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Development of a purification procedure for the placental protein 14 involving metal–chelate affinity chromatography and hydrophobic interaction chromatography

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Abstract

Placental protein 14 was isolated from the biological material of patients undergoing legal abortions. The major part of ballast protein was removed by ion-exchange chromatography on DEAE-Sepharose and CM-Sepharose. Albumin was separated by chromatography on Blue-Sepharose. Complete purification was obtained by metal–chelate affinity chromatography on Nickel–Chelate Sepharose and hydrophobic interaction chromatography on Phenyl-Sepharose and Octyl-Sepharose. The protein was not exposed to denaturing agents or extreme pH.

Keywords: Placental protein 14

1. Introduction

Study of placental proteins is of great importance for determination of the pathogenesis of the feto-placental system malfunction. Placental protein 14 (PP14) is the protein specific for the human reproductive system [1,2], playing a substantial part in female reproduction and endometrial function [3,4]. It has been also identified as an immunosuppressive factor [5]. In the first trimester of the gestational period PP14 is synthesized in decidual tissue from which it is secreted into the amniotic fluid. In the second and third trimesters of the gestational period the quantity of PP14 in placenta and amniotic fluid decreases ten-fold [2]. PP14 is synthesized in small quantities in the secretory-phase endometrium [2,5]. PP14 is possibly implicated in the preparation for pregnancy and in the normal development of the feto-placental complex [2–5].

Joshi and co-workers [7,8] found PP14 to comprise of two identical subunits with molecular masses between 25 and 27 kDa, so that the molecular mass of the whole molecule was estimated to be about 50 kDa. Using thin-layer chromatography on Sephadex, the PP14 molecular mass was found to be 25 kDa, while using SDS–polyacrylamide gel electrophoresis, PP14 was also identified as a protein with a molecular mass of 42 kDa [9,10]. PP14 is a glycoprotein, containing 17.5% cardohydrate [9]. The isoelectric point of PP14 is about 4.7 [7,9].

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Different combinations of gel-permeation, immunosorption, hydrophobic, ion-exchange and affinity chromatography on Con-A–Sepharose were used for isolation of PP14 [6,9,11,12]. The aim of this research was to improve the methods of purification of PP14 by using metal–chelate and hydrophobic interaction chromatography. These methods are highly selective and suitable for preparative purposes [13–16].

2. Experimental

2.1. Materials

DEAE-Sepharose CL-6B, CM-Sepharose CL-6B Cibacron Blue F3GA-Sepharose CL-6B, epoxy-activated Sepharose 6B, Phenyl-Sepharose CL-4B and Octyl-Sepharose 4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Preparation of the nickel–chelate gel was carried out essentially as described by Poráth et al. [13].

2.2. Samples

The biological material was obtained from patients undergoing legal abortion (6–9 weeks gestational period). No pretreatment was performed to homogenize the material. All the material obtained from abortion was filtered through several layers of gauze. The remaining solid particles and clots were removed by centrifugation (5000 *g*, 8 min). Thus, the mixture of blood, amniotic fluid and tissue exudates was the basic material used for further procedures. Solutions used in all experiments contained 0.02% sodium azide.

The sample (volume 2 l) was desalted by ultrafiltration on a Pellicon tangential flow filtration cell (Millipore, Bedford, MA, USA) on PTGC membrane (10 000 Da cut-off), filtration surface 0.5 m^2. Retentate flow-rate was 2 l/min and filtrate flow-rate was 0.2 l/min.

The pH was adjusted to 5.2 with 0.5 *M* acetic acid. After standing overnight at 4°C the precipitate was removed by centrifugation (5000 *g*, 8 min).

2.3. Analytical procedure

The PP14 content was measured by radial immunodiffusion in agar gel using rabbit anti-PP14 monospecific immune serum. The stabilized amniotic fluid with a known concentration of PP14 (90 mg/l) was used as a standard. The immune serum was received through rabbit immunization with enriched preparation of PP14. This preparation was obtained with the use of adsorption chromatography and ammonium sulfate precipitation in The Laboratory for Affinity-Based Sorption, Institute for Physicochemical Medicine, Moscow. [2]. The antiserum was adsorbed with normal adult serum. The adsorbed antiserum thus obtained was proved to be specific for PP14. The rabbit anti-PP14 serum gave a single line of precipitation in agar gel and immunoelectrophoretic tests against fetal serum. No precipitation was observed when the rabbit anti-PP14 serum was tested against normal adult plasma. The stabilized amniotic fluid and the rabbit anti-PP14 monospecific immune serum used for PP14 content measurement were kindly provided by Dr. D.D. Petrunin.

