

Isolation and characterization of eight pregnancy-associated glycoproteins present at high levels in the ovine placenta between day 60 and day 100 of gestation

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Abstract – Pregnancy-associated glycoproteins (PAG), structurally related to aspartic proteinases, are expressed in the outer epithelial cell layer (chorion/trophectoderm) of the ungulate placenta. The aim of the present study was to isolate as many PAG molecules as possible from placentae collected between day 60 and day 100 of gestation and to characterize their amino-terminal amino-acid sequences. Three heterologous radioimmunoassays were used to monitor PAG immunoreactivity throughout the isolation procedures. Sequential use of DEAE-cellulose, gel filtration, and CM ceramic chromatographies led to the isolation of several fractions rich in PAG immunoreactivity. The fractions with a large amount of proteins were also purified by chromatofocusing. The analysis of immunoreactive fractions by SDS-PAGE, Western blotting and two-dimensional electrophoresis followed by amino-terminal microsequencing on PVDF membranes allowed to identify eight different ovPAG with apparent molecular masses ranging from 55 to 66 kDa and isoelectric points from 4.0 to 6.8. The N-terminal sequences were determined and their comparison to those previously identified revealed that four of them are identical to those encoded by previously known cDNA, while the additional four sequences appear to be novel since they have not yet been described.

ovine / pregnancy-associated glycoprotein / placenta / multiple forms / N-terminal microsequencing

1. INTRODUCTION

Aspartic proteinases (EC 3.4.23) constitute one of four main classes of endopeptidases. They form a diverse family of proteins present in a wide variety of organisms including retroviruses, fungi, plants, and vertebrates [1]. Over the past decade, new

additions to this family called pregnancy-associated glycoproteins (PAG), have been identified in the placentae of ruminant species [2]. They have been found to share more than 50% amino-acid sequence identity with pepsinogen, pepsin, cathepsin D, and cathepsin E. Unlike other members of the aspartic proteinase family, most PAG

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seem to be inactive enzymes because of amino acid substitutions in and around the catalytic site [2].

Pregnancy-associated glycoproteins were first described as acidic 67-kDa glycoproteins with different isoelectric points. They were purified from cattle placenta and are also found in the maternal blood serum soon after embryo implantation [3]. These glycoproteins were later proven to be closely related to bovine PSPB (Pregnancy Specific Protein B) [4], isolated as early as 1982 by Butler et al. [5]. In sheep, PAG called ovPAG [2], SBU-3 [6], or oPSPB [7] were identified mainly by three different approaches. In 1993, Atkinson et al. [6] used a monoclonal antibody (anti-SBU-3) recognizing a placenta-specific carbohydrate epitope [8] to immunopurify placental antigens from placentomes of pregnant sheep uteri at various stages of gestation. Surprisingly, the three glycoproteins purified by affinity chromatography (57-, 62-, and 69-kDa) are similar but differ from other PAG at their amino-termini (61–78% similarity). This highlighted at an early date the surprising size and diversity of this glycoprotein family in sheep. Xie et al. [9] confirmed this diversity when, using a series of chromatographic procedures, they purified four additional molecules from the medium after culture of explants from day-100 ovine placenta. These molecules were named according to their molecular masses: ovPAG₅₅, ovPAG₆₀, ovPAG₆₁, and ovPAG₆₅. We very recently, isolated three novel molecules (ovPAG-55, ovPAG-57, and ovPAG-59) with different amino-acid sequences from ovine fetal cotyledons (on or after day 100) [10]. These proteins show similarity to all PAG identified earlier, especially with regards to the cleavage point (Arg or Ile), the consensus sequences for the carbohydrate sulfation site (Pro-Leu-Arg), and N-glycosylation site.

In parallel with the biochemical isolations, nine full-length ovine PAG-encoding cDNA were identified by screening cDNA libraries obtained at different stages of pregnancy with cDNA probe mixtures and

by applying reverse-transcription PCR. The PAG identified in this way include ovPAG-1 [2], ovPAG-2 [11], and ovPAG 3 to 9 [12]. In addition, ribonuclease protection assays and in situ hybridization have yielded more information on PAG-family proteins [13]. Firstly, ovPAG-2 appears to be the only PAG expressed on day 13. On day 16, ovPAG-5 and ovPAG-7 are detectable. The onset of expression of ovPAG-3, -6, -8, and -9 occurs later in pregnancy. Secondly, PAG fall into two groups: PAG expressed predominantly in binucleate cells (ovPAG-1, -3, -4, -5, -6, -7, -8 and -9) and a PAG (ovPAG-2) whose mRNA is detected throughout the trophoblast [13]. So far, only one molecule (ovPAG₆₅) of the nine identified cDNA has been successfully column-purified [9].

To our knowledge, attempts to purify PAG molecules expressed very early in pregnancy have been unfruitful probably due to the high proportion of lipoproteins present in young placenta, blocking the chromatographic gels used for fractionation [14]. In addition, it is unknown whether very early expressed PAG might be produced in sufficient amounts to make them good candidates for the development of pregnancy tests for sheep. Therefore, the aim of this study was to isolate different PAG proteins from ovine placenta collected earlier than in our previous work (between day 60 and day 100 of gestation), and to characterize them in terms of their molecular masses, isoelectric points, and N-terminal amino-acid sequences.

