Purification and Characterization of a Bovine Pregnancy-Associated Glycoprotein

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ABSTRACT

A 67 000 Mₐ bovine pregnancy-associated glycoprotein (bPAG) has been isolated from fetal cotyledons and purified to homogeneity by HPLC. The purification was monitored by a double immunodiffusion test and by RIA in conjunction with an antiserum raised against a crude fraction of placenta-specific antigens. The molecular weight of bPAG was estimated to be 67 000 by SDS-PAGE. The isoelectric points (pl) of the four isoforms, determined by high-resolution analytical electrofocusing in polyacrylamide gel, were 4.4, 4.6, 5.2, and 5.4. The carbohydrate content of the bPAG consisted of approximately 10.02 ± 1.09% neutral sugar and variant amounts of sialic acid (from 0.29 ± 0.06% in the most basic isoform to 2.1 ± 0.31% in the most acidic isoform). A specific antiserum was raised against the purified bPAG. A specific RIA showed that the bPAG was antigenically unrelated to BSA, alphafetoprotein (AFP), and human schwangerschafts-spezifischen (pregnancy-specific) β₁ glycoprotein (SP₁). According to some characteristics (e.g. the molecular weight), the purified bPAG may correspond to a form of the pregnancy-specific protein B previously described by Sassar and colleagues (Biol Reprod 1986; 35:936-942).

INTRODUCTION

The mammalian placenta is a source of a wide range of proteins and hormones. In most cases these compounds are homologous or identical to products synthesized by tissues of the normal nonpregnant adult, i.e. CG and LH, placental lactogen (PL) and prolactin + growth hormone (GH), steroids, and prostaglandins [1]. In 1970, Tatarinov and Masryukевич [2] identified a new protein from sera of pregnant women that was later purified from extracts of full-term placenta [3]. This placental protein, named beta-globulin or Schwangerschafts-spezifischen (pregnancy-specific) β₁ glycoprotein (SP₁), was considered to have no analog in the nonpregnant adult and to be strictly specific to the placenta. However, further studies have shown that the glycoprotein is not restricted to the placenta or even to pregnant women. It is produced in a range of normal tissues [4-7] and is now often classified as an oncostat protein [8, 9] since it is also a characteristic product of many tumors [10-12]. Although the biological function of SP₁ is unclear, it exhibits immunosuppressive properties [13, 14]. The SP₁ family of proteins exhibits a range of molecular weights and isoelectric forms [15, 16], and several related SP₁ genes appear to be expressed in the human fetal liver [17]. In 1982, Butler et al. [18] identified by serological procedures an apparently pregnancy-specific protein (PSPB) from bovine placental membranes that has been considered to be a ruminant equivalent of the human SP₁. This protein, like SP₁, seems to be heterodisperse and to have immunosuppressive properties [19]. Although specific antisera have been raised against PSPB, the protein still remains poorly characterized. Our laboratory has recently reported in abstract form the isolation of pregnancy-associated proteins from bovine and ovine cotyledons [20, 21]. Here we describe the purification and characterization of a group of related bovine pregnancy-associated glycoproteins (bPAG) of about 67 000 Mₐ, with pl ranging from 4.4 to 5.4.

MATERIALS AND METHODS

Production of the First Antisera

Placental extracts were fractionated by precipitation with ammonium sulfate. The 0-40% and 40-80%-saturated ammonium sulfate fractions were dialyzed against 0.01 M Tris-HCl buffer (pH 7.6), and chromatographed on DEAE-Sephadex A25 (Pharmacia LKB–Biotechnology, Uppsala, Sweden). The columns were eluted in five steps by addition of NaCl (0.05 M, 0.1 M, 0.15 M, 0.2 M, and 0.45 M) in elution buffer (Tris-HCl 0.05 M, pH 8). Each step was further purified by affinity chromatography to eliminate several proteins including BSA, hemoglobin, immunoglobulins that are not pregnancy-specific, and bovine placental...
lactogen. To perform affinity chromatography, immunoglobulins of specific antisera against these proteins were coupled to activated Sepharose 4B (Pharmacia) according to the procedure of Axen et al. [22]. The immunogel was equilibrated in Tris-HCl buffer (0.01 M, pH 7.6). Each DEAE step was loaded into the columns, which were eluted with the same buffer. The unadsorbed fractions were collected, concentrated by ultrafiltration, and lyophilized.

