N-terminal sequence analysis of human placental protein 14, purified in high yield from decidual cytosol

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Summary. Human placental protein 14 (PP14) has been purified in high yield from first trimester decidual cytosol. High-performance liquid chromatography on anion exchange, gel filtration and reverse-phase chromatography were used. The protein obtained is approximately 97% pure with an overall recovery of about 50% from the original tissue extract. The first 24 amino acids of the N-terminal were found to be Met-Asp-Ile-Pro-Gln-Thr-Lys-Gln-Asp-Leu-Glu-Leu-Pro-Lys-Leu-Ala-Gly-Thr-Glu-His-Glu-Met-Ala-Met. PP14 has been characterized in this study to be a dimeric glycoprotein of M_r 60 000, with homologous subunits having an M_r of 28 000.

Keywords: human; PP14; decidual cytosol; h.p.l.c. techniques

Introduction

Human placental protein 14 (PP14) was first isolated from soluble extracts of term placenta. It is a glycoprotein of about 17.5% carbohydrate, exhibiting $\alpha_1\alpha_2$ -electrophoretic mobility, with a pI of 4.5 (Bohn *et al.*, 1982). The development of a radioimmunoassay for PP14 (Bolton *et al.*, 1983) allowed detection in the peripheral blood of pregnant and non-pregnant females, amniotic fluid, placenta and endometrium, and high concentrations were found in first trimester decidua. Endometrial and decidual culture studies have demonstrated that these tissues secrete PP14 (Julkunen *et al.*, 1986a). So far, PP14 in males has only been detected by RIA in the seminal plasma (Bolton *et al.*, 1986). Several groups have independently isolated PP14 and have reported variations in its molecular weight, pI and whether PP14 is a monomeric or a dimeric protein. In addition, the described purification procedures, using various starting tissues, involve many steps, with consequent loss of material and possible effects on the molecular characteristics of the protein (Petrinin *et al.*, 1978; Joshi *et al.*, 1980a; Sutcliffe *et al.*, 1980; Bohn *et al.*, 1982; Bell, 1986b; Huhtala *et al.*, 1987).

A 3-step method is described for the purification of PP14 from decidual cytosol by high-performance liquid chromatography, which overcomes the deficiencies described above.

Materials and Methods

Preparation of decidual cytosols. Decidual tissues, by permission of the ethical committee, were obtained from patients undergoing first trimester termination at Guy's Hospital, London. By microscopic control ($\times 10$), decidua was separated from the placental and fetal tissues, washed in 0.9% (w/v) NaCl to remove excess blood, then stored at -20°C . Decidual cytosol was prepared by macerating the thawed tissue with a McIlwan histological tissue chopper,

and suspending in chilled 0.01 M-Tris-HCl pH 7.2 buffer at a concentration of 500 mg wet weight of tissue per ml of buffer. Homogenates were centrifuged at 80 000 *g* at 4°C for 60 min and supernatants were stored as 1-ml samples at -20°C.

Quantitation and detection of PP14. Fractions positive for PP14 were quantified utilizing radioimmunoassay (Bolton *et al.*, 1983), using purified PP14 (Lot no. 120/134) and antiserum (Lot no. 201ZA), obtained from Behringwerke AG (Marburg, FRG). PP14 was iodinated by the chloramine T method (Greenwood *et al.*, 1963), and subsequently purified by affinity chromatography using concavalin A-Sepharose (Pharmacia, Milton Keynes, U.K.). A 30% binding of labelled PP14 was obtained by diluting the antiserum 1:6000. Protein elution profiles were identified by u.v. detection at 280 nm. When tested against amino acid analysis, the RIA gave 90% of the calculated value for PP14, with respect to quantitation.

Total solubilized protein determinations. These were performed at each step of the purification schedule, using the colorimetric method described by Bradford (1976). Bovine serum albumin was used as the reference standard protein.

One-dimensional SDS-PAGE. A 12-track SDS discontinuous gel slab was used with a 10% acrylamide (containing 0.27% bis-acrylamide) resolving gel pH 8.9, and a 3.5% acrylamide (0.1% bis-acrylamide) stacking gel pH 6.8, as described by Laemmli (1970). To test for purity, samples of fractions positive for PP14, detected by RIA, and molecular weight markers were prepared by mixing with an equal volume of 0.125 M-Tris-HCl pH 6.8 buffer containing 5% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, and 0.002% (w/v) bromophenol blue. Gels were fixed in 20% ethanol and 3.5% perchloric acid in water, then stained with 0.04% (w/v) Coomassie Blue G (Sigma, Poole, U.K.) in fixing solution. Molecular weight markers used to calibrate the gel were phosphorylase (97 400), bovine serum albumin (66 000), ovalbumin (44 000), carbonic anhydrase (29 000) and lactalbumin (14 000) (Sigma, Poole, U.K.).

