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Identification of Pregnancy-Associated Glycoprotein (PAG) on Jawarandu Goat Cotyledons

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ABSTRACT

Pregnancy-Associated Glycoprotein (PAG) is a specific glycoprotein which associated to pregnancy. It is secreted by embryo as a signal of pregnancy before implantation. The aim of the study was to identify the molecular weight of PAG in Jawarandu goat. Placentas were obtained after labor to made the cotyledon extract. The study conducted through four stages, namely the extraction, purification performed using acid precipitation (H_3PO_4 1 M, pH 4.7) and salt precipitation ($(NH_4)_2SO_4$ 40% dan 80%), filtration using Sephadex G-75®, and identification the molecular weight of PAG using SDS-PAGE. Protein bands appeared on SDS-PAGE in the K-8 column showed two protein bands with molecular weight namely 43.61 kDa and 28.21 kDa. These two molecule could be used as a marker of early pregnancy in Jawarandu goat.

Keywords: PAG, Cotyledons, Jawarandu Goat, SDS-PAGE

Identifikasi Pregnancy-Associated Glycoprotein (PAG) pada Kotiledon Kambing Jawarandu

ABSTRAK

Pregnancy-Associated Glycoprotein (PAG) merupakan molekul glikoprotein yang berkaitan dengan kebuntingan. Molekul PAG disekresikan sebagai salah satu sinyal kebuntingan dari embrio kepada induk sebelum proses implantasi. Penelitian ini bertujuan untuk mengidentifikasi bobot molekul PAG pada kambing Jawarandu. Identifikasi PAG diperoleh dari ekstrak kotiledon yang dikumpukan setelah induk melahirkan. Proses penelitian dilakukan dengan empat tahap, yaitu ekstraksi, purifikasi larutan menggunakan presipitasi asam (H_3PO_4 1 M, pH 4.7) dan garam ($(NH_4)_2SO_4$ 40% dan 80%), filtrasi larutan menggunakan Sephadex G-75®, dan identifikasi bobot molekul PAG menggunakan SDS-PAGE. Bobot molekul protein yang muncul pada SDS-PAGE pada kolom K-8 menunjukkan adanya dua pita protein dengan bobot molekul 43,61 kDa dan 28,21 kDa. Molekul tersebut diduga dapat digunakan sebagai penanda kebuntingan dini pada kambing Jawarandu.

Kata kunci: PAG, Kotiledon, Kambing Jawarandu, SDS-PAGE

INTRODUCTION

Nowadays, various techniques have been applied for detecting early pregnancy such as ultrasonography, progesterone assay, radiography (Medan *et al.*, 2004; Zamfirescu *et al.*, 2011) and DEEA GestDect (Samsudewa *et al.*, 2008) as early as 21 days or even less after insemination,. However, those methods remained unsatisfaction on farm (Setiatin *et al.*, 2009). Therefore, it is necessary to develope simple and accurate

methods of early pregnancy detection which is applicable on farm. Early pregnancy detection could be performed by the endocrine component of the maternal (Gnatek *et al.*, 1989) and physiological and biochemical interactions in uterus (Ko *et al.*, 1991). One of the biochemical compounds which signing early pregnancy formed in proteins called as Pregnancy-Associated Glycoprotein (PAG) (Lopez-Gatius *et al.*, 2007; Gajewski *et al.*, 2008; Zamfirescu *et al.*, 2011).

Pregnancy-Associated Glycoprotein is a molecule produced by tropoblastic cells (Perenyi *et al.*, 2002), part of the aspartic protein synthesized by the superficial epithelial layer (mono and binucleic) of placental cells (Karen *et al.*, 2003; Sousa *et al.* 2006). Karen *et al.* (2003) stated that glycoprotein is a good marker of successful conception. Glycoproteins which synthesized by the placenta secreted into the fetal circulation (Zoli *et al.*, 1992). Keren *et al*. (2003) explained that ovinePAG was detected at 3 weeks after insemination. This molecule keep secreted along gestation period (Setiatin *et al*., 2009), and remained until 4-5 days after parturition (El Amiri *et al*., 2007). This moleculs could be detected through maternal blood (El Amiri *et al*., 2004; Echternkamp *et al*., 2006; Sousa *et al*., 2006) and milk (Patel *et al*., 1997; Gajewski *et al*., 2008), but its existence have not been proven yet on urine.

