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EFFICIENT PURIFICATION OF HUMAN PLASMA β_2-MICROGLOBULIN FROM THE HAEMODIALYSATE OF A PATIENT WITH CHRONIC RENAL FAILURE BY USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Human plasma β_2-microglobulin was isolated in a good yield (more than 80%) from the haemodialysate (blood ultrafiltrate) of a patient with chronic renal failure. The isolation procedure consisted of Sephadex G-100 gel filtration and two steps of high-performance liquid chromatography: reversed-phase high-performance liquid chromatography and gel permeation chromatography. The purified β_2-microglobulin was homogeneous by sodium dodecyl sulphate polyacrylamide gel electrophoresis and two-dimensional electrophoresis.

INTRODUCTION

Human β_2-microglobulin (β_2-m) is a single polypeptide chain of 11 800 molecular weight, and is normally present in trace amounts in both plasma and urine [1]. It may play an important role in the immune system because of its structural similarities to an immunoglobulin domain C_H3 [2] and its involve-

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ment in class 1 antigens of the major histocompatibility complex [3]. Previously, we isolated human plasma β_2-m from the blood ultrafiltrate of a patient undergoing therapeutic haemodialysis by a combination of Blue-Sepharose 4B, ion-exchange and gel chromatography [4]. Recently, reversed-phase high-performance liquid chromatography (RP-HPLC) and gel permeation chromatography (GPC) have been used in protein separations in the bioanalytical field [5], and these techniques are also becoming an important means of preparative purification for low-molecular-weight proteins [6].

The present study demonstrates a rapid and efficient method for the purification of β_2-m from the haemodialysate of patients with chronic renal failure and the chromatographic behaviour of the purified protein.

MATERIALS AND METHODS

A purified preparation of standard β_2-m was isolated as previously described [4].

Chemicals

Antiserum to human β_2-m was obtained from Seikagaku Kogyo (Tokyo, Japan). Sephadex G-100 (superfine) and the standard proteins used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS—PAGE) were from Pharmacia (Uppsala, Sweden). Acetonitrile and trifluoroacetic acid (TFA) for HPLC, acrylamide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylenediamine (all special grade for electrophoresis), Tris base, glycine and ammonium persulphate were from Wako (Osaka, Japan). Coomassie Brilliant Blue R-250 was purchased from Nakarai (Kyoto, Japan). Ampholines (pH 3.5—10 and pH 3.5—5) were from LKB Producter (Bromma, Sweden). The hollow fibre cartridges of an artificial kidney (type TAF 80S) were products of Terumo (Tokyo, Japan).

HPLC conditions

The HPLC system used in this study was composed of a Shimadzu liquid chromatograph LC-4A, spectrophotometric UV detector SRD-2AS and Chromatopac C-R2AX recorder (Shimadzu, Kyoto, Japan). RP-HPLC was carried out using a Shimadzu Shimpack C_{18} column (15 × 0.6 cm I.D.) with a particle size of 5 μm at a flow-rate of 1.5 ml/min. Solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The elution programme consisted of a 30-min linear gradient from 15% to 100% B. Detection was carried out at 280 nm. GPC was performed using a TSK-GEL 2000SW column (60 × 0.75 cm I.D.) (Toyo Soda, Tokyo, Japan) with a mobile phase of 50 m*M* potassium phosphate buffer containing 50 m*M* sodium sulphate at pH 7.0 and flow-rate of 0.8 ml/min. Detection was at 230 nm. The chart recorder speed was 30 cm/h.

Two-dimensional electrophoresis under non-denaturing conditions

Two-dimensional electrophoresis was mainly carried out according to the method of Manabe et al. [7]. First-dimension isoelectric focusing was performed on a precast LKB Ampholine 4% polyacrylamide gel column (8 ×

3.5 mm I.D.) with a pH range of 3.5—10. Electrophoresis was run at 2 mA constant current per gel column for 8 min and then at 220 V constant voltage for 3 h at 4°C.