2. MATERIALS AND METHODS

2.1. Extraction and fractionation

Sheep foetal cotyledons (1 580 g) were collected from pregnant uteri at the slaughterhouse. The stage of pregnancy was assessed by measuring the crown-rump length of the foetuses [15]. As in previous works, the placenta were collected after day 100 [9, 10]; in this study the period was focused on days 60 to 100. Total protein

(TP) concentrations of all fractions were determined by the Lowry method [16]. The immunoreactivity of fractions obtained throughout the purification procedure was monitored by three heterologous radio-immunoassays (RIA-1, RIA-2, RIA-3) using a highly purified boPAG (bovine PAG-1) prepared in our laboratory [3] as the standard and tracer and combined with three different antisera raised respectively against ovine PAG (R495) [17] or caprine PAG (caPAG₅₅₊₅₉ (R708) and caPAG₅₅₊₆₂ (R706)) [18]. The cotyledons were cut into small pieces and washed with saline to remove blood. The tissues were homogenized in potassium phosphate buffer (0.10 M, pH 7.6) containing a cocktail of proteinase inhibitors as described by El Amiri et al. [10]. The extract was subjected to acid precipitation (pH 4.5) and then to ammonium sulfate fractionation. The latter was performed by adding ammonium sulfate to achieve 40% saturation. After centrifugation, the pellet was discarded and additional ammonium sulfate was added to obtain an 80%-saturated solution. The 40%-80%-precipitate (6 800 mg total protein) was resuspended in 240 mL of 0.01 M Tris-HCl buffer (pH 7.6) and applied on anion-exchange chromatography (14 cm × 18 cm; DEAE-cellulose DE52; Whatman, Clifton, NJ). After the unbound proteins had washed through, the column was eluted in six steps of increasing ionic strength buffer by adding NaCl (20, 40, 80, 160, 320 mM, and 1 M). These fractions with immunoreactivity (40 and 80 mM) were subjected to gel filtration on a Sephadex G-75 (5 cm × 100 cm; Amersham Pharmacia, Uppsala, Sweden) equilibrated in ammonium bicarbonate buffer (0.05 M, pH 8). Twenty milliliters of sample containing 840 mg of total protein (fraction eluting at 40 mM NaCl from the DEAE column) or 500 mg of total protein (fraction eluting at 80 mM NaCl from the DEAE column) was loaded successively onto the column. The flow rate was 1 mL·min⁻¹ and fractions of 15 mL were collected. They were monitored by measuring the UV absorption at 280 nm and by means of heterologous

RIA. Next, fractions with high immunoreactivity were pooled and dialyzed against 10 mM ammonium acetate buffer (pH 5.2). The dialyzed proteins (150 mg from the 40 mM DEAE fraction and 200 mg from the 80 mM DEAE fraction) were run through a cation-exchange FPLC (1 cm × 3 cm; CM Ceramic HyperD F column; BioSeptra, Cergy-Saint Christophe, France). The proteins were then eluted with a linear gradient of NaCl (0–1 M NaCl in 10 mM ammonium acetate, pH 5.2).

When large amounts of proteins were available, the most immunoreactive fractions from CM Ceramic chromatography were subjected to chromatofocusing on a Mono P HR column (5 mm × 5 cm; Amersham Pharmacia). The column was equilibrated with start buffer (0.25 M bis-Tris, pH 6.3) and the proteins were eluted with Polybuffer 74 (Amersham Pharmacia LKB, Uppsala, Sweden) diluted 1:10 (v/v) with distilled water and adjusted to pH 4.0 with 0.1 N HCl. The flow rate was 0.7 mL·min⁻¹ and 1-mL fractions were collected. Ampholines were removed from the samples by saturating each fraction with 80% ammonium sulfate. Each protein peak was dialyzed, lyophilized, and subjected to SDS-PAGE on minigels (Bio-Rad, Hercules, CA).

2.2. Characterization

Immunoreactive fractions eluted from the CM Ceramic or Mono P HR columns were denatured in Laemmli buffer, then separated on 12% polyacrylamide gels in the presence of SDS and stained with Coomassie Blue R-250. Molecular weight standards, commercially available when the study was carried out (LMW Electrophoresis Calibration Kit; Amersham Pharmacia), were run simultaneously. Duplicates of the separated proteins were then transferred onto nitrocellulose membranes according to Towbin et al. [19] for Western blotting. The proteins were probed with the three above-mentioned polyclonal antibodies (R495, R708 and R706) and immunoreactive ovPAG were visualized.