Previous study have examined PAG as early pregnancy marker using RIA and ELISA. It has been applied as early pregnancy detection in dairy cattle at 21-28 days after artificial insemination (Xie *et al.*, 1996; Perenyi *et al.*, 2002; Piechotta *et al.*, 2010). Either Zoli *et al.* (1992) on bovinePAG (boPAG) which isolated from cotyledons could be detected accurately using RIA as early pregnancy detection with an accuracy of 94.65%. Similar results were shown by Piechotta *et al.* (2010) that used ELISA to detect PAG with a success percentage of 97.8%. In addition Perenyi *et al.* (2002) stated PAG either could be used to predict the health of fetus, placental abnormalities, embryonic mortality, and abortion.

Several types of PAG molecules could be isolated from the cotyledons using biochemical procedures (Sousa *et al.*, 2006). Some previous study have been carried out to identify the PAG of each breeds and species. Garbayo *et al.* (1998) showed the molecular weight of PAG in cattle and goat at 67 kDa and 55 kDa of 48-69 day of gestation. Karen *et al.* (2003) had isolated ovinePAG (ovPAG) with molecular weights ranging from 55-59 kDa at under 50 days of gestation. While Setiatin *et al.* (2009) obtained ovPAG in Garut sheep at 30.86 kDa of molecular weight at labor.

Some research showed that PAG have a various range of molecular weight among breeds and species. It is necessary to identify the molecular weight of PAG in Jawarandu goat to enlarge the data record. For that, the purpose of this study was to identify and determine the molecular weight of PAG in Jawarandu goats which could be used as starting effort to produce anti-PAG as early pregnancy detector.

MATERIALS AND METHODS

Materials

Jawarandu goat fetal cotyledons were collected after labor. As the previous study conducted by Setiatin *et al*. (2009) placentas were collected immediately after labor (non invasive). Cotyledons sample were obtained from 5 goats, yielding a total amount of cotyledon for 306,56 g. Material which used on this study were Phosphate Buffered Saline (PBS), H_3PO_4 1 M, KOH 1 M, $(NH_4)_2SO_4$, Tris-HCl 0,01 M, Sodium Sulfate Poly Acrylamide Dedocyl Electrophoresis (SDS-PAGE), acylamide, bis-acrylamide, TEMED, APS, aquabidest, Commassie Brilliant Blue, methanol, acetic acid, monogel, Board Range Standard® as molecular weight marker, Sephadex-G75®, and polyethylene glycol™ 6000 (Merck®). The tools which used were blender, stirrer, Refrigerated High Speed Centrifuge

(Hitachi®) and ultracentrifuge (Optima™ L-100, Beckman Coulter®), 50 ml centrifugation tube, electrophoresis kits, cool box, scaled pipet, and beaker glass, column filtration, and membrane dialysis tubing™ (Sigma-Aldrich®).

Methods

1. Extraction Placental cotyledons

Fetal cotyledons were separated from placental membrane then washed with 0.9% NaCl and stored at -20ºC (Barbato *et al.*, 2007). Extraction was performed using Setiatin *et al.* (2009) method. Cotyledons were mashed and homogenized for 10 minutes in Phospate Buffered Saline with a ratio of buffer to tissue of 3:1 (v/w), then stirred gently for 12 hours. Solution were then centrifuged using the High-Speed Refrigerated Centrifuge (5000×g, 4ºC, 30 min). Supernatant was separated from the precipitate, then dialyzed using membrane dialysis tubing (Sigma-Aldrich®) and polyethylene glycol 6000 (Merck™) until 1/3 of the initial volume.

2. Purification using Acid and Salts Precipitation

Acid precipitation was performed using 1 M H_3PO_4, adjusted to pH 4,5 and stirred gently for 2 hours then stored for 12 hours at 4ºC. The supernatant was then centrifuged at 27.000×g, 4ºC, for 30 minutes and separated from its precipitate. Its pH was readjusted using 1 M KOH up to 7,6. Sal $(NH_4)_2SO_4$ to 40% saturation. The solution was homogenized, and then stored for 12 hours to react. Supernatants were separated by ultracentrifuge (27.000×g, 4ºC, 30 minutes). Salt precipitation performed using $(NH_4)_2SO_4$ at 40% and 80% saturation. Dry ammonium sulphate was slowly added to the supernatant, then allowed to react for 12 hours at 4ºC, then separated the supernatant using ultracentrifuge (27.000×g, 4ºC, 30 minutes). Continued for salt precipitation at 80% saturation through the same way. The supernatant obtained from 80% salt precipitation was submitted for chromatography.

