Vol. 80, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1978 Pages 519-524

PURIFICATION OF β_2-MICROGLOBULIN BY GEL FILTRATION AND ISOELECTRIC FOCUSING

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Received November 7,1977

SUMMARY

Urine from persons with elevated excretion of β_2-microglobulin was concentrated and the proteins were separated by molecular sieve chromatography and isoelectric focusing in a granulated gel. A preparation of high purity was obtained.

INTRODUCTION

β_2-microglobulin[x)] is a small protein that is found in most body fluids (for review see ref. 1-3). It has in recent years attracted a growing interest in biology, immunology and medicine. The amino acid sequence of β_2-m is to a large extent homologous with regions of light and heavy chain immunoglobulins (4). Furthermore, it is found on cell surfaces where it makes up part of the "transplantation" HL-A antigens (see ref. 1-3). Elevated concentrations of β_2-m have been found in patients with various forms of cancer (5,6). One interesting fact is that β_2-m is excreted in increasing concentration in urine with increasing tubular malfunction, e.g. as caused by accumulation of cadmium in the kidneys (7-9). Increased concentration of β_2-m is also found in blood plasma with decreasing filtration capacity of the kidneys (10). Therefore determination of serum β_2-m might be superior to other methods for glomerular filtration rate determinations such as those with inulin and even determination of serum creatinine (10).

Most methods described earlier for purification of β_2-m from urine are four step procedures involving gel permeation and ion exchange chromato-

x) β_2-microglobulin = β_2-m

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graphy, and zone electrophoresis (1,7).

We have for some time been interested in β_2-m in studies of the effects of chemicals on the kidneys as well as in diseases (9,11,12). For such studies purified β_2-m is very useful. Since we have been acquainted with isoelectric focusing for many years, we have used the technique of focusing in flat bed granular gels because its high preparative capability (several grams of proteins may be applied) and simplicity in performance (13).

MATERIALS

Urine was obtained from elderly persons with tubular damage caused by occupational exposure to cadmium dust over several years. Those urine samples which were to be worked up within a few days were stored at +4°C. Otherwise samples were stored at -20°C. For dialysis Spectrapor membrane tubing 3 (Spectrum Medical Industries Inc., Los Angeles, USA) with an approximate cutoff limit of MW 3500 was used. Collodium bags 13200 (Sartorius-Membranfilter, Göttingen, W. Germany) were used for dialysis and concentration of volumes < 15 ml. Volumes > 15 ml were concentrated in a rotary evaporator, Büchi (Flawil, Switzerland). Ultrogel AcA54 for gel permeation chromatography, facilities for isoelectric focusing such as Ampholine carrier ampholytes, Multiphor and Ultrodex (especially purified Sephadex for isoelectric focusing in flat-bed granulated gel), constant effect Power Supply 2103, and cooling bath were from LKB-Produkter (Bromma, Sweden). Coomassie Brilliant Blue R 250 (ICI, Manchester, England) was used for staining. A pH electrode type LOT 403-30 (Ingold, Zürich, Switzerland) was used for pH determinations after isoelectric focusing. β_2-m was assayed in fractions by isoelectric focusing in polyacrylamide gel and by radioimmunoassay, Phadebas (Pharmacia, Uppsala, Sweden).

Buffer solutions: Tris (hydroxymethyl) - aminomethane - Acetate 0.002 M, pH 7.5; Tris - Acetate 0.1 M, pH 7.5 and Tris - HCl 0.06 M, pH 7.6.

METHODS AND RESULTS

The various steps for purification are indicated in the Table and are described in some detail in the following text.

The urine (1265 ml) was concentrated in a rotary evaporator at 35-40°C and 2.7 kPA. After the volume was reduced to approximately one tenth, the sample was dialyzed in dialysis tubing against 0.002 M Tris-acetate buffer at +8°C over night. Then the urine was further concentrated to about 5 ml. To decrease the salt concentration the sample was dialysed again for 4 hours with three changes of the outside buffer solution. It was then applied on an Ultrogel column. (See Fig. 1). Fractions were analyzed by analytical isoelectric focusing in polyacrylamide gel, essentially as described earlier (12). The Ampholine for focusing in polyacrylamide gel was: pH 3.5 - 10, 2.8 ml; pH 9 - 11, 0.4 ml; pH 5 - 7, 0.2 ml and pH 4 - 6, 0.2 ml thus giving a total Ampholine concentration in the gel of 2 % (v/v). Of each sample 50 µl was absorbed in pieces (5 x 8 mm) of a cotton-cellulose material (Paratex IV/80) and applied to the gel surface. Focusing time was 90 minutes starting with 400 volts