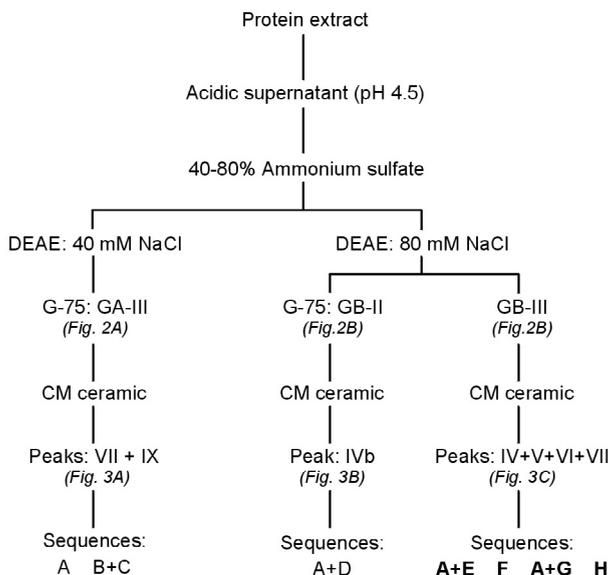


Figure 1. Diagram of ovPAG purification. The protein extract was fractionated by acidic and ammonium sulfate precipitation. The immunoreactive fraction precipitating between 40 and 80% saturation was run through a DEAE-cellulose column. The fractions eluting at 40 mM and 80 mM NaCl were subjected separately to gel filtration and CM ceramic chromatography. Letters A, B, C, D, E, F, G, H correspond to different N-terminal amino-acid sequences of immunoreactive proteins. The same letter appearing in different branches of the diagram means that the same N-terminal sequence was identified after the depicted procedures.

Immunoreactive fractions were also subjected to two-dimensional gel electrophoresis, using pH 3–10 Immobiline Drystrips (11 cm, Amersham Pharmacia, Uppsala, Sweden) for the first dimension followed by SDS-PAGE (12% acrylamide) as the second dimension. Spots of immunoreactive proteins were excised from the dried PVDF membranes and were N-terminally microsequenced. In some cases, when the protein isoforms overlapped and the spots were not clearly separated, the Edman degradation was performed on the proteins separated by 1D PAGE after transfer onto the PDVF membranes.

N-terminal amino-acid microsequences were obtained by gas-phase Edman degradation in a protein sequencer (Applied Biosystems Inc, Foster City, CA). The sequences obtained were compared with those of a databank (Fasta 3) [20] so as to detect homologies with known or deduced N-terminal sequences. They were then assem-

bled into multiple sequence alignments by pairwise comparisons with the PILEUP program of the Wisconsin Package, version 10.0, Genetics Computer Group (GCG, Madison, WI). A distance matrix was created by the old distances program (GCG). Then the sequences were compared again with those of 10 ovPAG molecules identified previously after a series of chromatographies [9, 10] or after immunoaffinity [6]. Only the alignment of the novel proteins is presented here. Alignments with the deduced N-termini of previously identified ovPAG are available from the authors up on request.

3. RESULTS

3.1. Extraction and fractionation

A schematic description of the PAG isolation is presented in Figure 1. Most immunoreactive proteins remained in the extraction

Table I. First steps of ovPAG purification: total protein (TP), immunoreactive protein, and ratio of immunoreactive to total protein.

Purification steps	TP ^a (mg)	PAG ^b (mg) [boPAG/TP ratio (%)]		
		RIA-1	RIA-2	RIA-3
Extraction	20 600	721 [3.5]	650 [3.1]	660 [3.2]
Acidic precipitation	10 380	540 [5.2]	497 [4.7]	580 [5.6]
Ammonium Sulfate (40–80%)	5 730	533 [9.3]	480 [8.5]	505 [8.8]
40 mM DEAE column fraction	840	170 [20]	80 [9.9]	152 [18.2]
80 mM DEAE column fraction	510	410 [80]	156 [30.7]	210 [41.2]

^a Determined by the Lowry method in BSA equivalents.

^b Determined by RIA.

supernatant after homogenization, and acidic precipitation. The ovPAG molecules were precipitated between 40%- and 80%-saturated ammonium sulphate. They were run through the DEAE-cellulose column. The ratios of immunoreactive to total protein in the material obtained in the above cited steps, as determined by three different RIA, are summarized in Table I. The majority of immunoreactive proteins were eluted from the DEAE-column at an NaCl concentration of 40 mM and 80 mM. Isolation was then continued independently for both fractions. The fraction eluted from the DEAE column in the presence of 40 mM NaCl was chromatographed on Sephadex G-75, the immunoreactive proteins were recovered in peak GA-III (fractions 77 to 90) (Fig. 2A). When the same procedure was applied to the "80 mM NaCl" fraction, two immunoreactive peaks were observed: GB-II (fractions 68 to 77) and GB-III (fractions 78 to 88) (Fig. 2B). Further purification of the pools GA-III (100 mg TP), GB-II (133 mg TP) and GB-III (154 mg TP) on CM Ceramic HyperD F chromatography gave highly immunoreactive peaks on the elution profiles (Figs. 3A, 3B and 3C). The GA-III yielded two immunoreactive peaks: VII (9.9 mg) and IX (9.5 mg) (black and hatched areas of Fig. 3A). Fractionation of the GBII and GBIII yielded a single immunoreactive peak IV_b (5.5 mg) in the former case (Fig. 3B) and four immunoreactive peaks IV

(17 mg), V (8.8 mg), VI (4.9 mg), and VII (18 mg) in the latter (Fig. 3C).

3.2. Characterization

Systematic analysis by means of SDS-PAGE, Western blotting, and 2D-electrophoresis of all CM ceramic immunoreactive peaks revealed several ovPAG. After SDS-PAGE, peak VII from Figure 3A revealed one major band with an apparent molecular weight of 58 kDa (Fig. 4A, lane 2) reacting strongly in Western blotting (Fig. 4A, lane 3) and showing several pI values (Fig. 4A, lane 3). Further fractionation of the same peak (6.8 mg) on a mono P column also revealed six variants (data not shown).