One-dimensional native PAGE. This utilized the same buffer systems and acrylamide concentrations as above, but omitted the 10% SDS and 10% 2-mercaptoethanol, on a 12-track discontinuous gel slab. Native gels were stained with Coomassie Blue G under the same conditions as for SDS-PAGE.

Anion exchange chromatography. The TSK DEAE 5PW ultrapac column (7.5 × 75 mm; LKB Bromma, Croydon, U.K.) was pre-equilibrated with 0.05 M-Tris-HCl pH 7.2. Decidual cytosol was thawed and passed through a 0.02 µm filter (Minisart NML, Sutton, U.K.) and 500 µl sample were injected onto the column. A linear 0.06 M-sodium chloride gradient was applied at a flow rate of 0.2 ml/min and 1-min fractions were collected.

Gel filtration. A 600 µl sample of the peak PP14-containing fractions from the DEAE 5PW column were applied to a Superose 12 HR 10/30 column (*V* = 25 ml; Pharmacia, Milton Keynes, U.K.). The column was previously equilibrated and subsequently eluted with 0.05 M-Tris-HCl containing 0.15 M-NaCl pH 7.2 at a flow rate of 0.5 ml/min for 60 min, and 0.5-ml fractions were collected.

Reverse-phase chromatography. A TSK ODS 120T 5 µm ultrapac column (4.6 × 250 mm; LKB Bromma) was equilibrated with 30% (v/v) acetonitrile in water containing 1% (v/v) trifluoroacetic acid. PP14 peak fractions (1 ml) from gel filtration were loaded and the proteins were eluted on a linear 30–80% (v/v) acetonitrile gradient containing 1% (v/v) trifluoroacetic acid. A flow rate of 1 ml/min was applied and 1-ml fractions were collected.

Analysis of the amino acids of the N-terminal of PP14. Purified PP14 was reduced and alkylated with 4-vinyl pyridine as described by Fullmer (1984). The sample was desalted by elution on a Brownlee C8 aquapore RP300 column (2.1 × 220 mm, Anachem, Luton, U.K.), which had been equilibrated with distilled water containing 0.1% (v/v) trifluoroacetic acid. A linear gradient of 0–90% (v/v) acetonitrile in water containing 0.1% trifluoroacetic acid was applied at a flow rate of 0.2 ml/min for 70 min, and 100-µl fractions were collected.

The N-terminal chain sequence analysis of PP14 was performed using the Applied Biosystems 477A sequencing system (Hewick *et al.*, 1981).

Results

Total solubilized protein and PP14 concentrations were estimated at each step of the isolation procedure and percentage recovery (yield) was calculated. One-dimensional SDS-PAGE analysis (Fig. 1) was performed on the PP14 peak fractions and total solubilized protein:PP14 ratios were calculated to assess purity (Table 1).

Protein elution profiles of each of the chromatographic steps as identified by u.v. detection at 280 nm and PP14 concentrations estimated by RIA are indicated in Fig. 2. PP14 was eluted from the DEAE 5PW column at about 0.25 M-NaCl. Haemoglobin was a major contaminant of the decidual cytosol, most probably due to the vascularity of the starting material. When an elution buffer of pH 7.2, equivalent to the pI of haemoglobin was selected, haemoglobin passed through

Fig. 1. One-dimensional SDS-PAGE of the peak fractions of the three chromatographic steps of the purification of PP14 from decidual cytosol. Peak fractions detected by RIA and u.v. at 280 nm. Lane 1 = molecular weight markers; Lane 2 = decidual cytosol; Lane 3 = anion exchange chromatography; Lane 4 = gel filtration; Lane 5 = reverse-phase chromatography.

Table 1. Estimations of total solubilized protein (TSP), by the colorimetric method described by Bradford (1976), and PP14 concentrations, by RIA, performed on the crude decidual cytosol and at each of the three chromatographic steps of the purification schedule

	TSP (mg)	PP14 (μ g)	TSP:PP14 ratio	% Yield	% Purity*
Decidual cytosol	24.94	838.6	1:0.04	100	—
Step 1: anion exchange	1.37	780.3	1:0.57	93	57
Step 2: gel filtration	0.61	507.2	1:0.83	60	83
Step 3: reverse-phase	0.43	415.9	1:0.97	49	97

*Assessed by the TSP:PP14 ratio and by one-dimensional SDS-PAGE.

the column with minimum retention. The molecular weights of PP14 and haemoglobin are similar (60 000 and 64 000 respectively). These proteins would be difficult to resolve by gel filtration. Elimination of haemoglobin at this initial stage simplified subsequent manipulations.

