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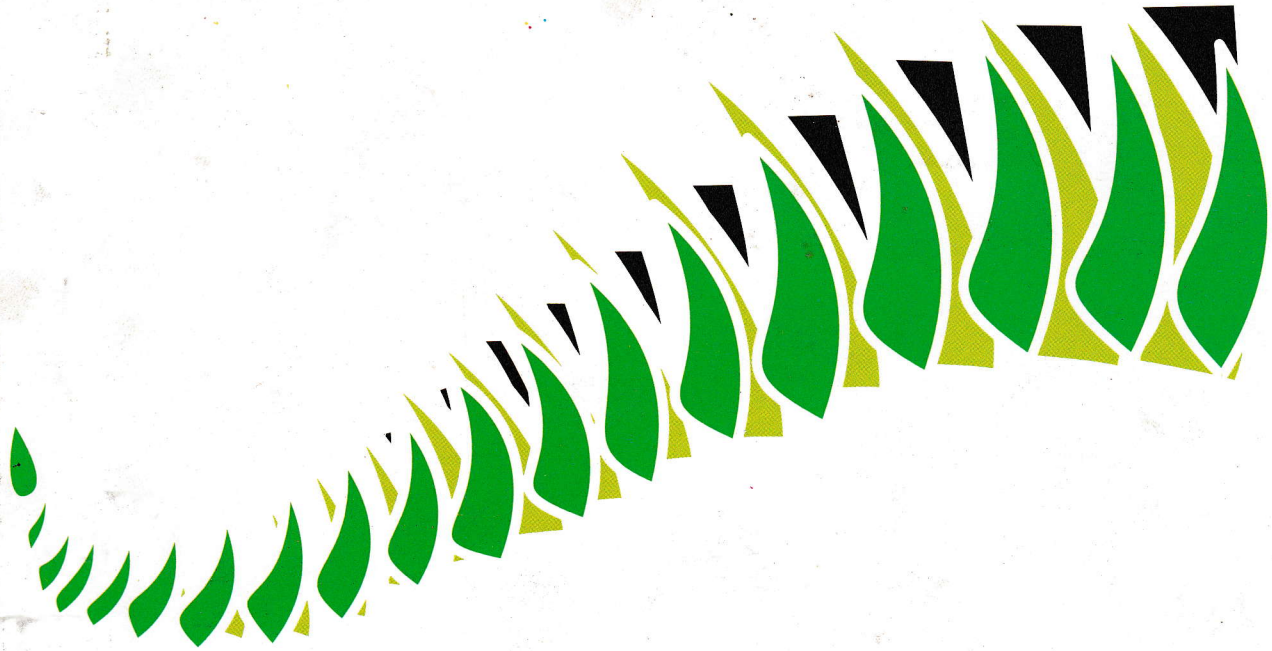

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ANTIBODY POLYCLONAL PRODUCTION ON RABBIT ANTI-OVINE PREGNANCY-ASSOCIATED GLYCOPROTEIN (Rabbit anti-ovPAG)

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ABSTRACT

The aim of the study was to produce polyclonal antibody (rabbit anti-ovPAG) which could detect PAG in the urine of pregnant ewes. Twelve rabbits were immunized against ovPG DEAE-TrisHCl (DT), DEAE-NaCl 20mM (DN2), DEAE-NaCl 40mM (DN4), DEAE-NaCl 80mM (DN8), DEAE-NaCl 160mM (DN16), DEAE-NaCl 320mM (DN32) and DEAE-NaCl 1M (DN1) and NaCl 0.9 % as a placebo. The 0.5 ml of isolate (purified from ovine cotyledon) was emulsified in equal volume with complete and incomplete Freud's adjuvant. The mixture of each isolate and adjuvant was injected at mutiple sites along the dorsal area of rabbits by subcutaneous route. Blood were collected from marginal ear vein, starting before first injection (baseline) and every 14 days. Rabbit anti-ovPAG were measured using Modified ELISA Technique. By using Western Blot Technique, DN32 showed the best immune response among others and also could differenciate ovPAG in the urine of pregnant ewes It could be concluded that ovPAG DN32 is a specific source of rabbit anti-ovPAG production. Protein of ovPAG at molecular weight 31 kDa is a pregnancy protein marker of garut sheep and could be developed as a major protein for producing antibodi.

Keywords : cotyledon, ELISA, ovPAG, polyclonal antibody, rabbit anti-ovPAG

INTRODUCTION

Pregnancy associated glycoprotein (PAG) is a member of aspartic proteinase family secreted from trophoblastic nucleic acid (Wooding, 1992; Perényi *et al.*, 2002; El Amiri *et al.*, 2003; Hughes *et al.*, 2003) that are expressed in the outer epithelial cell layer (chorion or trophectoderm) of the ungulate placenta (Green *et al.*, 2000; El Amiri *et al.*, 2004). Pregnancy-associated glycoprotein could be detected in blood using radioimmuno assay (RIA) at the attachment of placenta during development of fetomaternal sincysium (Ranilla *et al.*, 1994; Verberckmoes *et al.*, 2004). Of this, PAG could be used as pregnancy indicator and also feto-placental well-being (Karen *et al.*, 2003; Boscos *et al.*, 2003).

