as described in the Experimental section.

| Test No. | Injected BSA (mg) | Recovered BSA (mg) | Yield (%) |
|---|---|---|---|
| 1 | 6.83 | 5.61 | 82.1 |
| 2 | 6.83 | 5.00 | 73.2 |
| 3 | 6.83 | 5.17 | 75.8 |
| Mean ± S.D. | | | 77.0 ± 3.74 |

TABLE II

RECOVERY OF TOTAL BIOTINIDASE ACTIVITY

Biotinidase activity was measured as described in the Experimental section.

| Test No. | Total activity (nmol/min) | | Yield (%) |
|---|---|---|---|
| | Injected | Recovered | |
| 1 | 24.1 | 16.3 | 67.6 |
| 2 | 2.04 | 1.12 | 55.0 |
| 3 | 0.37 | 0.25 | 67.0 |
| 4 | 0.37 | 0.28 | 74.0 |
| 5 | 8.64 | 8.50 | 98.0 |
| 6 | 4.20 | 3.09 | 74.0 |
| Mean ± S.D. | | | 72.6 ± 13.0 |

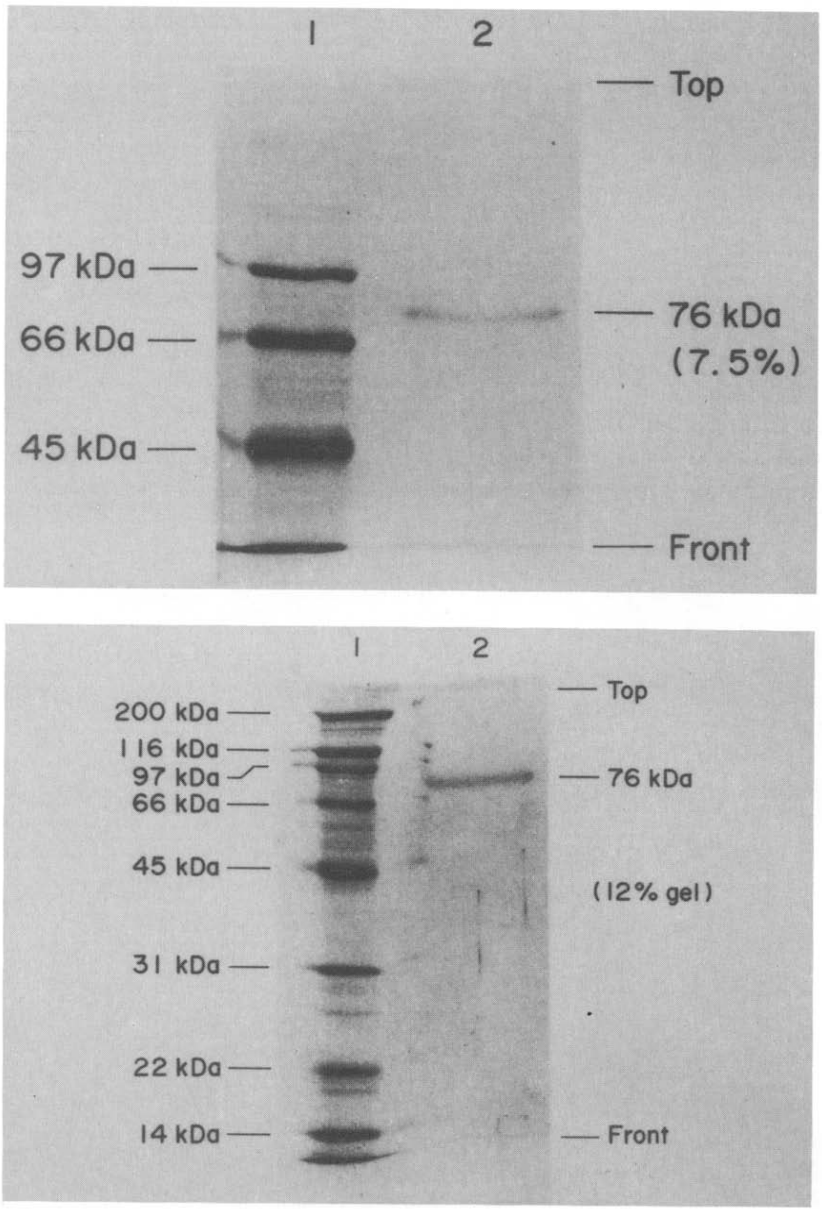


Fig. 4. SDS-PAGE analysis of purified human serum biotinidase. Acrylamide concentrations of 7.5% (upper panel) and 12% (lower panel) were used. Coomassie blue staining was used for protein detection. A 0.003-mg sample of biotinidase was analysed.

Therefore, this separation system was applied to biotinidase purification and HPGPC was used after the hydroxyapatite step (Fig. 1). The results of six recovery tests for total biotinidase activity from the column are shown in Table II. Although different total activities were tested, the recoveries were fairly constant. Furthermore, the mean recovery of total enzyme activity (72.6%, Table II) was similar to the protein recovery value of BSA (77.0%, Table I), which suggests that the elution conditions were gentle enough to retain enzyme activity.

As shown in Fig. 4, purified human serum biotinidase gave a single band of 76 kDa on the

SDS-PAGE system of Laemmli [6]. The purification step was completed within 1 h. Therefore, this HPGPC method was shown to be convenient and reproducible for application to the final step of biotinidase purification. Because of the relatively high yield of protein and enzyme activity, the reported HPGPC method seems potentially applicable to other protein purifications.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Health and Welfare, Japan, and Sumitomo Pharmaceuticals Co., Ltd., Osaka, Japan. One of the authors (C.D.F.) is on a post-doctoral fellowship from the Science and Technology Agency (S.T.A.), Japan.

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