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Short Communication

Rapid method to isolate serum amyloid P component from human plasma

Characterization of the isolated protein

Z. Urbányi

Department of Microbiology, G. Richter Ltd., P.O. Box 27, 1475 Budapest 10 (Hungary)

D. Medzihradszky

Department of Pharmaceutical Chemistry, UCSF, San Francisco, CA 94122 (USA)

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ABSTRACT

A rapid and reproducible method to isolate serum amyloid P component from healthy human plasma has been developed. It uses affinity chromatography on an agarose column followed by anion-exchange chromatography. It was found that the isolated compound has a significantly different isoelectric point (p*I* 5.7) from that reported previously (p*I* 4.1). The new data are in good agreement with calculated values determined from the amino acid composition of the protein.

INTRODUCTION

Serum amyloid P component (SAP) is a member of the pentraxin family and consists of ten non-covalently associated subunits, each with a relative molecular mass of 25 000. These subunits are arranged as two face-to-face cyclic pentameric discs [1,2]. SAP has a number of biological actions, including modulation of the immune response [3,4], and interaction with connective tissue proteins [5] and DNA [6]. It also has a role in amyloidosis [7,8]. Animal plasma proteins with a high degree of structural similarity to human SAP, such as the hamster female protein, were also previously described as acute phase proteins with immune modulator effect [5,6,9]. In order to study further the immune modulation effects of SAP, we needed the protein in adequate amounts and purity. Human serum containing SAP at *ca.* 40 mg/l [1] is a suitable source of this protein.

EXPERIMENTAL

Chemicals

Chemicals were obtained from Reanal (Buda-

Correspondence to: Z. Urbányi, Department of Microbiology, G. Richter Ltd., P.O. Box 27, 1475 Budapest, Hungary.

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pest, Hungary). Sepharose 6B was purchased from Pharmacia-LKB (Uppsala, Sweden), Sepabeads FP-DA05 was supplied by Mitsubishi (Tokyo, Japan). The protein concentration was measured by using the Schleicher & Schuell Minifold method. Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Neville [10]. Gel electrophoresis under "native conditions" and isoelectric focusing were performed according to the instructions of Pharmacia-LKB [11,12].

Isolation of SAP

All experiments were carried out at 4°C. A 100-ml volume of a cold buffer containing 1 *M* Tris and 1 *M* $CaCl_2$ (pH 7.0) was added to 1000 ml of fresh, cooled human plasma (with citrate salt). The solution was stirred overnight, then centrifuged at 5000 *g* for 20 min. The pellet was discarded, and 0.1 mol of crystalline $CaCl_2$ was added to the supernatant. The solution was stirred again overnight and centrifuged as described above. The pellet was discarded again and the supernatant (*ca.* 1100 ml) was saved.

Affinity chromatography

Sepharose 6B (112 ml) was suspended in a minimal volume of distilled water, which was then added to 1100 ml of supernatant and stirred for 1 h. The mixture was poured into a 23 cm × 2.5 cm I.D. chromatographic column with a 1000-ml filling adaptor, and the gel was filtered at the bottom of the column. The plasma containing unbound plasma proteins was removed from the column with a peristaltic pump. After all the plasma had entered the column, the column was washed with 0.1 *M* Tris–0.15 *M* NaCl–0.1 *M* $CaCl_2$ (pH 7.8) until no further change of absorbance at 280 nm was detected. SAP was eluted by 0.1 *M* Tris–0.15 *M* NaCl–4 m*M* EDTA buffer (pH 7.8) at a flow-rate of 63 ml/h. The UV absorbance of each fraction was measured at 280 nm (Fig. 1) and aliquots were analysed by SDS-PAGE. Fractions containing the appropriate molecular mass band were pooled, and the buffer was changed by diafiltration to 0.01 *M* sodium phosphate (pH 7.5).

