Microsequencing of three Pregnancy-Associated Glycoproteins isolated from sheep placenta

B. El AMIRI[1-2], B. REMY[1], N.M. SOUSA[1], J. SULON[1], N. GERARDIN-OTTHIERS[3], H. BANGA-MBOKO[1], H. DESBULEUX[1] and J.F. BECKERS[1]

① Physiology of Reproduction, Faculty of Veterinary Medicine, ULg, B41, B-4000, Liège, Belgium
② INRA, CRRA Saïs et Moyen Atlas, Meknès, BP 578, Maroc
③ Laboratory of Biochemistry, Institute of Chemistry, ULg, B6, B-4000, Liège, Belgium

Introduction

In sheep, the pregnancy length is about 150 days, the placenta is localised cotyletonary. The presence of binucleate cells is characteristic (fig.1).

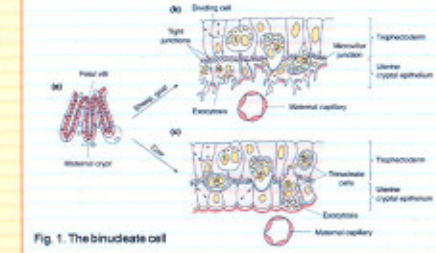

Fig. 1. The binucleate cell

Adapted from Wooding, 1992 and Green et al. 1998 (1,2)

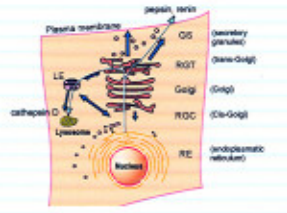

Fig. 2. Intracellular sorting of mammalian aspartic proteases. Schematic diagram of a cell including main organelles and transport pathways.

Adapted from Metcalf & Fusek, 1995 (3)

- In the last decade, using biochemical approach, the PAGs were first described and characterized in *Bos taurus* as acidic glycoproteins constituted by 380 aa and showing 4 molecular isoforms with 67 kDa molecular mass.
- The Pregnancy-Associated Glycoproteins (PAGs) constitute a large family of molecules synthesized in the superficial layers of the trophoblast and for part of them released in the maternal blood circulation.
- The PAGs belong to the aspartic proteinase family like pespsinogen, prochymosin, progastricsin, cathepsin D, cathepsin E, renin, napsin, memapsin, etc...
- The process of secretion in the maternal blood circulation is still unknown, however we can propose the general pathway described by Metcalf & Fusek (1995) (2) for cathepsin D, renin and pepsin to explain the hypothetical routes taken by different PAGs [Fig. 2].
- Most of the PAGs are considered to be enzymatically inactive because of mutations around their active site (3).
- Molecular biology studies identified different molecules: 21, 11 and 9 PAG cDNAs in cow (4), goat (5) and in ewe placentas (6), respectively.
- The same studies showed that PAG expression can vary spatially and temporally throughout gestation (4, 5).

Aim

The aim of this study was to investigate the characteristics of members of proteinase family extracted from sheep placenta

Material and Methods

Two different groups of placenta were used

Group I: placenta removed at third trimester of gestation ⟷ **Group II: placenta removed from 66 to 100 days**

The PAG purification procedure was based on that previously developed and described by Zoli et al. (8) for the bovine proteins.

The purification follow-up

Heterologous RIA
- boPAG-1 as tracer and standard
- caPAG$_{55-59}$ and ovPAG as antisera

Total protein (TP) determination Lowry method (9)

Extraction of soluble proteins

- 0.01 M potassium phosphate buffer pH 7.6 containing PMSF (0.2 mM)
- Pepstatin (1 mM)
- Leupeptin (1 mM)
- Na-EDTA (0.2%)

Isolation

Acidic precipitation:
pH 4.5 with H_3PO_4 0.5 M

Ammonium sulfate precipitations:
- 0-40% and 40-80% of saturation

Anion exchange chromatography
- DEAE-cellulose in 0.01 M Tris-Hcl, pH 7.6
- Eluted with 0.02, 0.04, 0.08, 0.16, 0.32 and 1 M Nacl concentration

