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Isolation of pregnancy-associated glycoproteins (PAG) from water buffalo (*Bubalus bubalis*) placenta by use of *Vicia villosa* bound agarose affinity chromatography

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ABSTRACT: The present study describes the isolation and characterisation of new PAG molecules extracted from mid- and late-pregnancy placentas in the water buffalo (*Bubalis bubalis*). After extraction, acid and ammonium sulphate precipitation and DEAE chromatography water buffalo PAG (wbPAG) were enriched by *Vicia villosa* agarose (VVA) affinity chromatography. As determined by Western blotting with anti-PAG-sera, apparent molecular masses of immunoreactive bands from VVA peaks ranged from 59.5 to 75.8 kDa and from 57.8 to 80.9 kDa in the mid- and late- pregnancy placenta respectively. Amino-terminal microsequencing of proteins allowed the identification of three distinct wbPAG sequences wich have ben deposed in the SwissProt database: RGSXLTIHPLRNIRDFFYUG (Acc. n. P85048), RGSXLTILPLRNIID (P85049) and RGSXLTHLPLRNI (P85050). Their comparison to those previously identified revealed that two of them were new since they have not been described yet. Our results confirm the suitability of VVA chromatography in enrichment of multiple PAG molecules expressed in buffalo placenta. Productions of specific antisera can be very useful in immonoistochemical and immunocyitochemical studies of PAG expression in fetomaternal interfaces. Purified native PAG are also required for development on specific immoassays (RIA/ELISA) currently used for pregnancy diagnosis and physiological investigation in farm animal.

Key words: Water buffalo, Pregnancy-associated glycoprotein, Vicia villosa agarose, Placenta.

INTRODUCTION - Pregnancy-associated glycoproteins (PAG) constitute a large family of glycoproteins specifically expressed in the outer epithelial cell layer (chorion/trophectoderm)

of the placenta in several Eutherian species (Green *et al*., 2000; Szafranska *et al*., 2001) They are members of the aspartic proteinase family, having high sequence homology with each other as well as with pepsin, pepsinogen, chymosin, cathepsin D and E (Xie *et al*., 1991) The present study was conducted to verify whether multiple kinds of PAG-related molecules are expressed in *Bubalus bubalis* placenta. It was also of interest to know whether there were differences in PAG N-terminal microsequences obtained from mid-pregnancy and late-pregnancy placentas. Finally, obtained sequences were compared to those from other Eutherian species.

MATERIAL AND METHODS - Uteri were collected from pregnant buffalo cows (*Bubalus bubalis*) immediately after slaughter. The fetal cotyledons were immediately dissected away from caruncular tissue, washed with 0.9% NaCl and stored at -20°C until use. The stage of pregnancy was precisely determined based on the day of artificial insemination of females. The fetal cotyledons coming from 5 placentas collected at mid-pregnancy (5 months) and 2 placentas collected at late-pregnancy (8 months) gestational ages were treated separately.

Table 1. Microsequence analysis of the 62 kDa, 68 kDa, 70 kDa, 73 kDa and 75 kDa buffalo PAG.Amino acid microsequence analysis was performed by automated Edman degradation. *Indicates mid-pregnancy origin of buffalo placenta. **Indicates late-pregnancy origin of buffalo placenta.

Protein	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
wbPAG$_{62kDa}$*	R	G	S	X	L	T	H	L	P	L	R	N	I							
wbPAG$_{68kDa}$*	R	G	S	X	L	T	H	L	P	L	R	N	I							
wbPAG$_{70kDa}$*	R	G	S	X	L	T	I	L	P	L	R	N	I	I	D					
wbPAG$_{73kDa}$**	R	G	S	X	L	T	I	H	P	L	R	N	I							
wbPAG$_{75kDa}$*	R	G	S	X	L	T	I	H	P	L	R	N	I	R	D	F	F	Y	V	G
Consensus	R	G	S	X	L	T	-	-	P	L	R	N	I	-	D					