Molecular mass of protein purified from garut sheep cotyledon is 30.8 kDa smaller than El Amiri *et al.* (2004) did on sheep foetal cotyledons at 55-65 kDa. Some ovPAG were gained during purification namely DEAE-TrisHCl (DT); DEAE-NaCl 20mM (DN2) ; DEAE-NaCl 40mM (DN4); DEAE-NaCl 80mM (DN8); DEAE-NaCl 160mM (DN16); DEAE-NaCl 320mM (DN32) and DEAE-NaCl 1M (DN1). From these, two of them were chosen as ovPAG sources on producing polyclonal antibody (anti-ovPAG), namely DN16 and DN32 (Setiatin *et al.*, 2009).

Polyclonal antibody was produced by

injecting purified ovPAG mentioned above, through rabbit subcutaneously (Ayad *et al.,* 2007). Polyclonal antibody could detect the biological material bounding on PAG (Green *et al.,* 2005). Therefore, rabbit anti-ovPAG could be used as the agent for detecting PAG in the urine. Rabbit anti-ovPAG determined using monogel SDS-PAGE method followed with Western Blot. This method was applied for detecting the polyclonal antibody in blood (Barbato *et al.,* 2007; Bella *et al.,* 2009) whereas its concentration was measured using modified ELISA technique.

The aim of the study was to produce polyclonal antibody (rabbit anti-ovPAG) which could detect PAG in the urine of pregnant ewes

MATERIALS AND METHODS

Polyclonal Antibody Production

There were 12 NZW rabbit divided into 6 groups based on ovPAG sources (Table 1) gained through ovPAG purification of cotyledon extract through Sephadex-G75 and DEAE-cellulose column, namely S, DT, DN8, DN16 and DN32 (Setiatin *et al.* 2009). Before immunization, all isolates were prior to Freud's complete adjuvant (Sigma®) and Freud's incomplete adjuvant (Sigma®) to bound ovPAG (Goldsby *et al.,* 2000; Erb and Hau, 1994; Hendriksen and Hau, 2003).

There were four types of blood, namely baseline collected before first immunization (B), FCA, FICA1 and FICA2. Ovine Pregnancy-Associated Glycoprotein (ovPAG) was immunized subcutaneously through back of NZW rabbit. At first immunization, each rabbit in every group had the mixture of 0.5 ml of isolate and 0.5 ml of Freud's complete adjuvant called FCA (Sigma®). At the 14-d, the first booster contained 0.5 ml isolate and 0.5 ml of Freud's incomplete adjuvant called FICA1 (Sigma®). Two weeks after that, rabbit had second booster, immunized with the same composition called FICA2. Clearly, every two weeks, bloods were taken by disposable syrink into anticoagulant glass. Blood was collected through *A. auricularis* and *V. auricularis* with maximum volume was 20% of body weight. (Ayad *et al.,* 2007).

There were four types of blood namely Baseline collected before first immunization (B), FCA, FICA1 and FICA2. Samples were centrifuged at 2,500 rpm for 15 minutes, then the blood plasma was collected and stored at – 20°C After this, rabbit anti-ovPAG was measured using modified ELISA.

Measurement of Rabbit anti-ovPAG Concentration using Modified ELISA Technique

Isolate ovPAG namely S, DT, DN8, DN16 and DN32 were added with carbonate-bicarbonate buffer (coating-buffer) at 1:10. It was added with 100 µl at every well, then was incubated at 4°C overnight. The plate was washed by PBS Tween 0.1 % four times. Blotto 5% (5 g skim milk added with PBS 0.1%) was filled into every well at 300 µl, and was incubated at 37°C for 60 minutes (Silva *et al.,* 2007). Then, plate was washed with PBS *Tween* 0.1 % four times (Crowther, 2001; Green *et al.,* 2005; Silva *et al.,* 2007).

Simultaneously, rabbit anti-ovPAG was diluted in blotto 5% (1 : 50), it was added with 100 µl blotto 5% into every well, then was incubated at room temperature for 90 minutes. After washing the plate, every well was added with 100 µl the mixture Goat anti-rabbit IgG peroxidase (Sigma®) in blotto 5% (1:2000), and it was incubated at 37°C for 60 minutes. ELISA plate was washed with PBS Tween 0.1% four times.