Pool IX from Figure 3A analyzed by SDS-PAGE showed two major bands (58 and 61 kDa) (Fig. 4B, lane 5), both being immunoreactive on Western blot (Fig. 4B, lane 6), and both having isoforms with different pI (pI 4.0 to 5.1 and 4.1 to 6.2 for 58 and 61 kDa, respectively), as shown after 2D PAGE (Fig. 4B, lane 7). Further fractionation of this pool on a mono P column confirmed the presence of several isoforms for each molecular weight (data not shown).

When peak IV_b from Figure 3B was subjected to SDS-PAGE, three major bands appeared (Fig. 4C, lane 8), two of them (55 and 66 kDa) being highly immunoreactive

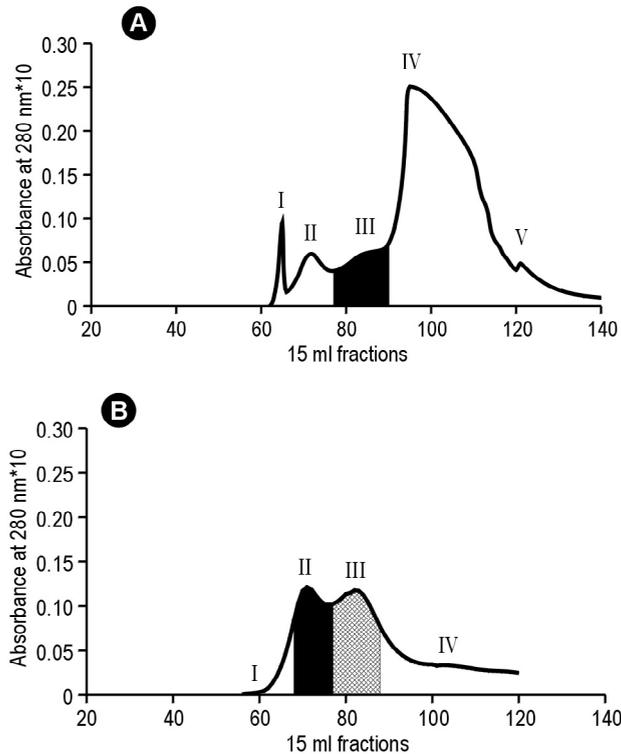


Figure 2. Gel filtration profiles of ovine placental proteins collected between day 66 and day 100 of gestation. (A) Elution profile of the “40 mM NaCl” fraction. (B) Elution profile of the “80 mM NaCl” fraction. The black and hatched areas indicate the immunoreactive fractions. When the absorbance of a fraction was higher than 1, it was determined in a tenfold diluted sample.

on Western blotting (Fig. 4C, lane 9). Both immunoreactive proteins gave different isoforms with different pI after 2D PAGE (Fig. 4C, lane 10).

Figure 4D shows the results obtained for peaks IV, V, VI, and VII from Figure 3C after either Coomassie blue staining (lanes 11–14) or Western blotting (lanes 15–18). The first approach revealed two major bands (59 and 66 kDa) for peak IV (lane 11), one major band (60 kDa) for peak V (lane 12), two major bands (60 and 59 kDa) for peak VI (lane 13), and two major bands (55 and 80 kDa) for peak VII (lane 14). All these major bands cross reacted in Western blotting (lanes 15–18) except the 80-kDa band generated from peak VII. In this case, a

major band detected after SDS-PAGE did not appear on the Western blot (Fig. 4D, lanes 14 and 18); in addition a 60-kDa molecule which was not detected with Coomassie Blue appeared clearly in the Western blot. This peak was also fractionated further on the mono P column (data not shown).

NH₂-terminal residues were determined on spots separated by one or two dimensional electrophoresis after the proteins were electroblotted on PVDF membranes. Table II summarizes the origin of the eight isolated ovPAG, their characteristics (molecular masses and pI), their accession numbers, and their highest identities with known PAG. Four different pools shared the same NH₂-terminal aa sequence (P83493). Screening of

