Isolation and Partial Characterization of a Pregnancy-Associated Leporine Serum Protein in the Rabbit

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ABSTRACT. A pregnancy-associated leporine protein-2 (PALP-2) was isolated from serum of pregnant rabbits by gel filtration, ion exchange and affinity chromatography. The purified PALP-2 migrated like β globulin and had a molecular size of approximately 45,000. The isoelectric point of PALP-2, determined by isoelectric focusing, was 5.5. This protein was species-specific; no reaction being seen with pregnancy-associated serum proteins in term maternal serum of human and rat. The immunoreactivity of PALP-2 was unaffected by heat treatment at 57°C for 30 minutes, variably changed on incubation at 65°C for 30 minutes, and stable at 4°C for a week or at –20°C for 24 weeks.

In our laboratory, existence of two pregnancy-associated leporine proteins, pregnancy-associated leporine serum protein-1 (PALP-1) and pregnancy-associated leporine serum protein-2 (PALP-2), has been demonstrated, and their immunochemical characterization and the serum concentrations have been described [16]. The pregnancy-associated protein PALP-1 is detectable only in a serum of pregnant rabbit, whereas PALP-2 is present, though at much lower concentration, also in a serum of non-pregnant rabbit. This paper describes the isolation and partial characterization of PALP-2.

MATERIALS AND METHODS

Animals sera: Pregnant New Zealand white rabbits were exsanguinated under pentobarbital anesthesia between the 28th and the 30th day of gestation. The serum obtained was used both for immunization to raise antiserum and for purification of PALP-2.

Antiserum: Immunoglobulin of the rat anti-PALP sera, precipitated in 50% sat-

uration ammonium sulfate solution, was separated by the affinity column of cyanogen bromide-activated Sepharose-4B [1] coupled with male rabbit serum. The immunization procedure has been described earlier [16]. By this negative affinity chromatography the rat antibody specific to PALP-2 was purified. The extent of adsorption was tested by two-dimensional antibody-antigen crossed electrophoresis [13].

Purification of PALP-2: Dialyzed late pregnancy serum (3 ml) was applied to a column (1.5 cm $\times$ 9 cm) of Sepharose 4B to which 40 mg of IgG fraction obtained from the rat anti-PALP-2 serum had been coupled. The column was equilibrated with 0.03 M phosphate-buffered saline, pH 7.4. The sample was eluted with the same buffer at a flow rate of 15 ml/h at 4°C. The eluate from this column was passed through a UV monitor (AL-TEX Instruments). When 280 nm readings began to fall, the column was washed with 0.03 M phosphate-buffered saline, pH 7.4, and then the same buffer was applied to the column until the 280

nm reading in the effluent fell to less than 0.02. The bound PALP-2 was eluted with 150 ml of 0.5 M NaCl-0.1 M glycine-HCl buffer, pH 2.6, at a flow rate of 15 ml/h at 4°C, followed by 400 ml of 0.03 M phosphate-buffered saline, pH 7.4. Protein fractions eluted from the column, 6 ml each, were measured spectrophotometrically at 280 nm and then neutralized by 0.5 M NaCl-0.1 M carbonate-bicarbonate buffer, pH 8.3. The eluted PALP-2 fraction was dialyzed at 4°C against four changes of 10 fold excess of 0.03 M phosphate buffer, pH 7.4.

This material was then applied (at 100 ml/h) to a column (1 cm×30 cm) of DEAE-Sephadex A-50 equilibrated with 0.03 M phosphate buffer, pH 7.4. After washing the column with the same buffer, the bound protein was eluted at 29 ml/h with a linear gradient of 200 ml of 0.03 M phosphate buffer, pH 7.4 and 200 ml of 0.5 M-NaCl/0.03 M-potassium phosphate pH 7.4. The elution of protein was monitored by 280 nm and the peak fractions were concentrated.

In the last step, the material containing PALP-2 was gel filtrated on a 1 cm×30 cm Sephadex G-150 column (flow rate

15 ml/h, 3 ml fractions). The fractions enriched with PALP-2 were pooled and concentrated by a collodion bag. The concentrated material was stored at -30°C until analysis.