Polyacrylamide gradient (4—17%, w/v) gels (8.2 × 8.2 × 0.27 cm) were prepared and electrophoresis in the second dimension was performed with the non-denaturing electrophoresis buffer. The non-equilibrated isoelectric focusing gels were applied directly to the top of the second gradient gels and electrophoresed at 30 mA per gel for 3 h at 4°C.

Staining and photography

The gels were stained overnight in 0.025% Coomassie Brilliant Blue R-250 in 7% (v/v) methanol, and destained in 7% (v/v) acetic acid by shaking at room temperature. These gels were photographed with a 35-mm camera using Fuji Minicopy II film.

SDS—PAGE

SDS—PAGE was carried out according to the method of Laemmli [8] in a 15% polyacrylamide slab gel containing 1% SDS.

Protein assay and immunodiffusion analysis

Protein concentration was determined by the Bio-Rad protein assay method [9]. The β_2-m content of each chromatographic step was determined by enzyme immunoassay [10]. Immunodiffusion was carried out in 1% agar dissolved in 0.15 *M* sodium chloride containing 0.1% sodium azide [11].

Sample preparation

A litre of ultrafiltrate was obtained from a chronic patient undergoing therapeutic haemodialysis by the extracorporeal ultrafiltration method with a TAF 80S artificial kidney [12].

RESULTS

The haemodialysis ultrafiltrate from the patient was dialysed against deionized water using a Spectrapor 6 membrane and lyophilized to a powder. The material was dissolved in 0.01 *M* potassium phosphate buffer, pH 7.0, containing 1 *M* sodium chloride, applied to a Sephadex G-100 column (135 × 3.5 cm I.D.), equilibrated with the same buffer, and eluted (Fig. 1). Analysis of fractions using Ouchterlony's test indicated that fractions 70—80 contained the protein with antigenic identity to reference β_2-m. Fractions containing β_2-m (fraction A) were pooled and lyophilized as mentioned above. RP-HPLC, employing acetonitrile as organic modifier and TFA as the ion-pairing reagent, was used for the fractionation of β_2-m. As shown in Fig. 2, under these conditions, fraction A was further resolved mainly into two peaks, a and b. The amount of fraction A loaded on the column was 1 mg at a time. Peak a was a main component comprising approximately 80% of fraction A. It was then subjected to GPC on a TSK-GEL 2000SW column, and further separated from a minor contaminant (Fig. 3). The major component was confirmed to be β_2-m

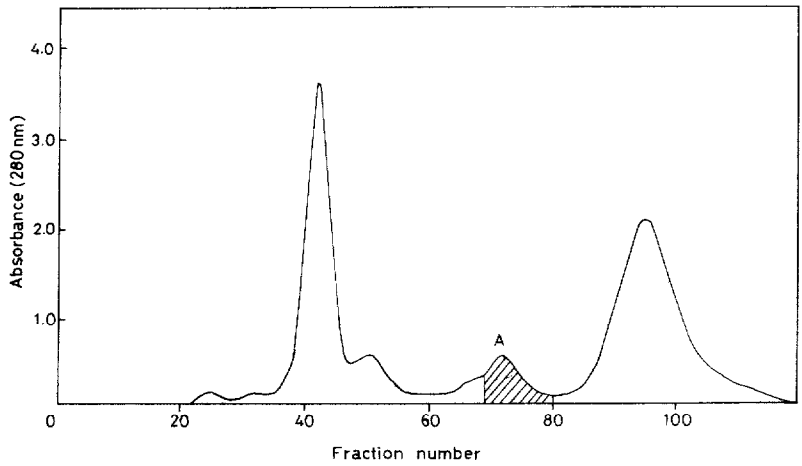

Fig. 1. Chromatography on a Sephadex G-100 column of concentrated material (1.020 mg) from the blood ultrafiltrate. The column (135 × 3.5 cm) was equilibrated with 0.01 *M* potassium phosphate buffer, pH 7.0, containing 1 *M* sodium chloride. Fractions of 13 ml were collected at 40-min intervals. Protein concentration was measured by reading the absorbance at 280 nm. The location of β_2-m (shaded area) in the effluent was detected by Ouchterlony's test.