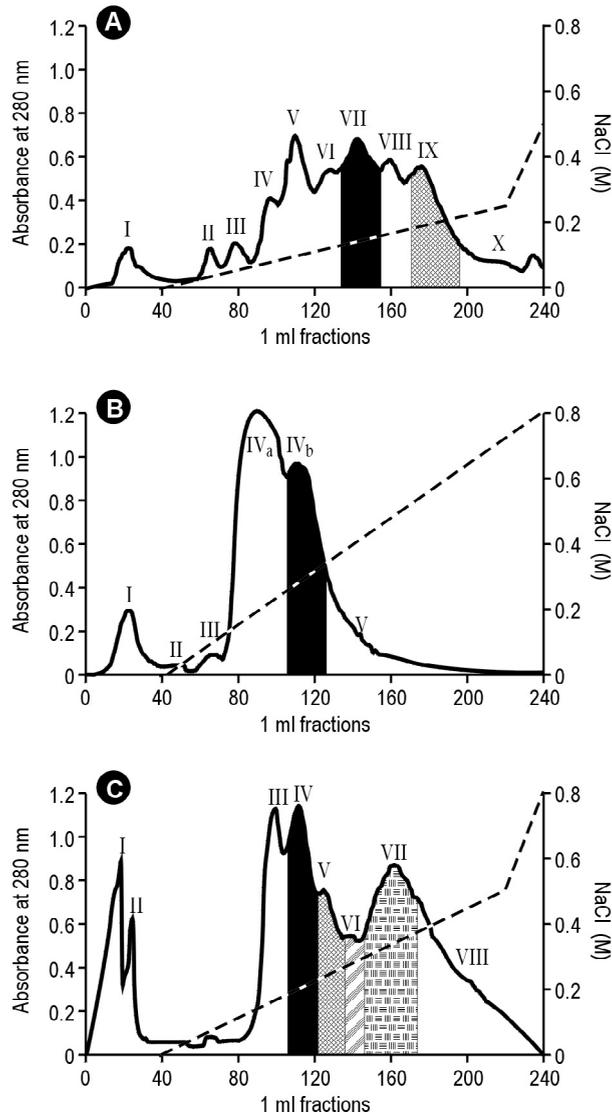


Figure 3. FPLC chromatographic elution profiles of GA-III (A), GB-II (B), and GB-III (C). The CM Ceramic HyperD F column (1 cm × 3 cm) was equilibrated in ammonium acetate buffer (10 mM, pH 5.2). Broken lines indicate the salt gradients. Black and hatched areas indicate the locations of the fractions showing the highest immunoreactivity: (A) peaks VII and IX; (B) peak IV_b; (C) peaks IV, V, VI, VII.

the EMBL and SWISS-PROT data banks revealed high sequence identity between the ovine PAG isolated in the present work (Tab. II) and numerous PAG of ovine (ovPAGs/SBU-3), caprine (caPAG), and

bovine (boPAG) origin (data not shown). Moreover, it showed that the amino-terminal sequences A (P83493), C (P83495), D (P83496), and E (P83497) shared 100% identity with known PAG while the additional

Table II. Apparent molecular masses, isoelectric points, and N-terminal microsequences of the highly immunoreactive proteins obtained after CM ceramic chromatography.

CM peak	Sequences	Identified as	Accession numbers	Mr*	PI**	Percent identity
^a VII	RGSNLTIHPLRNTKDL-VYLG	A	P83493	58	4.5;4.7;4.9;5.4;5.7;6.2	100% ovPAG-6
^a IX	RGSNLTIHPLRNTNDIDY	B	P83494	61	4.0;4.1;4.7;4.8;5.1	83% ovPAG-6 [§]
	RGSNLIHPLRNIRDTFYV-GNIT	C	P83495	58	4.1;4.7;4.8;5.1;5.4;6.2	100% ovPAG-4
^b IV _b	RASNLTIHPL	D	P83496	66	4.5;5.0;5.6;5.9	100% ovPAG-1
	RGSNLTIHPLRNTKDK	A	P83493	55	4.5;4.8;5.5;5.7;6.2	100% ovPAG-6
^c IV	RGSNLTIHPLRNTKDK	A	P83493	59	4.7;4.8;5.0;5.2;5.3	100% ovPAG-6
	RDSNVLTILPLRNMKDK	E	P83497	66	4.7;4.8;5.0;5.2;5.3	100% ovPAG-3/ ovPAG-7
^c V	RGSNLTIHPLRNTKDR-VYIG	F	P83498	60	4.6;5.1;5.4;5.6	90% ovPAG-6 [§]
^c VI	RGSNLTIHPLRNTKDK	A	P83493	60	6.1;6.2	100% ovPAG-6
	RGSNLTIHPLRNIKDLVYL-GNMGIG	G	P83499	59	6.1;6.2;6.5;6.7;6.8	88% ovPAG-6 [§]
^c VII	RVSNLTIHPLRNIIIDLRYVG	H	P83500	55	4.6;4.7;4.8;4.9	80% ovPAG-1 [§]

* Molecular weights (Mr) expressed in kDa were estimated after one-dimensional SDS-PAGE.

** Isoelectric points (pIs) were estimated after two-dimensional SDS-PAGE.

^a Peaks isolated from GA-III after CM ceramic.

^b Peaks isolated from GB-II after CM ceramic.

^c Peaks isolated from GB-III after CM ceramic.

[§] Novel ovPAG.

four sequences B (P83494), F (P83498), G (P83499), and H (P83500) were different from known PAG. The latter sequences were compared with the 10 ovPAG previously isolated biochemically (Figs. 5I, 5II, 5III and 5IV). A comparison of all these ovPAG to each other revealed 60% to 95% identity in the sequenced region. Especially their identities to those we recently isolated from placenta removed at ≥ 100 day of gestation ranges from 55 to 93%. All sequences display the consensus sequence: R-SN- - I-PLRN, except sequence D, which counts only 10 residues. All mature forms of isolated ovPAG have Arg as the N-terminal amino acid.

4. DISCUSSION

This manuscript describes the isolation and characterization of eight different PAGs

from sheep placentae removed between 60 and 100 days of gestation. It is novel in that the studies were completed at a stage of gestation that has yet to be examined. Our study confirmed one of the most interesting features of PAGs, the switching of gene expression [13, 21]. Some PAG are thought to be expressed first and then to be replaced at a later time by others. It is interesting to remember that a similar regulation at the transcriptional level is also thought to be responsible for the switching of genes likewise for pepsinogen F [22].