Further purification of PALP-2 was attempted using three steps of chromatography outlined in Fig. 1.

Immunoelectrophoretic methods: Double immunodiffusion was performed as described by Ouchterlony [18]. One- and two-dimensional antibody-antigen crossed electrophoresis were performed as described by Laurell [14].

Polyacrylamide-gel electrophoresis: Non-dissociating gel electrophoresis with 7.5% (w/v) polyacrylamide gels was carried out as described by Ornstein and Davis [17, 8]. Sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described by Dunker and Rueckert [9]. The following molecular-weight marker proteins were used: myoglobin (17,000), chymotrypsinogen A (25,000), Ovalbumin (45,000), bovine serum albumin (67,000).

Isoelectric focusing: Flat-bed isoelectric focusing was performed in polyacrylamide gels with Pharmalyte of pH range

Fig. 1. Purification protocols for PALP-2.

3-10. The anodal and cathodal buffers were 0.04 M aspartic acid and 1 M NaOH, respectively. Samples were applied directly to the gel surface of 110×230 mm plate (1 mm thick) through the cutouts in the applicator, and were run for 2 h at 30 W constant power. After completion of focusing, the gel was fixed in 5% sulfosalicylic acid plus 10% trichloroacetic acid for 60 minutes and was placed in a destaining solution of methanol: acetic acid: distilled water (3:1:6) for 30 minutes. The gel was then stained for 4 hours in 0.2% Coomassie brilliant Blue R-250 in methanol: acetic acid: distilled water (3:1:6).

RESULTS

No reaction was found between rat anti-PALP-2 serum and nonpregnant rabbit serum as a source of antigen. However, the antiserum gave one precipitate in a crossed immunoelectrophoresis when maternal serum was used as an antigen (Fig. 2).

Figure 3 illustrates an affinity chromatography using a column saturated by serum PALP-2. Applying glycine-HCl elution, PALP-2 was removed. The fraction of PALP-2 was pooled and dialyzed against 0.03 M phosphate buffer, pH 7.4. Dialyzed elute was applied to a DEAE Sephadex A-50 column. PALP-2 was tightly bound to the column and could be eluted with 0.2 M NaCl. The eluate was then subjected to gel filtration in a Sephadex G-150 column.

Double diffusion tests between purified PALP-2 fractions from the gel filtration and the original maternal serum revealed a complete fusion reaction. The protein was judged to be pure from the observation of a single band on polyacrylamide gel electrophoresis, homogenous sedimentation behaviour and a straight line obtained after equilibrium sedimentation.

Fig. 2. Crossed immunoelectrophoresis of term pregnancy serum as antigen and rat anti-PALP-2 serum diluted 8:100 in the second dimension gel.

Fig. 3. Affinity chromatography of pregnancy serum on a Sepharose 4B column coupled with rat antiserum PALP-2. Arrow indicates change of buffer. A₂₈₀ was measured spectrophotometrically. Hatched fractions were pooled and used for further preparation.

tation. The molecular weight determined by SDS-polyacrylamide gel electrophoresis was approximately 45,000 (Fig. 4). Isoelectric focusing performed in the pH between 3 and 10 showed one main band at pH 5.5 (Fig. 5).

Heating the pregnancy serum to 57°C for 30 minutes did not change these antigens. However, PALP-2 became nonreactive at 65°C (Table 1). The immunore-

activity of PALP-2 was stable for a week at 4°C and for 24 weeks at -20°C. All antigens were stable at pH range between 2 and 9 at 4°C for 30 minutes.

When tested against potent rat antibodies to rabbit pregnancy-associated proteins, none of the rat or human late pregnancy serum showed any reaction by immunoelectrophoresis or by immunodiffusion. Correspondingly, rabbit antibodies to human pregnancy-associated proteins were nonreactive against rabbit

pregnancy sera.