Ten adult rabbits weighing 3–4 kg were immunized according to the method of Vaitukaitis et al. [23]. One milligram of each semi-purified protein fraction was reconstituted in 1 ml distilled water, emulsified in an equal volume of Freund’s complete adjuvant, and then injected into a rabbit. Injections were given intradermally at multiple sites along the back, which had previously been shaved. The rabbits received boosts of fresh antigen (1 mg) at 2-wk intervals. Three months after the first injection, blood was collected from the marginal ear vein and allowed to clot for 12–24 h at room temperature; the sera thus obtained were removed and stored at −20°C until used.

To remove antibodies against more common proteins that were not pregnancy-specific, antisera were adsorbed with tissue extracts from nonpregnant cows (liver, kidney, skeletal muscle, and plasma) before their use in a double immunodiffusion test (DID). Then a semispecific RIA was developed to monitor BPAG. This RIA provided the basis for the assay employed in all subsequent purification processes.

**Purification**

**Collection of cotyledons.** Uteri were collected from pregnant cows within 20–30 min of slaughter. The fetal cotyledons were immediately dissected away from caruncular tissue, extensively washed with 0.9% NaCl, and stored at −20°C until processed. Only cotyledons from fetuses between 2 and 6 mo of age as determined by the crown-rump length measurement [24, 25] were used.

**Extraction.** One kilogram of fetal cotyledon tissue was thawed, finely minced, and homogenized in 0.01 M potassium phosphate buffer (KH2PO4 + KCl, 0.10 M; pH 7.6) with a ratio of buffer to tissue of 5:1 (v:w). Phenylmethylsulfonylfluoride (0.2 mM) and a sodium EDTA (0.2% w/v) were added at the time of homogenization. The homogenate was gently stirred for 2 h. It was then centrifuged at 27 000 × g for 1 h and the pellet was discarded. The supernatant solution was adjusted to pH 4.5 with 0.5 M H3PO4 and stirred overnight. The sample was then centrifuged at 27 000 × g for 1 h and the supernatant was retained. Its pH was readjusted to 7.6 with 0.5 M KOH.

**Ammonium sulfate precipitations.** Dry ammonium sulfate was slowly added to the stirred supernatant to obtain a 40% saturated solution. Proteins were then allowed to precipitate for 4 h and were discarded by centrifugation. Additional ammonium sulfate was added to achieve 70%-saturated solution, and the solution was stirred for 1 h. After centrifugation, the precipitate was retained, dissolved in 1 L of 0.01 M Tris-HCl buffer (pH 7.6), and extensively dialyzed against the same buffer. The solution was then centrifuged at 36,000 × g for 30 min, and the precipitate was discarded.

**DEAE-cellulose and gel filtration chromatographies.** The fraction isolated by 40–70% saturated ammonium sulfate was chromatographed on DEAE-cellulose (Whatman DE 52, Clifton, NJ). The column had previously been equilibrated with 0.01 M Tris-HCl buffer (pH 7.6). After the unbound proteins had washed through, the column was eluted in five steps of increasing ionic-strength buffer by adding NaCl (0.02 M, 0.04 M, 0.08 M, 0.1 M, and 0.2 M). Bovine PAG was monitored in all steps by RIA. The fractions with antigenic activity were collected, pooled, and ultrafiltrated. The concentrated solutions were submitted to gel filtration on Sephadex G-75 column (Pharmacia) equilibrated in 0.05 M ammonium bicarbonate buffer (pH 8). Thirty milliliters of sample was loaded onto the column (5 cm × 100 cm). Fractions of 15 ml were collected, and protein concentration was monitored by UV absorption at 280 nm. The bPAG-positive fractions were pooled, dialyzed against 0.005 M ammonium bicarbonate buffer (pH 8), and lyophilized.