The purity of PP14 was ascertained by immunoelectrophoresis in agarose gel containing antibodies against human adult serum [17].

SDS–polyacrylamide gel electrophoresis was carried out in 10% polyacrylamide slab gel [18].

The elution profile was recorded by measuring absorbance at 280 nm (Uvicord S, Pharmacia-LKB, Uppsala, Sweden).

The concentration of total protein in fractions containing PP14 was measured by the Bradford method.

2.4. Chromatography

All chromatographic experiments were performed at 20–22°C.

The sample (volume 2 l) containing about 196 g of total protein and about 200 mg of PP14 was applied to the column with DEAE-Sepharose (10×25 cm, gel volume 2 l), which was equilibrated with 0.025 *M* acetate buffer, pH 5.2. The column was washed first with the equilibration buffer and then with 0.025 *M* acetate buffer, pH 4.7. PP14 desorption was

carried out with the same buffer, pH 4.0. Flow-rate was 25 cm/h. The pH of the eluate was 4.3.

The sample was further applied to a CM-Sepharose column (10×25 cm) previously equilibrated with 0.025 *M* acetate buffer, pH 4.3. The column was washed with the same buffer. PP14 was eluted with 0.025 *M* acetate buffer, pH 5.5, containing 0.1 *M* NaCl. The flow-rate was 25 cm/h.

The sample was concentrated up to 300 ml in a Pellicon ultrafiltration cell. The pH was adjusted to 7.4 with 0.5 *M* Na_2HPO_4. Buffer exchange was carried out by diafiltration against 6 volumes of 0.01 *M* Na-phosphate buffer containing 0.15 *M* NaCl, pH 7.4 in the same cell.

The sample was applied to a column with Cibacron Blue Sepharose (5×3 cm, gel volume 50 ml), equilibrated with 0.01 *M* Na-phosphate buffer containing 0.15 *M* NaCl, pH 7.4. From this column the material followed to the column with Nickel–chelate Sepharose (5×5 cm, gel volume 100 ml). Successively connected columns were washed with 0.01 *M* Na-phosphate buffer, pH 7.4, with 0.15 *M* NaCl. The flow-rate was 20 cm/h.

PP14 was adsorbed on the second column. It was eluted with 0.01 *M* phosphate buffer, pH 6.0, containing 1 *M* NaCl. The flow-rate was 20 cm/h.

The sample (volume 200 ml) was diafiltrated in a Pellicon ultrafiltration cell against 6 volumes of 0.01 *M* Na-phosphate buffer, pH 6.5.

Ammonium sulfate was added to a final concentration 1 *M*. The sample was applied to a Phenyl-Sepharose column (5×5 cm, gel volume 100 ml) equilibrated with 0.01 *M* Na-phosphate buffer, pH 6.5, containing 1 *M* ammonium sulfate. The column was washed with about 200 ml of the same buffer. The flow-rate was 16 cm/h.

The material thus obtained (volume about 400 ml) was concentrated up to 50 ml in a Minitan ultrafiltration cell (Millipore) with membrane PTGC (10 000 Da cut-off), filtration surface 240 cm^2 (four filter packets). Retentate flow-rate was 800 ml/min and the filtrate flow-rate was 40 ml/min.

The pH was adjusted to 8.0 with 0.5 *M* Na_2HPO_4. Buffer exchange was carried out by diafiltration against 6 volumes of 0.01 *M* Na-phosphate buffer, pH 8.0 in the same cell.

The sample was applied to an Octyl-Sepharose column (2.5×10 cm, gel volume 50 ml) equilibrated with the 0.01 *M* Na-phospate buffer, pH 8.0. The column was washed with this buffer and a buffer containing 10% 2-propanol. PP14 desorption was carried out with 0.01 *M* phosphate buffer, pH 8.0, containing 25% 2-propanol. The flow-rate was 16 cm/h.