Ion-exchange chromatography

SAP binds to most of the commonly used carbohydrate-based chromatographic supports, therefore an ion exchanger with a synthetic copolymer matrix must be chosen for this separation. A 20 cm × 2 cm I.D. column (63 ml) was prepared from Sepabeads FP-DA05 material in 0.01 *M* sodium phosphate buffer (pH 7.5). The sample (35 ml) was applied at a flow-rate of 50 ml/h and washed with three column volumes of the same buffer. A linear gradient of 0 to 0.5 *M* NaCl in six column volumes was used to elute the SAP, which was eluted at *ca.* 0.3 *M* salt concentration. The procedure was followed by UV absorbance measurement of the fractions (Fig. 2)

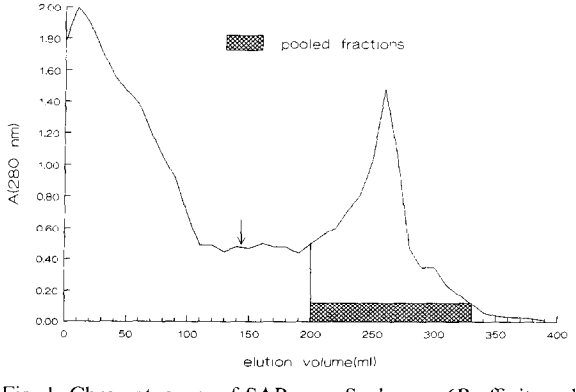

Fig. 1. Chromatogram of SAP on a Sepharose 6B affinity column (25 cm × 2.5 cm I.D.) as described in the text. The arrow shows where the buffer was changed.

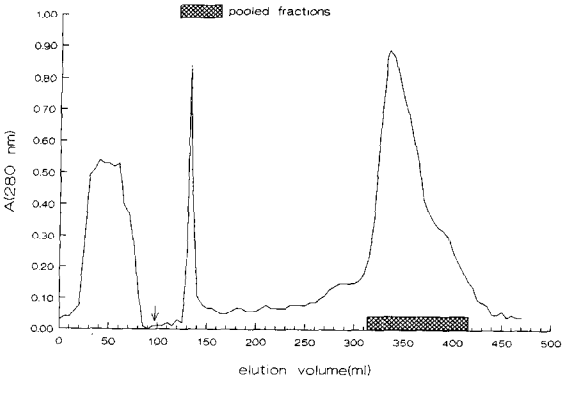

Fig. 2. Ion-exchange chromatograms on a Sepabeads FP-DA05 column. The chromatographic parameters are described in the text. The arrow shows the starting point of the gradient.

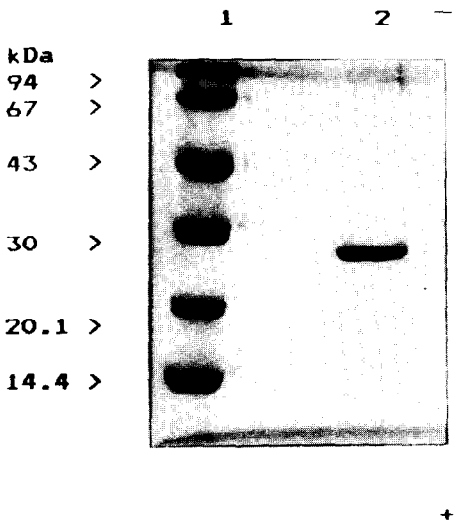

Fig. 3. Determination of subunit molecular mass of the isolated protein by using SDS-PAGE. Electrophoresis was carried out on a 200 × 100 × 1 mm vertical gel (T = 15%) at 200 V for 45 min using the Neville buffer system. The gel was stained with Coomassie Brillant Blue R-250. Lanes: 1 = Pharmacia-LKB LMW marker (phosphorylase b, 94 000; bovine serum albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; soybean trypsin inhibitor, 20 100; α-lactalbumin, 14 400; 2 = pooled fractions containing SAP.

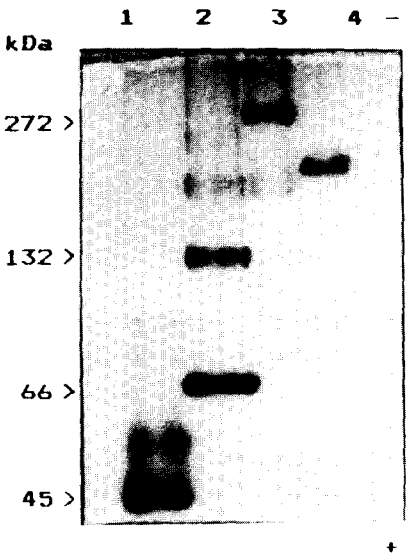

Fig. 4. Determination of native molecular mass of SAP by using native gradient PAGE. Gradient PAGE was carried out on a 200 × 200 × 2 mm vertical gel (T increased from 4 to 30%) at 100 V for 20 h in a Tris–borate–EDTA buffer system (pH 8.3). The gel was stained with Coomassie Brillant Blue R-250. Lanes: 1 = chicken egg albumin, 45 000; 2 = bovine serum albumin, 132 000 and 66 000 (dimeric and monomeric forms); 3 = jack bean urease, 272 000; 4 = SAP.

and SDS-PAGE. Fractions containing a single protein band at 24 000 (subunit M_r) as compared to a Pharmacia-LKB LMW kit were pooled and stored at 4°C (Fig. 3).