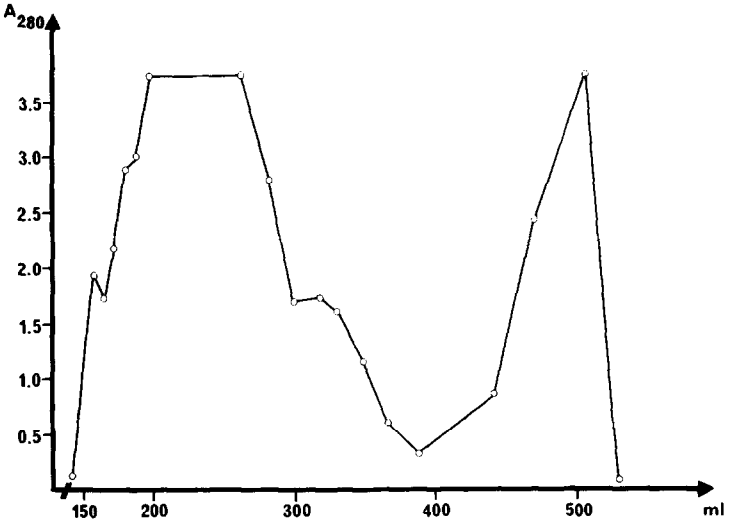

Fig. 1 Elutiondiagram of the proteins applied to a column with Ultrogel AcA 54, height 87,0 2.5 cm. Elutant: Tris-HCl buffer 0.6 ml/min. The absorbance at A_{280}nm is shown. β_2-m was found in the fraction volume 300-380 ml. In the first peak we also found albumin and orosomucoid.

and ending with 1000 volts with maximum power 40 W. Staining of proteins was by Procedure D of ref. 14. The position of β_2-m on the PAG was indicated by use of a pure standard (See Fig. 2). Quantification of some proteins from the first peak of the gel column was made with rocket immunoelectrophoresis in agarose gels (c.f. text of Fig. 1).

Fractions containing β_2-m were collected as a crude "β_2-m pool". The volume of this pool was reduced by concentration in the rotary evaporator. After dialysis (see above) isoelectric focusing in granulated gel (Ultrodex) was made as recommended earlier (13). 4 g of the gel was mixed with water and Ampholine solution (pH 5 - 7) in the proportions 95:5. The slurry was spread on a glass tray and excess water was evaporated to a predetermined evaporation limit. The protein sample (about 3 ml) was mixed with a small portion of the gel slurry and placed in the middle of the gel with a sample applicator. The electrode strips were placed at the short edges of the tray. The electrode solutions were: anode, 0.1 M H_3PO_4 and cathode, 0.1 M NaOH. The focusing time was 5 1/2 hours starting with 950 volts and ending with 1600 volts with maximum power 20 W.The positions of the focused bands were located on a paper print of the gel surface (13). A fractionating grid was then pressed into the gel. The pH was measured with a surface pH-electrode in the separated fractions. Thereafter the gel of each fraction was transferred to small plastic columns. The proteins were eluted from the Ultrodex gel with 2.5 ml 0.1 M Tris-acetate buffer for each fraction. The UV absorption at 280 nm was measured on each fraction (Fig. 3). The proteins were also analyzed by analytical focusing in polyacrylamide gel. β_2-m was found in four adjacent fractions and with a very high degree of purity as can be seen in Fig. 4. The yield of β_2-m was followed by radioimmunoassay after each step. The results of a typical experiment with calcula-

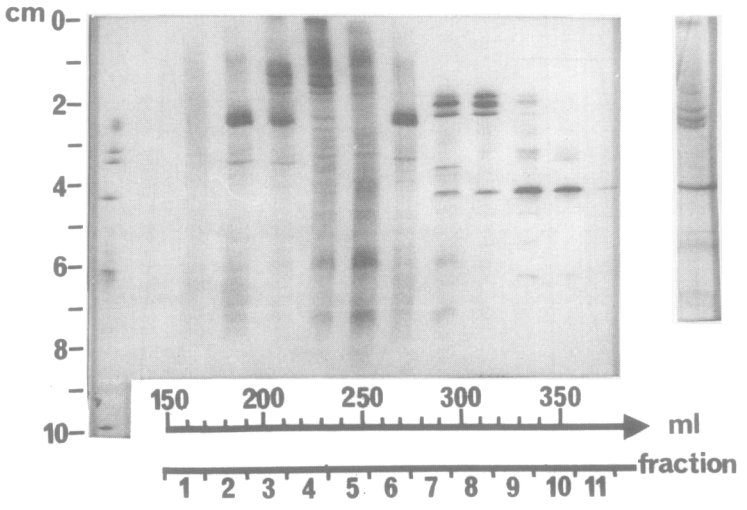

Fig. 2 Photograph of isoelectric focusing in a polyacrylamide gel of some fractions from the Ultrogel column after staining of protein bands. To the left a cm scale is shown with zero at the anode. To the right is shown a gel with separated bands of the original urine sample. β_2-m is the band at position 4.5 cm from the anode.