The lyophilized preparation was used to immunize two rabbits (R484 and R485), which received injections of 250 μg of protein homogenized in complete Freund’s adjuvant and boosted at 2-wk intervals.

**HPLC.** Ten-milliliter samples of lyophilized protein from the G-75 column were loaded onto a Mono S column (5 mm × 5 cm; Pharmacia) equilibrated in 0.01 M ammonium acetate (pH 5). After elution of the unbound proteins, a linear salt gradient (0–1 M NaCl) was applied at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected and assayed. Those with very high antigenic activity were pooled, dialyzed against 5 mM ammonium acetate (pH 8), and lyophilized. The most immunoreactive preparation appeared to be relatively pure when analyzed by SDS-PAGE and was used to immunize two rabbits (R496 and R497; 250 μg of protein with boosts at 2-wk intervals).

The Mono S preparation was submitted to chromatofocusing on a Mono P HR (5 mm × 20 cm) column (Pharmacia) equilibrated in 0.25 M biss-Tris-HCl buffer (pH 6.3). Approximately 2.5 mg of lyophilized proteins from the Mono S column was loaded onto the column. Proteins were eluted with polybuffer 74 (Pharmacia), diluted 1:10 (v:v) with distilled H2O and adjusted to pH 4.0 with 0.1 N HCl. Flow rate was 0.7 ml/min and fractions of 1 ml were collected.

Ampholines were removed from immunoreactive fractions by chromatography on a gel filtration column (1 cm × 35 cm) of Sephacryl HR S200 (Pharmacia) previously equilibrated with 0.05 M ammonium bicarbonate (pH 8). The immunoreactive fractions analyzed by silver-stained SDS-PAGE appeared to be pure. They were pooled, lyophilized, and used to immunize a rabbit (R498).
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**Characterization**

**SDS-PAGE.** One-dimensional SDS-PAGE analysis was carried out with and without mercaptoethanol (2.5%) essentially as described by Laemmli [26]. Slab gels were either stained with Coomassie Brilliant Blue R250 dye or silver-stained.

**Isoelectric focusing (IEF).** IEF analysis was carried out by using preformed Ampholine PAGplates (pH 4–6.5; LKB, Rockport, MD). Samples of bPAG (25 μg) were applied on sample wicks approximately 1 cm from the cathode. Focusing was carried out at 250 V and stopped after 3 h. A portion of gel was fixed in 11.5% trichloroacetic acid/3.5% sulfosalicylic acid overnight at room temperature and washed in destain solution (25% ethanol, 4% acetic acid) for 5 min. Staining was carried out with Coomassie Brilliant Blue R250 dye (0.115% w/v) dissolved in destain solution. Proteins in the remaining part of the gel were transferred onto nitrocellulose paper (Millipore Corporation, Bedford, MA) for Western blotting.

**Immunoblotting of transferred proteins.** Immediately after IEF, proteins in slab gel were transferred onto nitrocellulose paper in electrophoretic buffer containing 25 mM Tris base, 192 mM glycine, 0.1% w/v SDS, and 20% w/v methanol (pH 8.3) [27]. Transfer was carried out at a constant current of 20 mA overnight on the Biolyon Trans Blot System (Biolyon Réactifs et Matériels de Laboratoire, 69572 Dardilly Cédex, France). After transfer, the nitrocellulose paper was submitted to immunoblotting. Nitrocellulose membrane was washed in Trisbuffered saline (TBS) and then immersed in blocking solution (3% w/v BSA in TBS) for 30 min. It was then incubated in anti-bPAG (R498 1:500) for 3 h at 37°C. After this step, the nitrocellulose paper was incubated with goat anti-rabbit IgG-horseradish-peroxidase conjugate (1:200) (Bio-Rad Laboratories, Richmond, CA) for 2 h at 37°C. After the nitrocellulose paper was washed with TBS, peroxidase substrate was added (100 ml TBS, 60 μl H2O2, 20 ml methanol, and 60 mg chloronaphthol) (Bio-Rad) for approximately 10 min.