Gel filtration chromatography
- Sephadex G-75 (5x100cm)
- 0.05 M ammonium bicarbonate pH 8.0

Cation exchange FPLC
- CM ceramic column (Pharmacia) (1x3 cm)
- 0.01 M ammonium acetate pH 5.2

Characterization

- SDS-PAGE 12% with reducing agent
- IEF-SDS-PAG
- After transfer onto PVDF membrane, the NH_2-terminal amino acid sequence is determined using a pulse liquid-phase protein sequencer (Procise 492 Applied Biosystems Foster City, CA)

Results

Group I — Placenta at third trimester of gestation

Localization of the main immunoreactive fractions

Table 1: Total protein (TP) and ratio PAG:TP of fraction eluted after DEAE column

NaCl concentration	TP (mg)	Antisera: caPAG$_{55-59}$ Ratio PAG:TP	Antisera: ovPAG Ratio PAG:TP
0 M	478.7	0.48	0.9
0.02 M	692.8	0.9	8.9
0.04 M	116.9	**35.35**	**76.67**
0.08 M	317.2	**17.59**	**28.64**
0.16 M	620.6	0.8	1.9
0.32 M	186.6	1.64	1.7
1 M	35.1	2.26	4.1

Cation exchange elution profiles: CM Ceramic Column

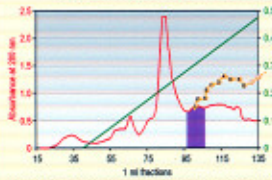

Fig. 3. FPLC chromatography elution profile of the step **0.04 M** NaCl DEAE on a CM column (1x3 cm) equilibrated in 0.01 M ammonium acetate buffer, pH=5.2. The green straight line indicate the salt gradient. The limited areas indicate the immunoreactive fractions.

The NH2-terminal microsequence analysis of one main spot from 0.04 M DEAE revealed the ***LPSSFDAREQ*** *sequence sharing high identity with human, rat and bovine cathepsin D precursor (90%) and with cathepsin B-like cysteine proteinase from Fasciola hepatica (77.78%). This sequence has never been sequenced in sheep placenta.*

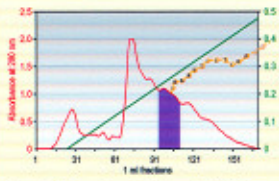

Fig. 4. FPLC chromatography elution profile of the step **0.08 M** NaCl DEAE on a CM column (1x3 cm) equilibrated in 0.01 M ammonium acetate buffer, pH=5.2. The green straight line indicates the salt gradients. The limited areas indicate the immunoreactive fractions.

The terminal microsequence analysis of one main spot from 0.08M DEAE revealed the ***ISSRVTILTIHP*** *sequence sharing high identity with caPAG$_{55}$, boPAG-17 and caPAG1 (Table 2).*

Table 2: sequence comparison between the ovPAG (**ISSRVTILTIHP**) and other pregnancy-associated glycoproteins

	I	S	S	R	V	T	I	L	T	I	H	P	Identity considering NH_2 terminal (12aa) (%)
caPAG$_{55}$	I	S	S	**Q**	V	**S**	X	L	T	I	H	P	75
boPAG-17	I	S	S	R	**Q**	**S**	N	L	T	I	H	P	75
caPAG-1	I	S	S	**Q**	V	**S**	N	L	T	I	H	P	75

Group II — Placenta from 66 to 100 days

Localization of the main immunoreactive fractions

Table 3: Total protein (TP) and ratio PAG:TP of fraction eluted after DEAE column