Electrophoretic analysis of the major immunoreactive pools separated by gel filtration, and ion exchange chromatography showed that each was composed of one to two PAG that differed in apparent molecular mass, isoelectric point and amino-terminal sequence (Fig. 1 and Tab. II). Such heterogeneity has been suggested to be the result

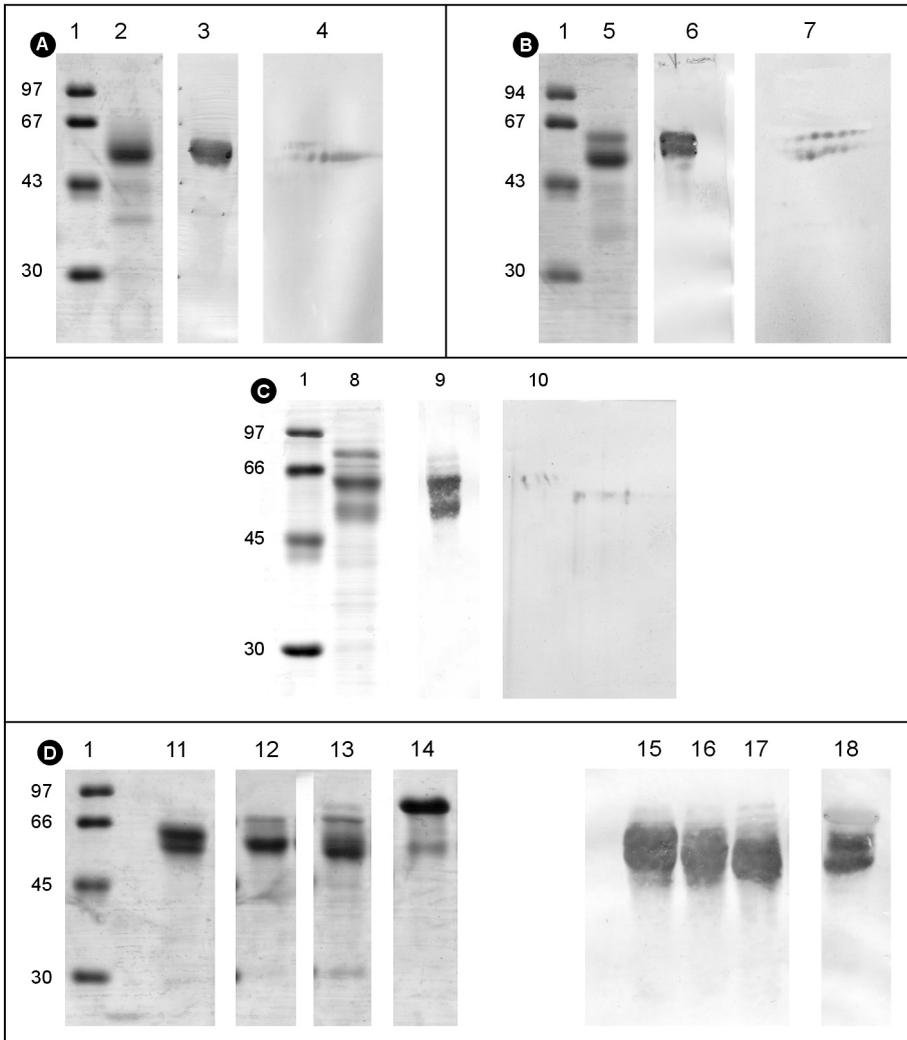


Figure 4. Electrophoresis analysis of the ovPAG contained in peaks emerging from the CM ceramic columns. In all panels, molecular weight standards ($\times 10^{-3}$) are identified on right slides (lanes 1). Panel (A): lanes 2, 3 and 4 represent respectively Coomassie bleu, Western blotting and two-dimensional isoelectric focusing of peak VII (from GA-III). Panel (B): lanes 5, 6 and 7 represent respectively Coomassie bleu, Western blot and two-dimensional isoelectric focusing of peak IX (from GA-II). Panel (C): lanes 8, 9 and 10 represent respectively one-dimensional SDS-PAGE, Western blotting, and two-dimensional isoelectric focusing of peak IVb (from GB-II). Panel (D): lanes 11, 12, 13, 14 respectively show one-dimensional SDS-PAGE of peaks IV, V, VI and VII (from GB-III). Lanes 15, 16, 17, 18 show the corresponding Western blots using R708.

I

Sequence B	R	G	S	N	L	T	I	H	P	L	R	N	T	N	D	I	D	Y	I:S*
ovPAG-55 _(p1832)	R	G	S	X	L	T	I	L	P	L	R	N	M	R	D	I	V	Y	72.72
ovPAG-57 _(p1833)	R	V	S	X	L	T	I	H	P	L	R	N	I	M	D	M			69.75
ovPAG-59 _(p1844)	R	G	S	N	L	T	I	H	P	L	R	N	I	R	D				87.87
ovPAG ₍₁₀₄₎					L	T	I	X	N	L									67.67
ovPAG ₍₂₀₄₎	R	G	S	X	X	R	I	H	P	L	R	N	H	R	D				67.67
ovPAG ₍₁₀₄₎	R	G	S	X	L	X	I	H	P	L	R	N	V	K	D	V	V	Y	67.72
ovPAG ₍₁₀₄₎	R	D	S	X	V	T	I	L	P	L	R	N	M	K	D	I	F	Y	61.67
SBU-3 ^{SH} _(DITREB)							I	X	P	L	R	N	T	K	H	L	V	Y	58.67
SBU-3 ^{SH} _(DITREI)			S	X	L	T	I	L	P	L	R	N	M	X	D	I	F	Y	69.69
SBU-3 ^{SH} _(DITREI)	Q	G	S	X	V	T	I	L	P	L	R	N	M	K	D	I	F	Y	61.72