**Determination of Carbohydrates and Sialic Acids**

The carbohydrate content of the purified bPAG was analyzed qualitatively by means of an enzyme immunoassay that employed an antibody-alkaline phosphatase conjugate (Glycan Detection Kit; Boehringer-Mannheim Biochemica, Mannheim, Germany). Human chorionic gonadotropin β (hCGB) was used as a positive control, and recombinant bovine methionine growth hormone (Met-bGH; Monsanto, St. Louis, MO) was the negative control. The quantitative determination of neutral sugars was carried out according to the method of Dubois [28], which utilizes phenol as the specific organic color-developing agent.

The procedure described by Warren [29] as modified by Aminoff [30] was used to determine sialic acid. This method, which uses thiobarbituric acid, determines only free sialic acid. Consequently, for the measurement of this sugar in bPAG, which is a protein, the sialic acid was first released by hydrolysis in 0.1 N H2SO4 at 80°C for 1 h. The N-acetylneuraminic acid (NANA) type IV (approx. 98%) for standard was purchased from Sigma Chemical Company (St. Louis, MO).

**RIA**

RIA was used to follow the bPAG throughout the purification procedure. Antisera R329, R484, R497, and R498, raised against semipurified protein, G-75, Mono S, and Mono P fractions, were used at the following initial dilutions: 1:2000, 1:150 000, 1:500 000, and 1:50 000, respectively, in RIA. Lypophilized immunoreactive fractions from Sephadiex G-75, Mono S, and Mono P were successively used both as tracers and standards. Their iodination was performed by the lactoperoxidase method described by Thorell and Johanson [31]. Highly purified BSA and alphafetoprotein (AFP) were purchased from Boehringer and Calbiochem (San Diego, CA), respectively. Bovine LH, bovine Pl (bLH and bPL), and porcine FSH (pFSH) were purified in our laboratory [32,33]. Equine chorionic gonadotropin (eCG) [Folligon] was purchased from Intervet (Intervet Belge S.A., Brussells, Belgium). The kit to assay human SP3 was purchased from Diagnostic Products Corporation (Dilbeek, Belgium).

**NH$_2$-Terminal Microsequence Analysis of bPAG**

Automated Edman degradations were performed with an updated 890B Beckman sequencer (Beckman Instruments, Palo Alto, CA) with the 0.33 M Quadrol program of Hunkapiller and Hood [34] in the presence of Polybrene (Aldrich-Chemie, Brussels, Belgium). Samples were extensively dialyzed against double-distilled water, lyophilized, and applied to the sequencer cup in 0.3 ml of double-distilled water containing 2% triethylamine.

The phenylthiohydantoin amino acid derivatives were quantitatively identified by reverse-phase, HPLC either on KS 100/6/4 Nucleosil 120-3C18 (MachereyNagel, Düren, Germany) in an acetonitrile gradient (0-90%, v/v) in 20 mM acetate buffer (pH 4.9) or on KS 250/6/4 Nucleosil 120-3C8 in 20 mM acetate buffer (pH 4.9): acetonitrile:1,2-dichloroethane 61.9:8.6:0.5 (v/v).

**RESULTS**

**Production of Antisera**

All rabbits immunized with semipurified placental proteins developed measurable antibodies when analyzed by DIF. Antisera from rabbits immunized with the second and third fractions from the DEAE column that had been loaded with 40–80% ammonium sulfate precipitate produced an additional precipitation line with a serum drawn from a full-term pregnant cow. Antisera to the Mono S (R496 and R497)
and Mono P (R498) fractions gave very high titers and could be used at final dilutions of 1:2 500 000 to 1:3 750 000 with highly purified $^{125}$I-radiolabeled bPAG (pool of Mono P fractions). No immunological cross-reactions were observed with gonadotrophic hormones (bLH, bPL, pFSH, eCG), with AFP (Fig. 1A) or with SP1 (Fig. 1B), and BSA.