3. Results and discussion

After the preliminary processing the sample (volume 2 l) contained about 196 g of total protein and nearly 200 mg of PP14. By chromatography on DEAE-Sepharose (Fig. 1) the material was purified from serum and tissue proteins with p*I* values lower than 4.7 [19]. By chromatography on CM-Sepharose (Fig. 2) the material was purified from haemoglobin

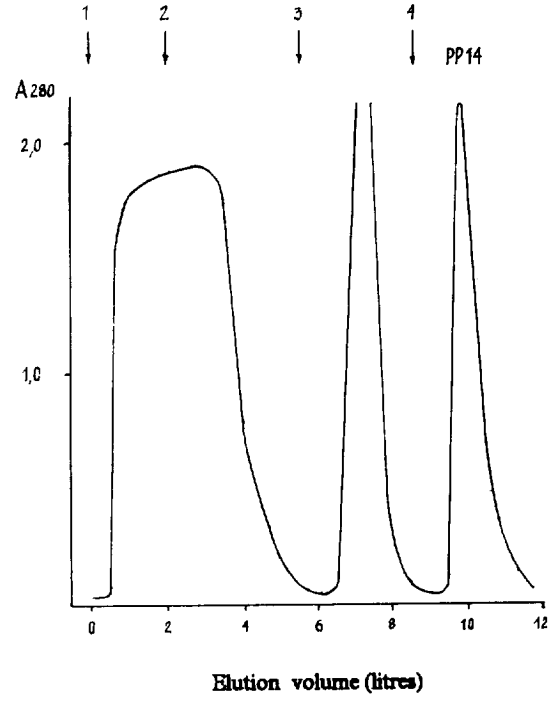

Fig. 1. Chromatography on DEAE-Sepharose CL-6B. Gel volume 2 l. (1) The sample containing PP14, pH 5.2. (2) Elution sequence: 0.025 *M* acetate buffer, pH 5.2; (3) 0.025 *M* acetate buffer, pH 4.7; (4) 0.025 *M* acetate buffer, pH 4.0.

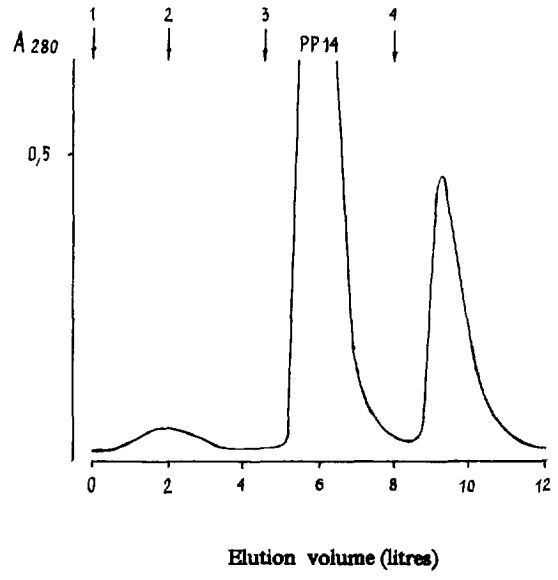

Fig. 2. Chromatography of PP14 containing fraction on CM-Sepharose CL-6B. Gel volume 2 l. (1) PP14 was applied in 0.025 *M* acetate buffer, pH 4.3. Elution sequence: (2) 0.025 *M* acetate buffer, pH 4.3; (3) 0.025 *M* acetate buffer, pH 5.5, containing 0.1 *M* NaCl; (4) 0.4 *M* sodium acetate, pH 8.0.

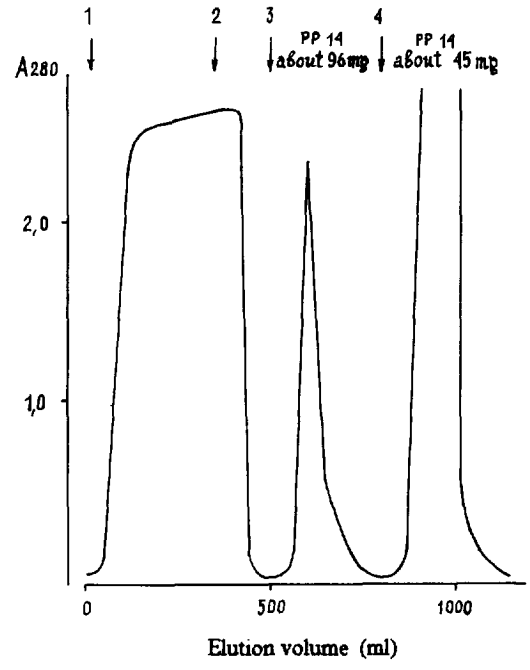

Fig. 3. Chromatography of PP14 containing fraction on nickel-chelate Sepharose 6B. Gel volume 100 ml. (1) PP14 was applied in phosphate-buffered saline (300 ml), pH 7.4. Elution sequence: (2) phosphate-buffered saline, pH 7.4; (3) 0.01 *M* Na-phosphate buffer, pH 6.0, containing 1 *M* NaCl; (4) 0.01 *M* Na-phosphate buffer, pH 6.0, containing 0.05 *M* EDTA.