NaCl concentration	TP (mg)	Antisera: caPAG$_{55-59}$ Ratio PAG:TP	Antisera: ovPAG Ratio PAG:TP
0 M	430	0.9	0.9
0.02 M	140	2.1	4.2
0.04 M	840	**9.95**	**20.2**
0.08 M	510	**30.67**	**60.39**
0.16 M	1600	0.5	1.2
0.32 M	250	2.6	4.8
1 M	46	4.3	4.3

Cation exchange elution profiles: CM Ceramic Column

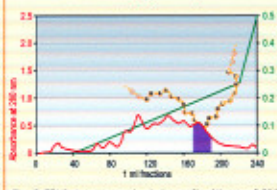

Fig. 5. FPLC chromatography elution profile of the step 0.08 M NaCl DEAE on a CM column (1x3 cm) equilibrated in 0.01 M ammonium acetate buffer, pH=5.2. The green straight line indicates the salt gradients. The limited areas indicate the immunoreactive fractions.

The terminal microsequence analysis of the most immunoreactive fraction from 0.04M DEAE revealed:
- *the* ***RGSALTQIPL*** *sequence sharing high identity with the caPAG$_{55}$ (70%)*
- *and* ***RGSNLIIHPL*** *equivalents to caPAG4 identified by molecular biology studies (Xie et al., 1997) and sharing high identity with many PAGs when considering the 10 aa of the NH_2-terminal sequence (Table 4)*

Table 4: sequence comparison between the ovPAG (**RGSNLIIHPL**) and other pregnancy-associated glycoproteins

PAGs*	R	G	S	N	L	I	I	H	P	L	Identity** (%)
ovPAG-4	R	G	S	N	L	I	I	H	P	L	**100**
boPAG-7	R	G	S	N	L	**T**	I	H	P	L	90
caPAG-7	R	G	S	N	L	**T**	I	H	P	L	90
boPAG-9	R	G	S	N	L	**T**	I	H	P	L	90
ovPAG-6	R	G	S	N	L	**T**	I	H	P	L	90
boPAG-4	R	G	S	N	L	**T**	I	H	P	L	90
caPAG-4	R	G	S	N	L	**T**	I	H	P	L	90
caPAG-11	R	G	S	N	L	**T**	I	H	P	L	90
boPAG-17	R	G	S	N	L	**T**	I	H	P	L	90
boPAG-5	R	G	S	N	**I**	**T**	I	H	P	L	80
ovPAG-7	R	G	S	N	L	**T**	**T**	H	P	L	80
boPAG-14	R	G	S	N	L	**T**	**T**	H	P	L	80
caPAG-5	R	G	S	N	L	**T**	**T**	H	P	L	80
boPAG-15	**H**	G	S	N	L	**T**	I	H	P	L	88.88
boPAG-19	R	G	S	N	L	**T**	**S**	H	P	L	80
ovPAG-1	R	**A**	S	N	L	**T**	I	H	P	L	80

* *PAG sequences were obtained from cDNA*
** *Identity considering 10 aa at the NH_2-terminal sequence*

The DNA sequence databases were screened and homologies computed at the NCBI (National Center for Biotechnology Information, Bethesda, USA)

Conclusions

In conclusion, this study identified one cysteine proteinase

LPSSFDAREQ (31.6 kDa, pI 5)

and three aspartic proteinases as Pregnancy-Associated Glycoproteins (ovPAGs):

- **ISSRVTILTIHP (57 kDa, pI 3.5 to 5.1)**
- **RGSALTQIPL (60.9 kDa, pI 4.8 to 5.3)**
- **RGSNLIIHPL (58 kDa, pI 5 to 5.3)**

Two of these ovPAGs have never been described earlier :

- **the ISSRVTILTIHP from the placenta removed at the last trimester of gestation**
- **the RGSALTQIPL from the placenta removed at 66 to 100 days of gestation**

Perspectives

The ISSRVTILTIHP and RGSALTQIPL molecules identified in this study, are to be purified till homogeneity in order to develop radio or enzyme immunoassay. These tools will allow the analysis of the temporal expression of the PAGs during gestation.

References

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