**Purification and Characterization**

**Chromatography.** Bovine PAG bound quantitatively to the DEAE column. It eluted at NaCl concentrations between 0.04 M and 0.2 M, with maximum amounts appearing at 0.08 M. Figure 2A shows the profile of eluted proteins from the Sephadex G-75 column. RIA monitoring showed that the bPAG was mainly recovered in fractions 35–40, which correspond to an apparent molecular weight in the range of 40 000–100 000. HPLC on the Mono S column gave a single major peak of bPAG (Fig. 2B) which gave one major and one minor band on SDS-PAGE with apparent molecular weights of 67 000 and 35 000, respectively (Fig. 3). On the Mono P column, proteins were eluted in four main peaks (I to IV; Fig. 2C), which were highly immunoreactive in Western blotting (Fig. 4B) and by RIA (Fig. 1C).

**SDS-PAGE, IEF, and immunoblot analysis.** Coomassie Brilliant Blue R250 dye and silver-stained SDS-PAGE (Figs. 5 and 6) show that each peak of Mono P was highly homogeneous. The major band of Mono S (Fig. 3), the four peaks of Mono P, and BSA (Fig. 5) have the same molecular weight, and the former are immunoreactive for R498 antiserum (Fig. 4B). On the other hand, even though the four variants had identical molecular weights, their apparent isoelectric points were estimated to be approximately 4.4, 4.6, 5.2, and 5.4 (Figs. 4A and 4B).

**Carbohydrate and Sialic Acid Determination**

The different peaks of Mono P contained carbohydrates, indicating that bPAG is a glycoprotein. As determined by the method of Dubois et al. [28] and Warren [29], the carbohydrate and sialic acid contents were respectively 10.02 ± 1.09% and 0.97 ± 0.1%. The sialic acid amounts of the four isoforms were 0.29 ± 0.06%, 0.65 ± 0.08%, 0.83 ± 0.08%, and 2.12 ± 0.31% (means of three assays) by weight, respectively.

**NH₂-Terminal Amino Acid Microsequence**

Direct microsequence analysis was carried out on a portion of bPAG. A total of 39 cycles of analysis gave the sequence shown in Figure 7. Genbank (Microgenie software, Beckman) searches for any sequence identity were unfruitful.

**DISCUSSION**

The results demonstrate that the 67 000 $M_r$ polypeptide component isolated and purified from bovine fetal cotyle-

dons is an acidic glycoprotein that appears to be associated with pregnancy. Critical steps in its purification included an initial extraction of tissue at acidic pH and subsequent high-performance anion exchange and IEF procedures.
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FIG. 2. Respective chromatographic profiles: (A) G-75 gel filtration elution profile of 0.08 M NaCl fraction of DEAE chromatography in 0.01 M Tris-HCl, pH 7.6. The stippled area indicates location of immunoreactive fractions. (B) HPLC on Mono S column (5 mm x 5 cm) equilibrated in 0.01 M ammonium acetate buffer, pH 5. The broken line indicates the slope of linear salt gradient and the stippled area indicates the bPAG immunoreactive fractions. (C) HPLC on Mono P column equilibrated in 0.025 M bis-Tris-HCl buffer, pH 6.3. The broken line indicates the slope of the pH gradient. Peaks I, II, III, and IV are bPAG-immunoreactive.

SDS-PAGE and IEF were carried out to estimate the relative purity and the apparent molecular weight and pI, respectively, of the protein. Coomassie Brilliant Blue R250 dye and silver-stained SDS-PAGE indicated that preparations from the four Mono P peaks are highly homogeneous and of similar molecular size (approximately that of BSA). Thus, the estimated molecular weight of each of the four fractions was about 67,000. Reduction with mercaptoethanol did not reduce the size of the bPAG band. This observation indicates that the molecule is probably composed of a single
peptide chain with no interchain disulfide bonds. Similar observations have been made for the human SP, [35].

