Isolation and Partial Characterization of a Bovine Amniotic Fluid Protein

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Abstract. An bovine, amniotic-fluid protein, (bAP), was purified from amniotic fluid by gel filtration, anion exchange chromatography and isoelectric focusing. The isolation was monitored by a double immunodiffusion with an antiserum raised against amniotic fluid antigens and absorbed with maternal and fetal sera. The molecular weight of an amniotic fluid protein isolated was estimated as 60,000 by SDS-PAGE, and the isoelectric point by isoelectric focusing was 3.4–3.8.

Key words: Bovine, Amniotic fluid, Pregnancy-associated protein.

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The soluble proteins in amniotic fluid are originated predominantly in maternal serum, and in small proportion in fetal serum and the placental membrane [1]. In common with proteins of fetal origin, the major species of proteins in the amniotic fluid are apparently synthesized by the endometrium/decidua [2]. Secretory proteins by the fetus has recently been investigated. Fay *et al.* [3] carried out immunoelectrophoresis with anti-human amniotic fluid antibody and isolated 2 fetal

antigens. A negative-affinity chromatography with anti-human serum antibody to the solid matrix, was introduced for the isolation of the proteins in amniotic fluid that do not derived from the maternal serum [4].

Only a few of non-maternal serum proteins have been isolated and characterized. In this study, an antiserum was raised against amniotic fluid antigens of cows and absorbed with maternal and fetal sera to detect a protein of amniotic origin.

Materials and Methods

Amniotic fluid and amniotic tissue

Amniotic fluid was collected by amniocentesis from 4 cows at the last month of gestation. The collected samples were cleared of cells and other non-soluble materials by centrifugation $10,000 \times g$ for 30 min at 4 C, stored at -20 C, and used for immunization.

Preparation of antisera

Five male Japanese white rabbits received intradermal injections of 5 ml of the amniotic fluid (containing 45 mg protein) emulsified in an equal volume of Freund's complete adjuvant at 2 weeks intervals for 10 weeks. Each antiserum obtained 7 days after the 5th injection was incubated with equal volumes of both pregnant and fetal sera for 2 h at 37 C and then overnight at 4 C, centrifuged at $10,000 \times g$ for 30 min and stored. All the animals immunized produced antibodies against the amniotic fluid antigens. But the absorbed antisera from 3 of 5 animals failed to react with amniotic fluid proteins by immunodiffusion test. A double-diffusion test was carried out using 1.2% agar in saline solution. Immunoelectrophoresis was performed in 1.2% agar in 0.06M-barbital buffer (pH 8.6).

Separation of the amniotic fluid protein

1) Ammonium sulfate precipitations: Amniotic fluid was fractionated by precipitation with ammonium sulfate between different concentration and saturation; 0–40%-, 40–65%-, 65–85%-, 85–100%-saturation. Each fraction was dialyzed against 0.05M Tris-HCl buffer (pH 8.0). Dried ammonium sulfate crystals were slowly added to obtain 40%-saturated solution. The samples were allowed to precipitate for 2 h, and the supernatant was discarded after centrifugation. Additional ammonium sulfate was added to make 65%-saturated solution, and the solution was stirred for 1 h. After centrifugation, the precipitate was dissolved in 0.05M Tris-HCl buffer (pH 8.0), and extensively dialyzed against the same buffer. The solution was then centrifuged at $10,000 \times g$ for 30 min, and the precipitate was discarded.

2) Gel filtration and ion exchange chromatographies: The fraction isolated by 40–65%-saturated ammonium sulfate was chromato-

graphed on Sephadex G-200 (Pharmacia). The column was equilibrated and eluted with 0.05 M Tris-HCl buffer (pH 8.0). Six ml of the sample was loaded onto the column (1.5×70 cm) and fractionated 5 ml of each protein concentration was monitored by UV adsorption at 280 nm. A bovine amniotic fluid protein (bAP) positive fractions were pooled and ultrafiltrated. The concentrated solution was chromatographed on DEAE-Sephadex (Pharmacia A-25). The column (2.2×24.5 cm) had previously been equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). After unbound proteins were washed through, the column was eluted with a 0–1.0 M-NaCl gradient in 0.05 M Tris-HCl buffer (pH 8.0). The bAP-positive fractions were pooled, dialyzed against 0.05 M phosphate buffer (pH 8.0), and lyophilized.

3) Isoelectric focusing: The preparative electrofocusing was done on the column (LKB 8100-1) with sucrose gradient solution contained 2% ampholytes with an operating range of pH 2.5–6.0 at 3 W and 4 C for 24 h. Protein concentrations were monitored at 280 nm.

4) Polyacrylamide gel electrophoresis under non-denaturing conditions: Purified and concentrated samples from amniotic fluid were applied to a 7.5% polyacrylamide disc gel with buffer consisted of 0.05 M Tris-HCl (pH 8.0). After the samples ran into the gel at 2 mA/gel for 15 min, the electrophoresis was developed for 2.5 h at 3 mA/gel. The gel was stained with coomassiebrilliant blue R-250/0.05% in 10% acetic acid and 45% methanol.