3. Filtration Chromatography of Extract Cotyledons and Measurement of Protein Concentration

The solution obtained from 40%-80% saturated ammonium sulphate was filtered on a Sephadex G-75® column which had been equilibrated using 0.05 M NH_4HCO_3 buffer. Fractions isolated from chromatography were collected in 3 ml sample tube until the solution was drained. The total protein concentrations were measured using spectrophotometer (λ=700 nm) to determine the highest protein. Absorbances of the fractions were collected in the graphic to determine the sample which had the highest protein concentration. Sample with the highest protein concentration was then used for identification of PAG molecular weight using SDS-PAGE.

4. Analysis of the PAG electrophoresis.

Running gel and stacking gel was made at a concentration of 14% and 4%. The composition of the gel separator was made with acylamide 40%, 2% bis-acrylamide, TEMED, APS 10%, dH_2O, Tris-HCl buffer (pH 8.8), and the stacking gel pH 6.8. Characterized using Standard Broad Range® molecular weight marker at 5-250 kDa. Electrophoresis performed at 100 volts for 90 minutes. Once completed, the gel was stained with Commassie Brilliant Blue. SDS-PAGE had 9 wells which used to identified 8 sample from each stage of extraction. Each well loaded with one sample solution from each stage for protein identification (Table 1).

Determination of molecular weight standardized using regression between molecular weight and the relative migration of markers (data are not shown). It resulted a regression formula as follow:

$Y = -1.35152X + 2.26128$; $R^2 = 0.935$

Y : molecular weight (kDa)
-1.35152 : coefficient of relative migration
X : relative migration of protein band
2.26128 : constanta
$R^2 = 0.935$: coefficient of determination

Table 1. Formation of Containing Sample Loaded to SDS-PAGE

Wells	Lane Code	Sample
1	K-8	Final extract
2	M	Marker
3	K-1	Rough cotyledon extract
4	K-2	Rough cotyledon extract
5	K-3	Rough cotyledon extract
6	K-4	Dialysis of rough cotyledon extract
7	K-5	Acid precipitation
8	K-6	Salt precipitation at 40% saturation
9	K-7	Salt precipitation at 80% saturation

Table 2. Protein Concentration of Each Extraction Stage

Extraction Stage	Code	Protein Concentration (ng/ml)
Rough Cotyledon Extract	K-1	0,425
Rough Cotyledon Extract	K-2	0,436
Rough Cotyledon Extract	K-3	0,451
Dialyzed Cotyledon Extract	K-4	1,306
H_3PO_4 Precipitation	K-5	0,876
40% $(NH_4)_2SO_4$ Precipitation	K-6	0,813
80% $(NH_4)_2SO_4$ Precipitation	K-7	0,747
Chromatographed Isolate (Precolumn SDS-PAGE)	K-8	0,085

RESULTS AND DISCUSSIONS

Precipitation of extract cotyledon solution using phosphate acid (pH 4.5) and ammonium sulfate at 40% and 80% saturation (pH 7.6) led to decreased protein concentration. Decreasing of protein concentration illustrated that large proteins had been bound during acid and salt precipitation (Table 2). Setiatin *et al.*, (2009) explained that precipitation was aimed to eliminate large protein, so it only remained small proteins in extracts of cotyledons. The final isolate from cotyledon extract contain 0,085 ng/ml of total protein concentration.

Cotyledon extract of Jawarandu goat showed some protein bands on SDS-PAGE. It was classified in to five different protein bands, mentioned as 100s kDa, 90s kDa, 80s kDa, 70s kDa 40s kDa, and 20s kDa (Figure 1). Those five groups of protein bands identified at lane K-1 to K-7. Acid and salt precipitation were succeed to decrease the protein concentration, but not eliminate protein content. Protein appeared in SDS-PAGE relatively had the low molecular weight, as described Gnatek *et al.* (1989) that conception produced a number of proteins with low molecular weight derived from the trophoblast. Explained further by Sumadiasa and Yuliani (2008) that the molecular weight of protein secreted by the goat embryo as early pregnancy signals varies, mentioned as 100, 95, 55, 43, 28, and 18 kDa.