[20]. During the two chromatographic steps the PP14 loss was not more than 25–30%.

Further purification was carried out on Blue-Sepharose [21]. Under the usual chromatographic conditions PP14 adsorption did not occur. PP14 was completely eluted during column washing with the starting buffer. About 18% of applied protein remained on the column.

The sample applied to the Nickel–chelate column contained 23 g of total protein and about 140 mg of PP14 (Fig. 3). The fraction eluted with 0.01 *M* phosphate buffer, pH 6.0, containing 1 *M* NaCl included 1.7 g of total protein and nearly 96 mg of PP14. The preliminary experiments showed that with 0.01 *M* phosphate buffer, (pH 7.4) containing 1 *M* NaCl, the protein was not eluted.

About 85% of applied protein was adsorbed on a Phenyl-Sepharose column. PP14 adsorption did not occur, and PP14 was almost completely eluted under the starting conditions (effluent volume 350 ml).

The results of the chromatographic separation on Octyl-Sepharose are shown in Fig. 4. The fraction eluted by the 25% 2-propanol contained 64 mg of total protein and 60 mg PP14. Almost 40% of PP14 remained on the column. The increase of 2-propanol concentration resulted in massive ballast protein desorption.

A summary of the purification of PP14 according to the methods described above is shown in Table 1.

PP14 displayed heterogeneous behavior in chromatography on Nickel–chelate Sepharose and on Octyl-Sepharose. The same was observed in adsorption chromatography on aluminium hydrophosphate and on silica gel [2]. PP14 is probably available in the sample as a mixture of different molecular forms.

SDS gel electrophoresis was carried out and the purified PP14 preparation gave an electrophoretically distinct main component with a molecular mass of about 55 kDa, and two minor bands (Fig. 5). With SDS gel electrophoresis in the presence of mercaptoetanol the preparation gave an electrophoretically

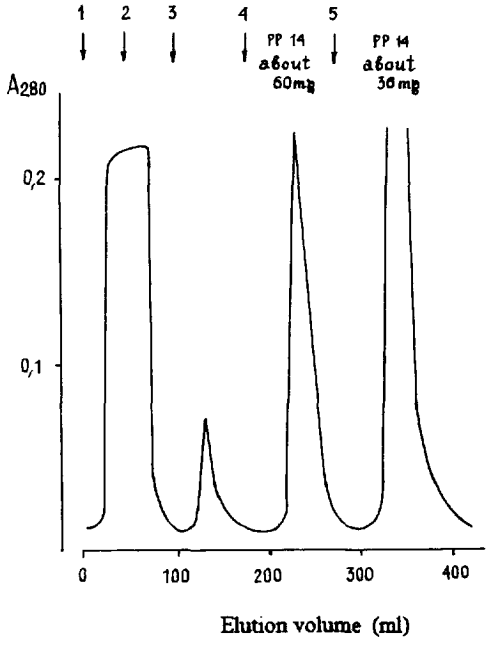

Fig. 4. Chromatography of PP14 containing fraction on octyl-Sepharose 4B. Gel volume 50 ml. (1) PP14 was applied in 0.01 *M* Na-phosphate buffer (50 ml), pH 8.0. Elution sequence: (2) 0.01 *M* Na-phosphate buffer, pH 8.0; (3) 0.01 *M* Na-phosphate buffer, pH 8.0, containing 10% 2-propanol; (4) 0.01 *M* Na-phosphate buffer, pH 8.0 containing 25% 2-propanol; (5) 0.01 *M* Na-phosphate buffer, pH 8.0, containing 40% 2-propanol.

distinct component with a molecular mass of about 25 kDa (Fig. 6). Obviously, under our experimental conditions PP14 may have been present in its dimeric form. Similar results have been reported by Joshi et al. [7].