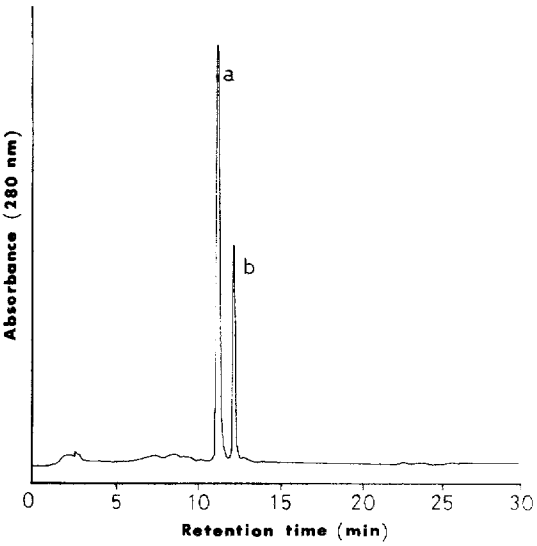

Fig. 2. Chromatogram of fraction A from Sephadex G-100 gel filtration. Chromatographic conditions: column, Shimadzu Shim-pack C_{18} column (15 × 0.6 cm I.D.); solvent A was 0.1% TFA in water and solvent B was 0.1% TFA in acetonitrile. The elution programme consisted of a 30-min linear gradient from 15% to 100% solvent B; flow-rate, 1.5 ml/min; detection, UV 280 nm; recorder chart speed, 30 cm/h.

by Ouchterlony's test. The molecular weight of the protein was determined on the TSK-GEL 2000SW column to be 12 000 with reference to the standard proteins, which were run simultaneously. This value was also supported by SDS—PAGE analysis. Fig. 4 shows SDS—PAGE patterns of each chromatographic step. The haemodialysate, fraction A separated by Sephadex G-100

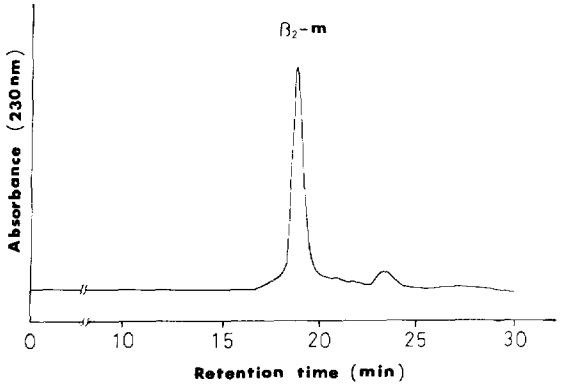

Fig. 3. Chromatogram of peak a from RP-HPLC. Chromatographic conditions: column, TSK-GEL 2000SW (60 × 0.75 cm I.D.); mobile phase, 0.1 *M* potassium phosphate buffer containing 0.05 *M* sodium sulphate at pH 7.0; flow-rate 0.8 ml/min; detection, UV 230 nm; recorder chart speed, 30 cm/h.