The PAG molecules of Jawarandu goat were contained in fraction obtained from Sephadex G-75 column chromatography (lane K-8). It showed two protein bands with molecular weight namely 43.61 kDa and 28.21 kDa. The molecular weight of PAGs in Jawarandu goat were smaller than the other caprinePAG (caPAG) isolated by Garbayo *et al.* (1998) which had molecular weight 55 kDa. Other PAG which isolated from goat cotyledons have 3 molecular weight at 55, 59, and 62 kDa (Sousa *et al*., 2006). Likewise, it were smaller than sheep which had molecular weight ranged between 55-59 kDa or 58-61 kDa (Karen *et al.*, 2003;

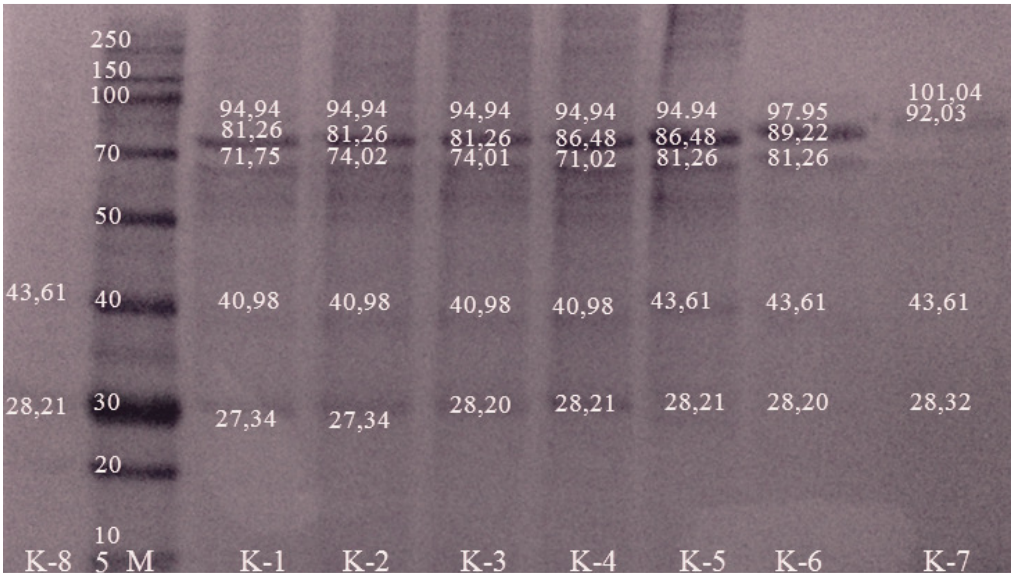

Figure 1. Identification the PAG of Jawarandu Goat using SDS-PAGE. (M = marker, K-1 – K-4 = rough cotyledon extract, , K-5 = acid precipitation, K-6 = salt precipitation (40%), K-7 = salt precipitation (80%), K-8 = Sephadex G-75® filtration)

El Amiri *et al.*, 2004) extacted at different gestation. Perenyi *et al.* (2002) conducted purification cotyledons at 50 days gestation age that produces a protein with a molecular weight of 67 kDa. While El Amiri *et al.* (2003) have isolated three types of PAG of fetal cotyledons at 60-100 days gestation and characterized by its molecular weight as ovPAG-55, ovPAG-5, and ovPAG-59.

One of the protein bands appeared have a molecular weight of 28.21 kDa. It showed nearly similar with Setiatin *et al.* (2009) who had found ovPAG in Garut sheep as 30.86 kDa. Other protein which appeared have molecular weight as 43,61 kDa. It was reported by Sumadiasa and Yuliani (2008) that protein with 43 kDa of molecular weight ever been found in goat embryo through in vitro fertilization. Those protein also nearly similar with Green *et al.* (1999) that found equinePAG (eqPAG) with molecular weight about 41 kDa in *Artiodactyle* (horse and zebra). PAG which had similar molecular weight composed by the same identity of amino acid sequences ranging from 60-90% among species (El Amiri *et al*., 2004), and 80% among breeds (Sousa *et al*., 2006). Both of those protein bands (28,21 and 43,61 kDa) have an possibility as early pregnancy marker in Jawarandu goat. Some placental protein found specifically and could be used to detect early pregnancy (Gnatek *et al.*, 1989; Garbayo *et al.*, 1998; Perenyi *et al.*, 2002).

This study have identified that Jawarandu goat have two PAG molecules, namely 20s kDa and 40s kDa. Those two molecules are having possibility to be used as biomarker of pregnancy detection kit. Further studies are needed to figure out the existence of PAG molecules on maternal blood, milk, and urine.

CONCLUSIONS

SDS-PAGE identified that caprinePAG (caPAG) of Jawarandu goat have two molecular weight, namely of 43.61 kDa and 28.21 kDa. These two molecules could be expected as the marker of early pregnancy in Jawarandu goats.

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