DISCUSSION

Two pregnancy-associated leporine serum proteins were immunochemically identified in our laboratory. One of these protein, pregnancy-associated leporine protein-2 (PALP-2), has a molecular weight of about 45,000 daltons. This protein, detectable in low concentration in the serum of non-pregnant female rabbit, increases its concentration during pregnancy, but is not found in the serum of male rabbit. The affinity chromatography with anti-PALP-2 Sepharose appears to be a promising method for purification of PALP-2. The final product

Fig. 4. Molecular weight of PALP-2 estimated by the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the maker proteins bovine serum albumin, ovalbumin, chymotrypsinogen and myoglobin.

Table 1. Heat-sensitivity of pregnancy-associated leporine protein-2

Temperature (°C)	Residual reactivity of antigens
57	+
65	-
75	-
85	-
100	-

Whole serum was exposed to the indicated temperature for 30 minutes.

Fig. 5. Thin-layer isoelectric focusing of PALP-2 in polyacrylamide gel on silanized glass plate. pH 3-10 carrier ampholytes. Sample application: 25 μ l/cm of a 0.5% solution. Focusing: 30 W, constant power (find voltage 2000 V) for 110 $\times$ 230 mm plate (1 mm thick). ●: PALP-2, pH 5.5.

was found to be free of contamination when examined immuno-electrophoretically.

There are several proteins associated with pregnancy. Stevens *et al.* [24] found a group of specific proteins that appeared in the uterine secretions of rabbit in early pregnancy. Five antigens which could not be found in blood sera were identified in rabbit uterine fluid by means of immunoelectrophoresis. These proteins have been named "blastokinin" [12] and "uteroglobin" [2]. These proteins are thought to have a functional role in embryonic development and implantation. Some of these uterine proteins appear to be uterine specific for many mammals [3, 7]. Shapiro *et al.* [22] reported both gel electrophoresis and immunoelectrophoresis revealed several protein species in rabbit oviductal fluid which were not present in rabbit serum. Sacco & Shivers [20] reported that several tissue specific proteins were detected in ovary, oviduct and uterus by Ouchterlony double diffusion method. The rabbit placental proteins reported by Chang [6] appeared to be absent in the maternal serum. The α 2-pregnancy-associated murine protein reported by Hau *et al.* [10] had a molecular weight of 70,000 and its isoelectric point was 4.0 as determined by means of crossed immunoelectrofocusing.

The PALP-2 appears to be a unique species different from any pregnancy-associated proteins reported in the literature. Also, it is distinct from the rabbit α -fetoprotein. The molecular weight of the latter, estimated by gel filtration, was about 69,000 and the *pI* was 6.0 [21] while the values for PALP-2 were 45,000 and 5.5, respectively. In addition, the antisera used in the present study did not crossreact with α -fetoprotein.

The PALP-2 exhibited no cross-reac-

tivity with human pregnancy zone protein [23], pregnancy-specific β 1-glycoprotein [5], steroid binding β -globulin [4] or pregnancy-associated antigens in rat serum [15].

Like the human pregnancy-associated proteins, PALP-2 seems to be a glycoprotein. The uniqueness of PALP-2 is also indicated by its characteristic resistance to pH extremes and high temperature.

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要 約

ウサギ妊娠関連蛋白の分離とその物理化学的性質：松下博治(麻布大学獣医学部生理学第一講座)——ウサギ妊娠関連蛋白の Pregnancy-associated leporine serum protein-2 (PALP-2) について、アフィニティークロマトグラフィー、イオン交換クロマトグラフィーおよびゲル濾過等を用いて精製を試みた。PALP-2 は電気泳動的には β mobility を示し、SDS ポリアクリルアミドゲル電気泳動法により分子量の測定で、約 45,000 の値を認めた。また等電点電気泳動法により得られた pI は 5.5 であった。一方、PALP-2 は、ヒトおよびラットの妊娠後期血清中における妊娠関連蛋白との免疫交叉反応を認めず、種特異性を示した。温度に対する PALP-2 の抵抗性は、57°C30分の処理にて抗原活性に影響を認めず、65°C30分の処理には抗原活性に変化を認めた。4°C 保存では一週間安定しており、-20°C 保存では6カ月間抗原活性に変化を認めなかった。