Chromatofocusing and electrofocusing of protein from the major Mono S fraction gave four distinct immunoreactive peaks or bands (Fig. 4B), each with different estimated pI. The second and third peaks were the most abundant. The variability in pI noted could be explained by the amount of sialic acid found associated with each peak. This variability in sialic acid content could result from different amounts of sialic acid added during synthesis or from subsequent loss of these relatively labile groups. However, this variability in sialic acid did not modify the apparent molecular weight. On the other hand, the immunoreactivity did seem to be modified. Indeed, as shown in Figure 1C, the immunoreactivity decreased from peak I (the most basic) to peak IV (the most acid). There was good correlation among sialic acid content, pIs, and immunoreactivity, indicating that these three characteristics may be closely linked.

Other workers have described a glycoprotein, PSPB, which was isolated either from fetal membranes [18], from cotyledons, or from cultures of whole cotyledons [36–38]. This protein exhibits a range of molecular weights and isolectric forms [38]. Although the product we describe has a molecular weight nearly identical to that of one of the multiple forms of PSPB ($M_r = 65$ 000), it is difficult to claim that the two polypeptides are identical without comparing their amino acid sequences or testing them immunologically. Some RIA results [36] seem to show a lack of identity between the two glycoproteins.

The bPAG N-terminal amino acid sequence, determined over 39 cycles, indicated the presence of only a single polypeptide. This sequence was not represented in any Gen-
FIG. 4. Electrofocusing in preformed ampholine PAG plates (pH 4–6.5, LKB): 25 µg of protein were loaded. (A) Lane 1 = pl standard; lanes 2–5 = Mono P peaks IV, III, II, and I; lane 6 = major peak of Mono S. (B) Corresponding Western blot treated with antiserum anti-bPAG (R498).
Fig. 5. Coomassie Brilliant Blue R250-stained SDS-PAGE containing 2.5% of β-mercaptoethanol (v/v); 30 μg protein was loaded. Lane 1 = BSA (Boehringer); lane 2 = 35 000 M, band (peak 0 in Fig. 2C); lanes 3-6 = Mono P peaks I, II, III, and IV.

Fig. 6. Silver-stained SDS-PAGE of the four major peaks of Mono P (lanes 1-4 = peaks IV-I) and Mono S peak fraction (lane 5). The gel was run with 2.5% of β-mercaptoethanol, and 2.5 μg of each protein was loaded.
Bank data and was clearly distinct from that of SP1 [39]. According to the 39 NH2-terminal amino acids, the bPAG appears to be unrelated to other placental proteins.

When the 125I-radiolabeled bPAG (pool of Mono P) and the R497 and R498 antisera at final dilutions of 1:3 750 000 were used, no inhibition of binding was observed with AFP, even at high concentrations. No cross-reactions were observed with several gonadotropic and placental hormones (bLH, pFSH, bP, bCG, and eCG). This suggests that the RIA is specific. In this respect, our results differ from those of Sasser et al. [36], who observed inhibition of binding to PSPB with bLH, oFSH, and oP.

The highly purified protein obtained and its specific high-titer antiserum can provide a highly specific and sensitive RIA. These reagents should allow for further studies of the pregnancy-associated protein to be conducted, e.g., the molecular cloning of its cDNA. In addition, these reagents will be useful in establishing the biological function(s) of bPAG and in providing evidence for or against nonplacental accessory sources of bPAG in females or in males. In this regard, bPAG or bPAG-like protein has been identified immunologically in bovine and ovine testes [40]. These reagents might also be useful for diagnosis of early pregnancy and determination of embryonic mortality in cattle and other domestic ruminant species.

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**FIG. 7.** NH2-terminal amino acid sequence of bPAG. Question marks indicate no assignment, and the bold characters indicate uncertainty in assignment.
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