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

K. A. Permana, E. T. Setiatin dan D. Samsudewa

Laboratorium Genetika, Pemuliaan, dan Reproduksi Ternak, Fakultas Peternakan dan Pertanian, Universitas Diponegoro
Kampus Tembalang, Semarang
E-mail: permana.ka@gmail.com

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 (H₃PO₄ 1 M, pH 4.7) and salt precipitation ((NH₄)₂SO₄ 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

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 signaling 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 molecules 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), \( \text{H}_3\text{PO}_4 \) 1 M, KOH 1 M, \( \text{(NH}_4\text{)}_2\text{SO}_4 \), 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
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 H3PO4, 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 (NH4)2SO4 to 40% saturation. The solution was homogenized, and then stored for 12 hours to react. Supernatants were separated by ultracentrifuged (27,000×g, 4ºC, 30 minutes). Salt precipitation performed using (NH4)2SO4 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 NH4 HCO3 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 acrylamide 40%, 2% bis-acrylamide, TEMED, APS 10%, dH2O, 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
Identification of Pregnancy-Associated Glycoprotein (PAG)… (Permna et al.)

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

<table>
<thead>
<tr>
<th>Wells</th>
<th>Lane Code</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K-8</td>
<td>Final extract</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>Marker</td>
</tr>
<tr>
<td>3</td>
<td>K-1</td>
<td>Rough cotyledon extract</td>
</tr>
<tr>
<td>4</td>
<td>K-2</td>
<td>Rough cotyledon extract</td>
</tr>
<tr>
<td>5</td>
<td>K-3</td>
<td>Rough cotyledon extract</td>
</tr>
<tr>
<td>6</td>
<td>K-4</td>
<td>Dialysis of rough cotyledon extract</td>
</tr>
<tr>
<td>7</td>
<td>K-5</td>
<td>Acid precipitation</td>
</tr>
<tr>
<td>8</td>
<td>K-6</td>
<td>Salt precipitation at 40% saturation</td>
</tr>
<tr>
<td>9</td>
<td>K-7</td>
<td>Salt precipitation at 80% saturation</td>
</tr>
</tbody>
</table>

### Table 2. Protein Concentration of Each Extraction Stage

<table>
<thead>
<tr>
<th>Extraction Stage</th>
<th>Code</th>
<th>Protein Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rough Cotyledon Extract</td>
<td>K-1</td>
<td>0.425</td>
</tr>
<tr>
<td>Rough Cotyledon Extract</td>
<td>K-2</td>
<td>0.436</td>
</tr>
<tr>
<td>Rough Cotyledon Extract</td>
<td>K-3</td>
<td>0.451</td>
</tr>
<tr>
<td>Dialyzed Cotyledon Extract</td>
<td>K-4</td>
<td>1.306</td>
</tr>
<tr>
<td>H$_3$PO$_4$ Precipitation</td>
<td>K-5</td>
<td>0.876</td>
</tr>
<tr>
<td>40% (NH$_4$)$_2$SO$_4$ Precipitation</td>
<td>K-6</td>
<td>0.813</td>
</tr>
<tr>
<td>80% (NH$_4$)$_2$SO$_4$ Precipitation</td>
<td>K-7</td>
<td>0.747</td>
</tr>
<tr>
<td>Chromatographed Isolate (Precolumn SDS-PAGE)</td>
<td>K-8</td>
<td>0.085</td>
</tr>
</tbody>
</table>

### 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). Setiati 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;...
El Amiri et al., 2004) extracted 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.

**REFERENCES**

Echternkamp, S.E., K.A. Vonnahme, J.A. Green, and S.P. Ford. 2006. Increased vascular