5) SDS-polyacrylamide gel electrophoresis: For one dimensional SDS gradient gel electrophoresis, less than 50 μ l of each fractionated sample was mixed with an equal volume of buffer containing β -mercaptoethanol (10%), SDS (10%), glycerol (20%), trizma (0.125 M, pH 6.8) and bromophenolblue (0.002%). Samples were heated for 1 min at 100 C and analyzed. Gels were stained for 30 min with coomassiebrilliant blue R250/0.05% in 10% acetic acid 45% methanol. Gels were calibrated with the proteins of known molecular weight.

Results

Absorption of the bovine amniotic-fluid antiserum with maternal and fetal calf sera removed

all but one band (Fig. 1) in the antisera generated in 2 of 5 rabbits. Immunoelectrophoresis using fetal serum and unabsorbed antiserum resulted in the formation of several precipitine bands, whereas no precipitine bands were visible when term pregnancy serum was tested against the absorbed antiserum (Fig. 2). When purified bovine amniotic fluid protein (bAP) was tested against the antisera on immunoelectrophoresis, one precipitine line was formed in the beta-region (Fig. 2). Although there is a wide variety of bAP concentrations among different samples of amniotic fluid, maximum concentrations were found during the term of pregnancy.

Amniotic fluid fractions of the 40–65% ammonium sulfate cut were pooled and subjected to gel chromatography. Fig. 3 shows the profile of eluted proteins from the Sephadex-G200 column. The fractions with the highest immunoreaction were pooled and analyzed by ion exchange chroma-

Fig. 1. Ouchterlony gel precipitin after diffusion of various body fluids against anti-bAP serum. 1, bovine amniotic fluid of late pregnancy; 2, bovine fetal serum of late pregnancy; 3, antiserum to bovine amniotic fluid absorbed with both maternal and fetal sera; 4, bovine maternal serum of late pregnancy.

Fig. 3. Gel filtration of the bovine amniotic fluid precipitated at 40–65% ammonium sulfate on a column (70 × 1.5 cm) of Sephadex G200 equilibrated with 0.05 M Tris buffer at pH 8.0, and containing 0.01 M NaCl. The stippled area indicates immunoreactive fractions.

tography. The bAP was quantitated by DEAE column. It eluted at NaCl concentrations between 0.4 M and 0.5 M (Fig. 4). The bAP-containing fractions eluted from DEAE-Sephadex were pooled, dialyzed and then concentrated by ultrafiltration on a collodion bag. Final purification was achieved by isoelectric focusing. The fraction from the DEAE-Sephadex column was applied to the column chromatofocusing. A typical chromato-

Fig. 2. Electrophoretic pattern at pH 8.6 of bovine amniotic fluid protein (bAP) with unabsorbed (a) and absorbed (b) rabbit anti-amniotic fluid antisera. 1, serum from fetus at age of 9-month; 2, purified bovine amniotic fluid protein; 3, serum from a dam at term pregnancy.

Fig. 4. An elution profile of bovine amniotic fluid protein from a DEAE-Sephadex column.

Fig. 5. Column isoelectric focusing of bovine amniotic fluid protein at pH 2.5–6.0 with a carrier of ampholytes.

gram profile is illustrated in Fig. 5. Under the condition of the gradient varying from pH 2.5 to 6.0, bAP was eluted between pH 3.4 and 3.8. The molecular weight of the separated bAP was estimated as 60,000 (Fig. 6).

Fig. 6. Determination of the molecular weight of the bovine amniotic fluid protein by SDS-polyacrylamide gel electrophoresis. The left lane, marker proteins.

Discussion

Since a precipitation band formation was found even between the absorbed antisera and the amniotic fluid, we concluded that the antigen found in this study in the term amniotic fluid is not originated in the maternal serum. Amniotic fluid consists predominantly of maternal serum proteins, only a small proportion is derived from the fetus, the placenta, and the endometrium [9]. Several proteins of non-maternal serum origin are demonstrated in the human amniotic fluid by a two-dimensional gel electrophoresis [10], or by a negative immunoaffinity chromatography [4].

A pregnancy-specific protein was detected in the bovine uterus at early pregnancy [5]. Butler *et al.* [6] identified by serological procedures an apparently pregnancy-specific protein (PSPB) from bovine placental membranes. Beckers *et al.* [7] reported the isolation of pregnancy-associated proteins from bovine cotyledons. The molecular weight of this protein was estimated as 67,000 by SDS-PAGE, with pI ranging from 4.4 to 5.4 [8].

The bAP antigen in this study had beta-globulin

mobility, the molecular weight by SDS-PAGE, was estimated to be 60,000 with a similar pI ranging from 3.4 to 3.8 as fetuin. However, the molecular weight of fetuin is reported as 48,400 [11].

The new protein of fetal origin will have potential value in prenatal diagnosis of fetal diseases.

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