Characterization of the isolated protein

The molecular mass of native SAP was determined by electrophoresis (Fig. 4) using a 4–30% polyacrylamide gel and was found to be about 250 000, as reported previously [1,2]. The isoelectric point (pI) of the denatured protein was found to be 5.7–5.8 (Fig. 5). The pI was also calculated by a computer program based on a combination of the charge of terminal amino acids and amino acid side-chains within the sequence [13]. The measured and the calculated pI values were in good agreement. Edman sequencing was carried out on an ABI 473A gas phase sequencer equipped with an on-line analyser. The first fifteen amino acids were identified and found to be identical with the SAP sequence [14].

RESULTS AND DISCUSSION

SAP can reversibly bind to insoluble carbohydrate polymers such as agarose, starch, etc., in the presence of calcium ions. Accordingly we worked out a rapid and reproducible method to isolate SAP from human plasma with a yield similar to that of other published procedures on that scale. SAP was bound to a Sepharose 6B column in the presence of $CaCl_2$ and was eluted with a buffer containing EDTA. In this step SAP was separated from most of the other plasma proteins. In the next step SAP was purified to homogeneity by ion-exchange chromatography. The average yield was 8.5 mg of pure SAP from 1000 ml of human plasma. The measured native and subunit molecular masses (250 000 and 24 000, respectively) were in accordance with the known values.

The pI of the SAP was determined to be 5.7–5.8. This is in good agreement with the calculated

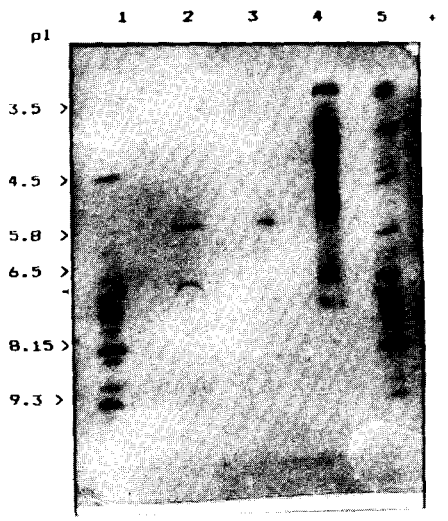

Fig. 5. Determination of the p*I* of SAP by isoelectric focusing. The isoelectric focusing was carried out at 5 W power until 2000 V h was reached (U_{max} = 1200 V) on a 105 × 85 × 1 mm horizontal gel (T = 4%) containing Pharmalyte (pH 3–10) and 6 *M* urea. Anode buffer, 1 *M* H_3PO_4; cathode buffer, 1 *M* NaOH. The gel was stained with Coomassie Brillant Blue R-250. A precipitated lane is visible at the sample application site (arrow) because of the poor solubility of SAP. Lanes: 1 = Pharmacia-LKB high-p*I* calibration kit (pH 5–10.5); 2 and 3 = SAP; 4 = Pharmacia-LKB low-p*I* calibration kit (pH 2.5–6.5); 5 = Pharmacia-LKB broad-p*I* calibration kit (pH 3–10).

value (5.7). Ohkubo *et al.* [15] have determined a significantly different p*I* value (4.1) for SAP. The methods for measuring the p*I* were substantially different. Ohkubo *et al.* [15] used chromatofocusing to determine the p*I* of the native protein, whereas we measured the p*I* of the denatured protein by isoelectric focusing in the presence of urea.

This discrepancy may reflect a p*I* difference between the native and denatured protein. Alternatively, the p*I* value determined by Ohkubo *et al.* [15] is practically the same pH at which SAP is released from an agarose column (*ca.* pH 4.0) [16], so the retention of SAP on the column until *ca.* pH 4.0 during chromatofocusing may also have been caused by the affinity bonds between SAP and the column matrix.

The SAP purified by this procedure was used in immunological studies (to be reported elsewhere).

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