PP14 was eluted from the Suparose 12 HR 10/30 column at fraction 27 (13.5 ml) and molecular weight markers were used as calibration standards. On reverse-phase (TSK ODS 120T), in which

Fig. 2. Protein elution profiles of the three chromatographic steps in the purification of PP14 from decidual cytosol. (a) Step 1, anion exchange chromatography; (b) Step 2, gel filtration; (c) Step 3, reverse-phase chromatography.

elution is related to the hydrophobicity of the components fractionated, PP14 was eluted at about 70% acetonitrile, suggesting that PP14 has many hydrophobic residues.

Purified PP14, reduced and alkylated with 4-vinyl pyridine, was eluted from the C8 reverse-phase column at about 60% acetonitrile. The major peak was loaded on to the Applied Biosystems 477A sequencer. Figure 3 represents the elution profile from the C8 reverse-phase column and the cyclic cleavage of the consecutive amino acid residues from the N-terminal of PP14. Analysis of the first 24 amino acids of the N-terminal of PP14 revealed the sequence as:

M D I P Q T K Q D L E L P K L A G T E H E M A M

(see Fig. 4 for the coding of the amino acid sequence).

Fig. 3. Elution profiles of N-terminal amino acid analysis of PP14: (a) profile of PP14, treated with 4-vinyl pyridine, from the Brownlee C8 reverse-phase column, and (b) concentrations of the consecutive amino acid residues in their cyclic cleavage from the N-terminal of PP14 (see Fig. 4 for the key to amino acids).

The molecular weight of PP14 isolated from Step 1 was estimated by gel filtration and native PAGE and indicated an M_r of 60 000. When PP14 was further purified on gel filtration (Step 2), SDS PAGE gave an M_r of 60 000 (Fig. 1, Lane 4). When the same material was eluted on reverse-phase chromatography with 70% acetonitrile and 1% trifluoroacetic acid, a single band corresponding to an M_r of 28 000 was obtained under the reducing and non-reducing conditions of SDS-PAGE. This indicates that the subunits of PP14 are not held together by disulphide bonds, and dissociate in a hydrophobic environment. A single band also suggests homologous subunits, a finding confirmed by N-terminal analysis in which only one set of sequence data was found.

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Fig. 4. A comparison of the N-terminal sequence analysis of PP14 with horse, dolphin and cattle β -lactoglobulin (β LG) and human serum retinol-binding protein (RBP). Boxed residues marked with — indicate homology with PP14; boxed residues marked with — indicate homology with the β -lactoglobulins and RBP. A = alanine; C = cysteine; D = aspartic acid; E = glutamic acid; F = phenylalanine, G = glycine; H = histidine; I = isoleucine; K = lysine; L = leucine; M = methionine; P = proline; Q = glutamine; R = arginine; T = threonine; V = valine; W = tryptophan; Y = tyrosine.

Discussion

Several research groups have isolated proteins which subsequently have been shown to be similar and in some cases identical to PP14 by both immunological and physico-chemical analysis. Each group has ascribed a name to their protein, thereby producing a chaotic nomenclature. PP14 has been shown to be antigenically identical with chorionic α_2 -microglobulin (CAG-2), sometimes referred to as placenta-specific α_2 -microglobulin (PAMG-2). CAG-2/PAMG-2 was isolated from first trimester placenta and is reported to have a molecular weight of 25 000 (Petrunin *et al.*, 1978). This molecule probably represents the subunit of PP14, produced by their isolation procedure. In the absence of sequence data for CAG-2, it is possible that this protein could represent a polymorphic form of PP14. Joshi *et al.* (1980a, b, 1982) isolated progesterone-dependent endometrial protein (PEP) from human decidua of early pregnancy and studied its distribution in related tissues and fluids. Radioimmunological and immunodiffusion techniques have shown PEP to be immunologically indistinguishable from PP14 (Julkunen *et al.*, 1986b). Alpha-uterine protein (AUP), also isolated from extracts of human decidua (Sutcliffe *et al.*, 1980), exhibits serological identity with PEP. Both AUP and PEP are dimeric glycoproteins with an M_r of 50 000, subunit 25 000, and of 48 000, subunit 28 000, respectively (Sutcliffe *et al.*, 1982), and are localized in the glandular and surface epithelium of the endometrium (Mazurkiewicz *et al.*, 1981). Like PP14 and PEP, pregnancy-associated endometrial α_2 -globulin (α_2 -PEG) (Bell, 1985), is a dimeric glycoprotein which binds concavalin A-Sepharose with similar molecular weight estimations (M_r 56 000, subunit 28 000). Marginal differences in pI have been reported, PEP being 4.9, PP14 4.6, and α_2 -PEG 4.7 (average), due to the presence of multiple pI forms (Joshi *et al.*, 1980b; Bohn *et al.*, 1982; Bell, 1986a, b; Bell & Patel, 1987). It has been agreed that PP14 and α_2 -PEG immunologically cross-react (Bell & Bohn, 1986). These two molecules are probably identical or perhaps α_2 -PEG represents a polymorphic form of PP14.