The purification procedure, included extraction of proteins at neutral pH, acidic and ammonium sulphate precipitations, anion exchange chromatography (DEAE) and *Vicia villosa* agglutinin affinity chromatography (VVA), was monitored by two different radioimmunoassay systems using a highly purified boPAG-1 as tracer and standard, and different antisera (AS#497 and AS#708+709) raised respectively against bovine PAG (boPAG$_{67kDa}$) and caprine PAG$_{55kDa+59kDa}$. The most immunoreattive fractions issued from anion and affinity exchange chromatographies were analysed by SDS-PAGE and Western blotting, before transfer to a polyvinylidene difluoride (PVDF) membrane for NH_2-microsequence determination. The sequence obtained were compareted to known protein sequences in the Swiss-Prot, TremblNew data banks. Total protein (TP) concentrations were determined by the Lowry method. The most immunoreattive fractions issued from anion and affinity exchange chromatographies were analysed by SDS-PAGE and Western blotting, before transfer to a polyvinylidene difluoride (PVDF) membrane for NH_2-microsequence determination. The sequence obtained were compareted to known protein sequences in the Swiss-Prot, TremblNew data banks.

RESULTS AND CONCLUSIONS - As determined by RIA-497, PAG-like proteins represented 0.68% and 0.34% of the total soluble proteins extracted from mid- and late-pregnancy buffalo placentas, respectively. In general, during the whole of the purification protocol, detection of immunoreactive proteins was improved by utilization of RIA-708+709.
Despite the relatively low PAG:TP ratio after DEAE chromatography, most of DEAE steps (0 M, 0.04 M, 0.08 M and 0.16 M NaCl) were treated by VVA affinity chromatography. Chromatographic profiles of 0.04 M, 0.08 M and 0.16 M DEAE fractions were very similar, irrespective of the placenta origin. A preliminary Western blot was carried out in order to detect immunoreactive PAG proteins issued from affinity chromatography. The apparent molecular masses of immunoreactive bands from VVA peaks ranged from 59.5 to 75.8 kDa (0.04, 0.08 and 0.16 DEAE steps from mid-pregnancy placentas). In late-pregnancy placentas, immunoreactive apparent molecular masses exhibited a slightly higher variation (from 57.8 to 80.9 kDa). Proteins issued from VVA peaks were electroblotted on PVDF membranes and submitted to Edman degradation on the basis of their Western blot reactivity and availability. The Table 1 summarizes the origin of the five N-terminal sequenced water buffalo PAG molecules (wbPAG), as well as their apparent molecular masses as determined after PVDF Coomassie staining.
There was no difference on N-terminal microsequence between $wbPAG_{75kDa}$ isolated from MP-Cot and $wbPAG_{73kDa}$ isolated from LP-Cot. This is consistent with previous results of Green *et al*. (2000) which showed that major changes of RNA expression of distinct PAG is mainly observed in early pregnancy and near term. Both $wbPAG_{75kDa}$ and $wbPAG_{73kDa}$ exhibited 100% identity (15 to 20 aa long) with boPAG-4 microsequence (Garbayo *et al*., 2000)The same identity was found with ovPAG-59, a protein isolated from ovine placentas older than 100 days (El Amiri *et al*., 2003) Surprisingly, none of the PAG sequences identified in water buffalo placentas collected between the 5^{th} and 8^{th} months of pregnancy corresponded to the major expressed $boPAG_{67kDa}$. Percentages of identity with $boPAG_{67kDa}$ varied from 80% ($wbPAG_{70kDa}$, $wbPAG_{73kDa}$ and $wbPAG_{75kDa}$) to 84.62% ($wbPAG_{62kDa}$ and $wbPAG_{68kDa}$).
Our results confirm both molecular mass and amino acid N-terminal sequence multiplicities within the buffalo PAG family. Five proteins presenting distinct relative molecular masses were submitted to Edman degradation (13 to 20 residues long). These sequences revealed the existence of three PAG never described in other species.
Production of specific PAG antisera can be very useful in immunohistochemical and immunocytochemical studies of PAG expression in fetomaternal interfaces (Zoli *et al*., 1992a; Klisch and Leiser, 2003; Wooding *et al*., 2005) Purified PAG are also required for development of radioimmunoassay and enzyme-linked immunosorbant assay techniques, currently used for pregnancy diagnosis and physiopathological investigations in farm animals (Zoli *et al*., 1992b; Green *et al*., 2005) Therefore, the present study provides a background on the use of lectin affinity chromatography for PAG purification. Naturally glycosylated PAG molecules can be obtained and used for further investigations clarifying the functional significance of differential glycosylated forms in the establishment and progression of pregnancy in Eutherian species.

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