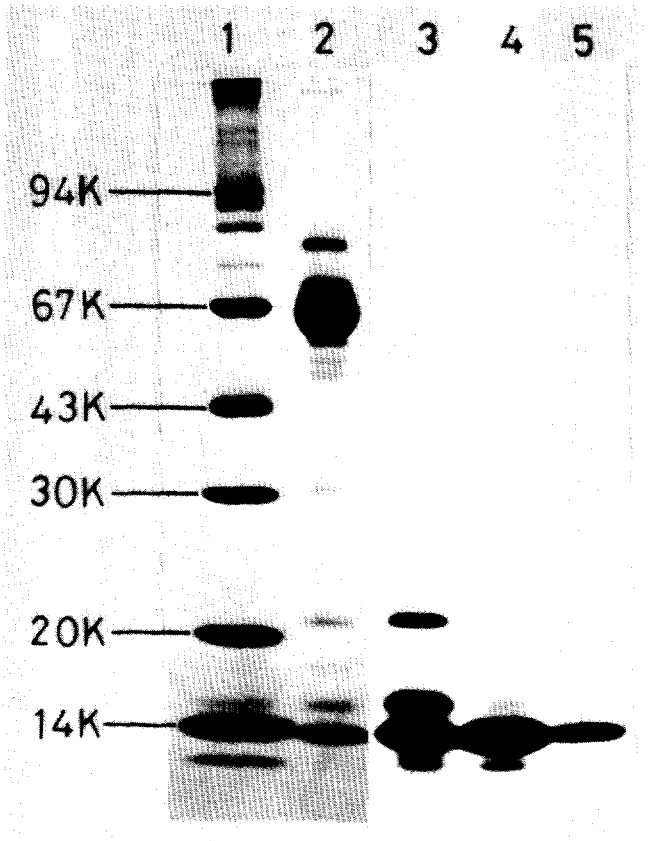

Fig. 4. SDS—PAGE (15%) of the materials from each purification step. (1) Marker proteins: phosphorylase *b* (molecular weight, MW = 94 000), bovine serum albumin (MW = 67 000), ovalbumin (MW = 43 000), carbonic anhydrase (MW = 30 000), soybean trypsin inhibitor (MW = 20 100), and α-lactalbumin (MW = 14 400). (2) Blood ultrafiltrate from patients with chronic renal failure. (3) Fraction A from Sephadex G-100. (4) Peak a from RP-HPLC. (5) β_2-m fraction from GPC.

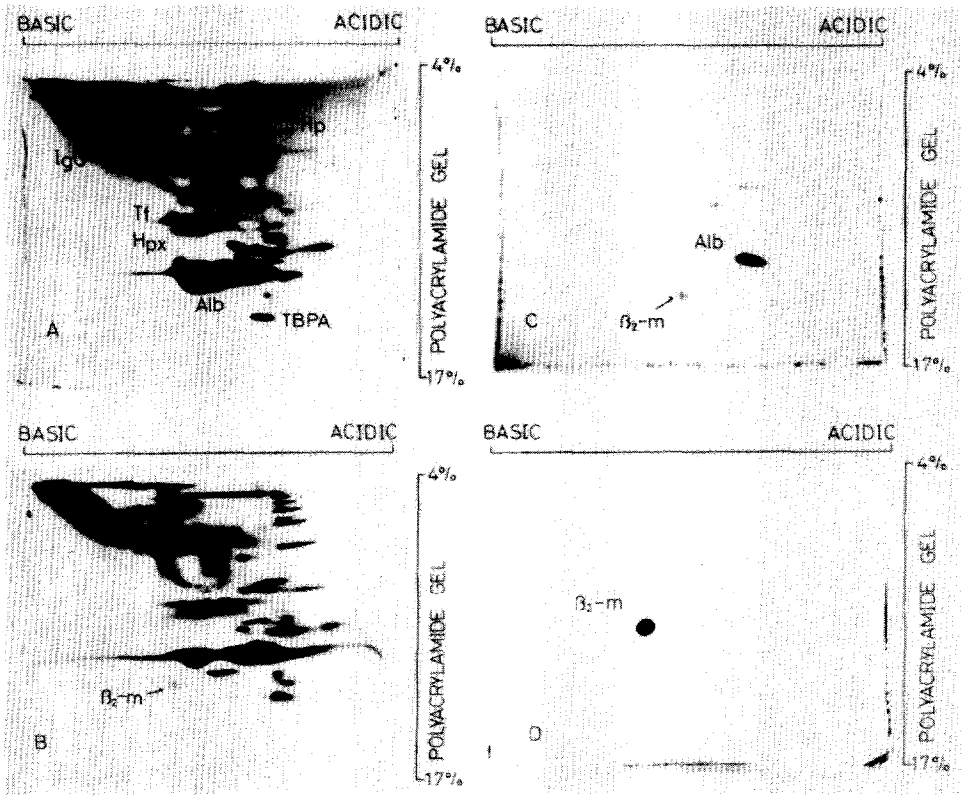

Fig. 5. Two-dimensional separation of human plasma proteins. Sample volumes were 5 μl for plasma, 20 μl for the blood ultrafiltrate. The photographs show the patterns of: (A) normal human plasma; (B) plasma from a patient with chronic renal failure; (C) the blood ultrafiltrate; (D) purified β_2-m. Abbreviations: IgG = immunoglobulin G; Hp = haptoglobin; Tf = transferrin; Alb = albumin; Hpx = haemopexin; TBPA = prealbumin.