The present studies were undertaken to purify PP14, define the molecular weight, some physico-chemical properties, and initiate studies of its primary structure. PP14 has been prepared from decidual cytosol to about 97% purity by a three-stage system of anion exchange, gel filtration and reverse-phase chromatography. By gel filtration and native PAGE, we estimate the molecular weight of PP14 to be about 60 000. Under the reducing and non-reducing conditions of SDS-PAGE, purified PP14 had an apparent M_r of 28 000, suggesting that PP14 is a dimeric protein. These results generally agree with those found for α_2 -PEG (Bell, 1986a, b) but differ from those of Julkunen *et al.* (1986a) who stated that PP14 was a single polypeptide chain. Bohn *et al.* (1982) suggested a molecular weight for PP14 of 42 000–43 000, by the technique of ultracentrifugation. The physico-chemical properties of their protein preparation and ours are identical. We are unable to explain the differences in molecular weight estimations between the respective preparations. The variations may be attributed to the methodological differences in the respective purification schedules and the tissues used to extract and isolate this protein.

Detection and quantitation of PP14 by RIA in the purification schedule utilized the PP14 antiserum and purified protein of Bohn *et al.* (1982). Immuno-electrophoretic techniques have identified that this PP14 antiserum (Lot No. 201ZA, Behringwerke AG, Marburg, FRG) exhibits specificity for placental protein 12 (PP12) and alpha-fetoprotein (AFP) (T. Fay, personal communication). The presence of PP12 and AFP in fractions positive for PP14, in the initial steps of the purification, could falsely elevate the PP14 values, thereby creating a negative bias with respect to the estimated yield. Parallelism of binding affinity with the PP14 antiserum was observed when our pure preparation of PP14 was serially diluted. Comparing quantitation of the RIA with amino acid analysis of PP14 purified in our schedule (monomeric PP14), the RIA gave about 90% of the calculated value for PP14. The 10% discrepancy may be explained by a methodology-associated bias. Therefore, it may be suggested that the PP14 antiserum recognizes a site on the monomer of PP14, and the RIA can precisely quantify both the monomeric and dimeric forms in terms of the real mass of the protein. Analysis of the first 18 amino acids of the N-terminal has shown identity with the sequence data determined by Huhtala *et al.* (1987). Some diversity occurs between the two sets of data further along the polypeptide chain. This can probably be explained by genetic polymorphism. The following results suggest that PP14 is a dimeric protein with homologous subunits. Firstly, PP14 is eluted at an M_r of about 60 000 by gel filtration, but of 28 000 under the reducing and non-reducing conditions of SDS-PAGE. Secondly, PP14 was eluted as a single peak on the C8 reverse-phase column after treatment with 4-vinyl pyridine. Finally, N-terminal sequence analysis revealed only one set of sequence data.

Significant homology exists between PP14 and β -lactoglobulins of a number of species, including horse, dolphin and cattle, and human serum retinol-binding protein, if the first residues of PP14 and the three β -lactoglobulins are aligned with residue 6 of retinol-binding protein (Pervais & Brew, 1985; Godovac-Zimmerman *et al.*, 1985; see Fig. 4).

As far as the function of PP14 is concerned, reports are both inconclusive and varied. The findings of homology in sequence data of PP14 and the β -lactoglobulins has not assisted since the physiology of the β -lactoglobulins is also unclear (Pervais & Brew, 1985). Binding studies attempting to show that PP14 is a retinol-binding protein are unconvincing (O. M. R. Westwood, unpublished observation). Bolton *et al.* (1987) have suggested an immunosuppressive function of PP14, purified by the techniques of anion exchange and gel filtration. Nomenclature still remains a problem, since the names suggested by the various groups are based on the tissue of origin rather than the structural or functional characteristics of the protein. With the first 24 amino acids of the N-terminal now characterized, we hope to undertake a structural analysis of both the protein and the gene.

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