II

Sequence F	R	G	S	N	L	T	I	H	P	L	R	N	T	K	D	R	V	Y	I	G	I:S*	
ovPAG-55 _(p1832)	R	G	S	X	L	T	I	L	P	L	R	N	M	R	D	I	V	Y			72.78	
ovPAG-57 _(p1833)	R	V	S	X	L	T	I	H	P	L	R	N	I	M	D						73.73	
ovPAG-59 _(p1844)	R	G	S	N	L	T	I	H	P	L	R	N	I	R	D						87.93	
ovPAG ₍₁₀₄₎					L	T	I	X	N	L											67.67	
ovPAG ₍₂₀₄₎	R	G	S	X	X	R	I	H	P	L	R	N	H	R	D						67.63	
ovPAG ₍₁₀₄₎	R	G	S	X	L	X	I	H	P	L	R	N	V	K	D	V	V	Y	L		74.79	
ovPAG ₍₁₀₄₎	R	D	S	X	V	T	I	L	P	L	R	N	M	K	D	I	F	Y	V	G		60.70
SBU-3 ^{SH} _(DITREB)							I	X	P	L	R	N	T	K	H	L	V	Y	L		69.77	
SBU-3 ^{SH} _(DITREI)			S	X	L	T	I	L	P	L	R	N	M	X	D	I	F	Y	R	G		61.61
SBU-3 ^{SH} _(DITREI)	Q	G	S	X	V	T	I	L	P	L	R	N	M	K	D	I	F	Y	V	G		60.75

III

Sequence G	R	G	S	N	L	T	I	H	P	L	R	N	I	K	D	L	V	Y	L	G	N	M	G	I	G	I:S*	
ovPAG-55 _(p1832)	R	G	S	X	L	T	I	L	P	L	R	N	M	R	D	I	V	Y								72.89	
ovPAG-57 _(p1833)	R	V	S	X	L	T	I	H	P	L	R	N	I	M	D	M	L									71.82	
ovPAG-59 _(p1844)	R	G	S	N	L	T	I	H	P	L	R	N	I	R	D											93.100	
ovPAG ₍₁₀₄₎					L	T	I	X	N	L																67.67	
ovPAG ₍₂₀₄₎	R	G	S	X	X	R	I	H	P	L	R	N														67.73	
ovPAG ₍₁₀₄₎	R	G	S	X	L	X	I	H	P	L	R	N	V	K	D	V	V	Y	L	X	G				79.90		
ovPAG ₍₁₀₄₎	R	D	S	X	V	T	I	L	P	L	R	N	M	K	D	I	F	Y	V	G	X				60.80		
SBU-3 ^{SH} _(DITREB)							I	X	P	L	R	N	T	K	H	L	V	Y	L	X	N	I	H	X	G		63.68
SBU-3 ^{SH} _(DITREI)			S	X	L	T	I	L	P	L	R	N	M	X	D	I	F	Y	R	G	X	I	T	I	G		57.70
SBU-3 ^{SH} _(DITREI)	Q	G	S	X	V	T	I	L	P	L	R	N	M	K	D	I	F	Y	V	G	X	I	T	I			54.79

IV

Sequence H	R	V	S	N	L	T	I	H	P	L	R	N	I	I	D	L	R	Y	V	G	I:S*	
ovPAG-55 _(p1832)	R	G	S	X	L	T	I	L	P	L	R	N	M	R	D	I	V	Y			61.72	
ovPAG-57 _(p1833)	R	V	S	X	L	T	I	H	P	L	R	N	I	M	D	M	L				81.94	
ovPAG-59 _(p1844)	R	G	S	N	L	T	I	H	P	L	R	N	I	R	D						87.87	
ovPAG ₍₁₀₄₎		V	S	X	L	T	I	X	N	L											67.67	
ovPAG ₍₂₀₄₎	R	G	S	X	X	R	I	H	P	L	R	N									67.67	
ovPAG ₍₁₀₄₎	R	G	S	X	L	X	I	H	P	L	R	N	V	K	D	V	V	Y	L		58.74	
ovPAG ₍₁₀₄₎	R	D	S	X	V	T	I	L	P	L	R	N	M	K	D	I	F	Y	V	G		60.75
SBU-3 ^{SH} _(DITREB)							I	X	P	L	R	N	T	K	H	L	V	Y	L		54.62	
SBU-3 ^{SH} _(DITREI)			S	X	L	T	I	L	P	L	R	N	M	X	D	I	F	Y	R	G		61.72
SBU-3 ^{SH} _(DITREI)			S	X	V	T	I	L	P	L	R	N	M	K	D	I	F	Y	V	G		61.78

Figure 5. Comparison of the N-terminal amino-acid sequences of the newly isolated ovPAG (from both the “40 mM NaCl” and “80 mM NaCl” fractions) with the 10 known molecules isolated biochemically from sheep placenta. (I) sequence B; (II) sequence F; (III) Sequence G; and (IV) sequence H. *Percent identity/similarity. The amino-terminal sequences of ovPAG-55, ovPAG-57, and ovPAG-59 are taken from El Amiri et al. [10]; those of ovPAG₅₅, ovPAG₆₀, ovPAG₆₁, ovPAG₆₅ are from Xie et al. [9], and those of the SBU-3 proteins are from Atkinson et al. [6]. Grey squares represent radical substitutions. Black squares represent conservative substitutions. The comparison is based on the region of highest similarity between sequences.

of carbohydrate content and the existence of more than one potential site of N-glycosylation. It was discussed in detail in our previous report [10], and in several earlier PAG purification attempts [6, 7, 9, 17, 23].