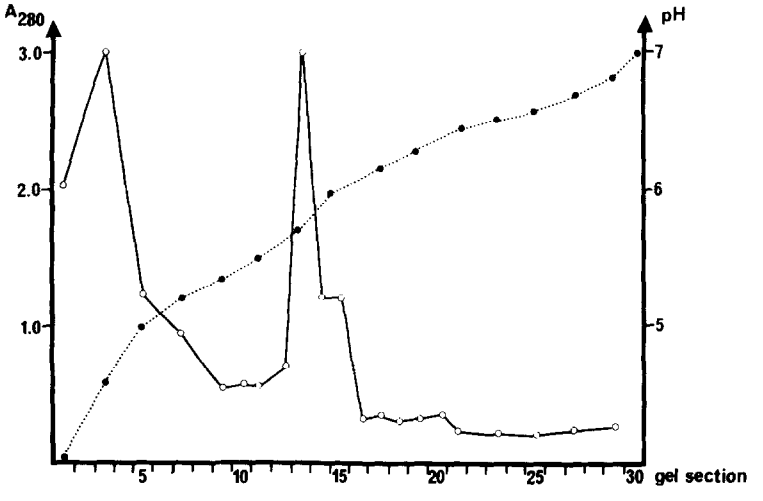

Fig. 3 Result of preparative isoelectric focusing in granulated gel of pooled fractions from the Ultrogel column showing absorbance of the fractions (o——o); and pH, (● .... ●).

tions of the yields are shown in the table. β_2-m was purified about 46 times.

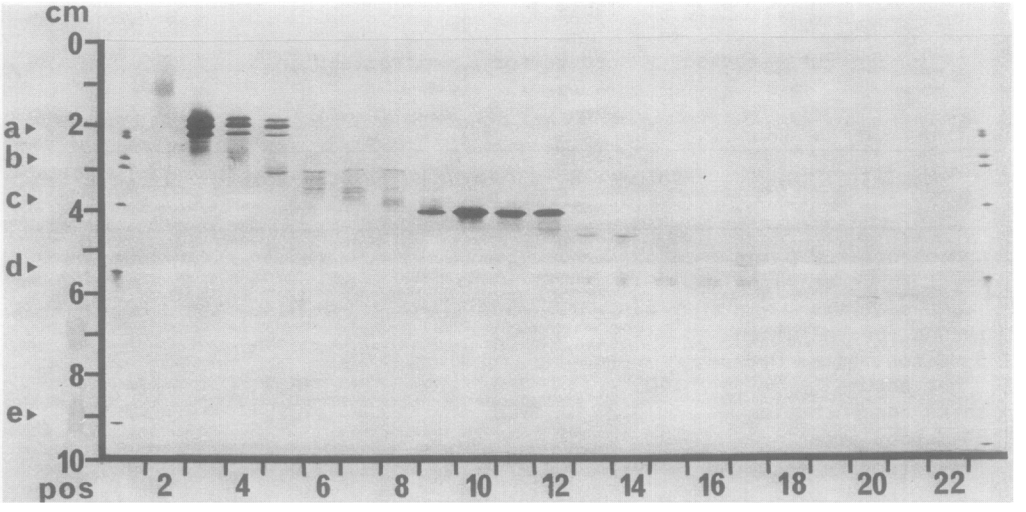

Fig. 4 Photograph of isoelectric focusing in polyacrylamide gel of fractions from preparative focusing in granulated gel after staining of the protein bands. The anode was at the upper edge. β_2-m was found in fractions 9-12. A standard protein mixture (12) is shown in position 1. The proteins in this mixture were: 'a', albumin; 'b', β-lactoglobulin (2 bands); 'c', β_2-microglobulin; 'd', hemoglobin and 'e', cytochrome c.

DISCUSSION

β_2-m was purified by use of simple procedures to a high degree of purity. For practical reason we have used as a starting material urine samples from workers exposed to cadmium over many years, because such urine may contain β_2-m in concentration more than a hundred times above the normal level (8,11). Concerning the final yield shown in Table 1 the following may be said. Firstly, in order to get as high purity as possible, we have discarded some fractions with β_2-m on both sides of the maximum peak after the gel filtration step, (see Fig. 1) and after isoelectric focusing. Secondly, as β_2-m is a very small protein (MW 11800) dialysis will cause losses in spite of using dialysis tubes that are claimed to retain even low molecular proteins better than ordinary dialysis tubings.

The β_2-m is intended for identification purposes when analyzing urine

Table 1

Purification scedule for β_2-microglobulin

Step	Volume ml	β_2-m concentr. mg/l	β_2-m total mg	Yield %
unconcentrated urine	1265	20	25.3	100
first concentration and dialysis	58	350	20.3	80
second concentration	4.9	3200	15.8	62
β_2-m pool after Ultrogel column	105	180	18.9	74
third concentration	3.2	3900	12.5	49
β_2-m pool after isoelectric focusing	20.5	390	8.0	32

samples from persons with suspected tubular kidney malfunctions. Furthermore, it may be useful in cancer research, e.g.in studies on tumor antigens as has been shown recently (15).

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