With immunoelectrophoresis the preparation gave a single precipitation line against antiserum to PP14

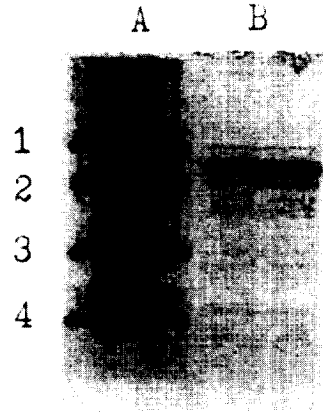

Fig. 5. SDS–PAGE. (A) Standard proteins. (1) bovine serum albumin, M_r 67 kDa; (2) ovalbumin, M_r 43 kDa; (3) chymotrypsin A, M_r 25 kDa; (4) ribonuclease A, M_r 13.7 kDa. (B) Purified PP14 preparation.

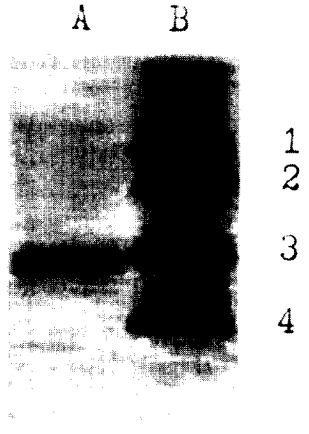

Fig. 6. SDS gel electrophoresis in the presence of mercaptoethanol. (A) Purified PP14 preparation. (B) Standard proteins. (1) bovine serum albumin, M_r 67 kDa; (2) ovalbumin, M_r 43 kDa; (3) chymotrypsin A, M_r 25 kDa; (4) ribonuclease A, M_r 13.7 kDa.

Table 1
Summary of PP14 purification procedure

Purification step	Total protein (mg)	PP14	Recovery (%)	Purification factor[a]
Starting material	196 000	200	100	–
DEAE-Sepharose	36 000	150	75	4.0
CM-Sepharose	28 300	146	73	5.1
Blue-Sepharose	23 300	141	71	5.9
Ni–chelate-Sepharose	1 710	96	48	55
Phenyl-Sepharose	262	94	47	348
Octyl-Sepharose	64	60	30	916

[a] Related to the purity of the starting material.

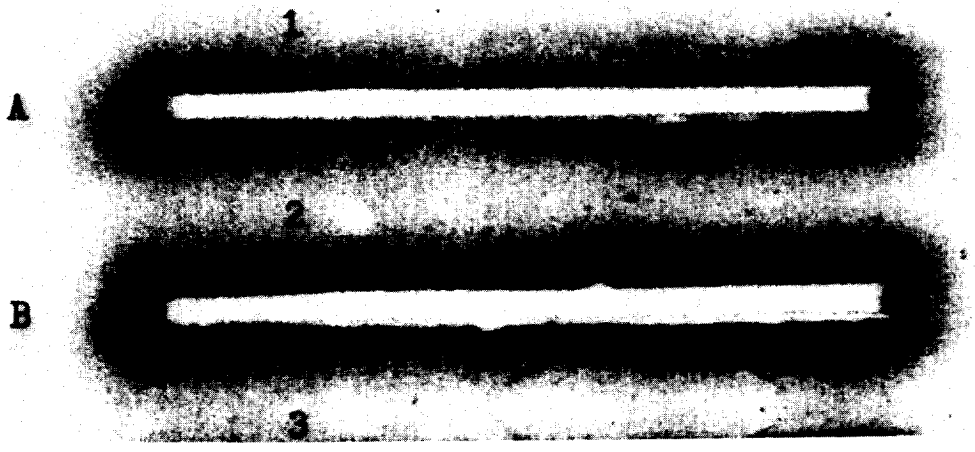

Fig. 7. Immunoelectrophoretic pattern of purified PP14 (wells 1 and 3) against antiserum to normal adult human serum (trough A) and against antiserum to PP14 (trough B). Well 2 is blank. Anode is to the right.

and it did not react with antiserum to normal human serum (Fig. 7).

The methods of PP14 purification by adsorption chromatography used before allowed preparations with a purity of not more than 90% to be obtained [2].

However, the use of immunosorption methods resulted in purity of up to 98%, but the yield was considerably lower than in the procedure described here [9,11,22].

The proposed procedure is reproducible and enables us to obtain considerable quantities of a highly purified PP14 preparation in one run. The material is not subjected to denaturing agents and the occurrence of structural malfunction is minimal.

Acknowledgments

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