The large number of different molecules identified in our study may be due to the careful choice of our purification scheme. First, we used three different RIA (Tab. I) to probe all fractions issued from the DEAE column, and notably used antibodies raised against caprine PAG [18]. In 2002, Perenyi et al. [24] stated that RIA-2 and RIA-3 were better for detecting PAG molecules released between days 30 and 70 in pregnant cows and suggested that the antisera used in these assays (raised against caPAG55+59 and caPAG55+62) are better for detecting PAG-family members. Using the same antisera in double radial immunodiffusion assays, we recently found them to detect more effectively than the antiserum used in RIA-1 ovine PAG present at relatively low concentration in crude sheep placental extracts [25]. This high immunoreactivity of sheep placental extracts and ovine PAG with antisera against caPAG is not surprising. Sheep and goat are phylogenetically close, even though they can not cross-breed, their PAG have been shown to share a common epitope or epitopes at their surfaces [26], allowing cross-species immunodetection. Furthermore, it has been shown that the sequences of sheep and goat PAG are inter-mixed in the phylogenetic tree and share considerable sequence identity [27]. Second, the use of a G-75 column in the purification protocol provided additional immunoreactive fractions. Indeed, if this step was not included probably some PAG would have escaped the fractionation.

Edman degradation provided eight different PAG, all starting with an Arg residue that derived from cleavage occurring between the Phe and Arg in the sequence ISF↓RG/DSN [9]. The isolated sequences include, on the one hand, molecules whose amino-terminal amino-acid sequences were identical to PAG identified by cDNA

sequencing (Tab. II). These are peptides A (P83493); C (P83495); D (P83496); and E (P83497), corresponding to ovPAG6 (O02726), ovPAG4 (O02724), ovPAG1 (Q28755), and ovPAG3 (O02723) or ovPAG7 (O02727), respectively. On the other hand, we isolated new proteins sharing high but not 100% identity with the amino-terminal amino-acid sequences from other known members of the PAG family. These are peptides B (P83494); F (P83498); G (P83499); and H (P83500). Such diversity has been shown on two levels: cDNA and proteins. Regarding the cDNA, the first clone identified by Xie et al. [2] was shown to encode a protein (PAG-1) structurally related to pepsinogens, but differing from them by substitutions close to the catalytic center that would probably render it inactive as a proteinase. In the following years, additional PAG cDNA were identified: ovPAG-2 [11] and ovPAG 3 to 9 [12]. At the protein level, Atkinson et al. [6] used placentomes corresponding to various gestational ages to immunopurify three different SBU-3 antigens. They noted high similarity (ranging from 61 to 78%) between these molecules and the PAG that were known at that time. In addition, they highlighted for the first time the surprising size and diversity of this family of glycoproteins in sheep. In 1997, Xie et al. [9, 12] confirmed the diversity of the ovPAG family by isolating four molecules with different amino-acid sequences. We recently isolated three different PAG from placentae removed at a very late gestational stage (≥ 100 days) which are different but shared similarities with those isolated in the present study.

The sequence E (P83497) identified in the present study was until recently, the only ovPAG sequence identified by both molecular cloning and purification procedures [9]. Although the predicted N-terminal sequences coded by both ovPAG-3 and ovPAG-7 cDNA correspond to the same N-terminal sequence as that of peptide E, it cannot be excluded that this molecule might not correspond to any of them.

Four different CM ceramic fractions were found to contain molecules characterized by the N-terminal peptide A (P83493), which presented the same predicted amino-terminal microsequence as that encoded by the 5' region of ovPAG-6 cDNA. These molecules are quite abundant and display different molecular weights and isoelectric points. Sousa et al. [14] have likewise found different molecular masses (57 to 67 kDa) and different isoelectric points (4.4 to 6.7) for a boPAG-1 (bovine PAG-1) sequence isolated after systematic analysis of all DEAE fractions. In addition, Garbayo et al. [18] found the same caPAG-55 molecule in two different fractions after DEAE chromatography.

The comparison of the isolated ovPAG sequences to each other showed identities ranging from 60 to 90%. These values were in the same range as those previously described [12]. In fact, all amino-terminal amino acids deduced from PAG cDNA differ from each other by 10 to 40%. For example, the deduced amino-terminal amino acid sequences of ovPAG-3, -4, -6, -7, and 9 differ by no more than 30%. In particular, ovPAG-4 and ovPAG-7 share more than 90% identity, but ovPAG-2 and ovPAG-5 show only about 60% both to each other and to other ovPAG [28].

In conclusion, we show here that between day 60 and 100 of pregnancy, the ovine placenta produces multiple PAG with considerably different amino-terminal amino acid sequences. Our present work has contributed to new knowledge regarding the relative abundance and processing of different PAG. We obtained four novel PAG proteins. The newly discovered PAG and antisera raised against some of them might be used to improve the sensitivity and specificity of pregnancy diagnosis in sheep and to isolate PAG molecules expressed at different times during pregnancy.

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