TABLE I

PURIFICATION OF HUMAN PLASMA β_2-m FROM THE BLOOD ULTRAFILTRATE OF A PATIENT WITH CHRONIC RENAL FAILURE

Procedure*	Total volume (ml)	Total protein (mg)	Total β_2-m** (mg)	Yield (%)
Blood ultrafiltrate	1730	1020	33	100
Sephadex G-100	182	58	31	94
RP-HPLC				85***
GPC				93***

*The blood ultrafiltrate was subsequently reconcentrated, subjected to chromatography on Sephadex G-100, pooled and concentrated as described under Materials and methods.
**Determined by enzyme immunoassay.
***Amounts injected and recovered from the columns were quantitated by enzyme immunoassay.

chromatography, peak a from RP-HPLC and the major component from GPC were lyophilized and dissolved in saline containing 1% SDS and 6 *M* urea. They were separately applied to a 6% stacking gel (pH 6.8), and then electrophoresed in a 15% slab gel at pH 8.8. The final product showed a single band on SDS—PAGE and formed a homogeneous precipitin band with anti-human β_2-m antiserum by Ouchterlony immunodiffusion.

Fig. 5 shows the two-dimensional electrophoretic patterns: Fig. 5A is from the plasma from a normal person and Fig. 5B that from a patient with chronic renal failure. Several spots of low-molecular-weight proteins including β_2-m are observed. Fig. 5C is the haemodialysate obtained by the TAF 80S artificial kidney. The distribution of these proteins was compared among several materials and their positions were confirmed to be very reproducible.

In Fig. 5D the purified β_2-m is shown as a single spot whose position is also shown by an arrow in Fig. 5B. Table I summarizes the purification procedure and the yields of this protein.

DISCUSSION

A high serum level of β_2-m has been reported in patients with impaired renal function [13], rheumatoid arthritis, Sjögren's syndrome [14], and malignant tumours [15]. More striking increases were shown in lymphoproliferative disorders [16]. The plasma content of β_2-m from patients undergoing chronic therapeutic haemodialysis was ten to twenty times higher than in normal subjects. We previously reported some clinical evaluations of the haemodialysis treatment using a protein-permeating haemofilter [17]. By this treatment, the blood ultrafiltrate from the patients was shown to contain most low-molecular-weight proteins, including β_2-m which accounted for 17 mg/l. Removal of the main contaminant, albumin, from the ultrafiltrate on a Sephadex G-100 column facilitated the next separation step of β_2-m.

The analytical HPLC techniques in the past have mostly been directed toward purification and determination of low-molecular-weight compounds, but in recent years HPLC on reversed-phase silica packings has been used to separate not only low-molecular-weight compounds but also low-molecular-weight proteins [5, 6]. To achieve the purification of β_2-m, RP-HPLC with a counter agent and GPC were used in this study. By combined use of RP-HPLC and GPC, highly purified β_2-m could be isolated from the fraction A of Sephadex G-100 chromatography with a recovery of over 80%. Although β_2-m is known to be unstable in urine at pH 5.5 or below [18], β_2-m purified by using RP-HPLC containing 0.1% TFA in acetonitrile as eluent retained immunological antigenicity and had a similar solubility compared with the previously reported protein [4].

The procedure described here provided an easy and rapid way to separate β_2-m. Although the amount of starting material used in this experiment was 1 mg in total, it may be possible to scale up the procedure several times according to the capacity of the HPLC columns. In addition, the combination use of HPLC may also provide other suitable chormatographic systems for the purification of not only β_2-m but also of other low-molecular-weight proteins.

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