The next step, every well was filled with 100 µl TMB or 3.3', 5.5' tetra methyl benzidine dihydrochloride (Sigma®), incubated at room

Table 1. Ovine Pregnancy Associated Glycoprotein and Its Concentration to New Zealand White Rabbit

ovPAG Sources	Rabbit (n)	Concentration (ng/µl)
Sephadex-G75 (S1 & S2)	2	181.00
DEAE-Tris HCl 0.01 M (DT.1 & DT.2)	2	171.00
DEAE-NaCl 80 mM (DN8.1 & DN8.2)	2	2.67
DEAE-NaCl 160 mM (DN16.1 & DN16.2)	2	44.33
DEAE-NaCl 320 mM (DN32.1 & DN32.2)	2	86.00
Control (C1 & C2)	2	0.00

temperature for 20 minutes until the color became blue. The reaction was stopped using 50 µl 2N H_2SO_4 at every well. The changing color from blue to yellow indicated that the reaction finished. Optical density of yellow color was measured using Microplate Reader Model 3550 (Biorad®) at wavelength 450 nm (Crowther, 2001; Cho *et al.*, 2003).

Rabbit anti-ovPAG Determination using Western Blotting Technique

Western Blotting Technique was applied to detect the specified reaction of rabbit anti-ovPAG to ovPAG in pregnant and nonpregnant urine of garut sheep. Urine was collected early in the morning and divided into alliquot, the ovPAG protein was separated by monogel SDS-PAGE vertical to molecular marker (Merck®) and subjected into Western Blotting. Protein migrated through running buffer and attached to the protein marker which had a proper molecular mass. Monogel SDS-PAGE was transfered into nitrocellulose membrane, washed with blotto 5% for 60 minutes. Blotting was formed by rabbit anti-ovPAG DN32 (1:50), incubated overnight. After washing with PBS Tween 0.1%, Western analysis by alkaline phosphatase (1: 5000) was incubated at 37°C for 60 minutes. After three times washing, nitrotetrazolium blue chlorine-98% (NBT) and 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BICP) were added as standard substrates, then was incubated for 20 minutes, and washed again with dH_2O (Goldsby *et al.*, 2000; Barbato *et al.*, 2007; Bella *et al.*, 2009; Majewska *et al.*, 2005; Huebner, 2004).

Data Analysis

Response immune and the specifity of rabbit anti-ovPAG were analyzed descriptively. Response immune was analysed by measuring the changing of optical density ovPAG to rabbit anti-ovPAG (B; FCA; FICA1, dan FICA2) gained during immunization. Based on response immune and total protein concentration, rabbit anti-ovPAG was chosen as an measuring ovPAG concentration in urine. Meanwhile, determination of rabbit anti-ovPAG to ovPAG in C; DN32; DN16; DN8; DT, and S collection during purification (Setiatin *et al.*, 2009) also in the pregnant and nonpregnant urine were analysed based on the relative migration of protein ovPAG.

RESULTS AND DISCUSSION

Immunogen has specific criteria known as foreign agent, has large molecular mass, has a complex chemical structure, dose injection, route and time, antibody could attach to its epitop, and capable to produce specific immune response. Rabbits had been chosen to produce rabbit anti-ovPAG because of different species, easy to be handled, had never contact with ovPAG produced from cotyledone garut sheep. Ovine Pregnancy-Associated of garut sheep has these criteria that is produced from different species, molecular weight was at 30.7–78 kDa, and never been injected to rabbit as antibody producer (Setiatin *et al.*, 2009).

B and or T lymphocyte could be detected if rabbit be known the epitop. Epitop is active binding site antigen (ovPAG) which could attach to B and T receptors. Immune response produced related to injecting dose and route during immunization. When mixed antibody could determine more than one epitop, called polyclonal antibody (Goldsby *et al.*, 2000; Abbas *et al.*, 2007).

The strength interaction between antigen and antibody depend on their affinity. The higher affinity the stronger ability of antibody to bound of antigen and this bound would stay longer. Cross-reactivity appeared when two antigens have identical epitop or antibody had specific binding site for one epitop also bound to other epitop which had similar chemical structure (Huebner, 2004).

Measuring *Rabbit anti-ovPAG* Concentration

There were variation of immune response produced by rabbit (n=12) during immunization, therefore antibodies produced during research must be selected of their capability before using as polyclonal antibody sources. There were 11 rabbits had good response to ovPAG based on optical density measurement using modified ELISA. Baseline titer was stated as based titer before first ovPAG injection. To elevate immunogenicity of ovPAG, isolate was added with adjuvant. Complete adjuvant containing *Mycobacterium sp* in Freud's Complete Adjuvant (Sigma ®) obtained once at first immunization. Moreover, at second and third immunization, rabbits were injected with dilution of ovPAG and incomplete adjuvant. These could stimulate B and T cell to to produce immune response, called rabbit anti-ovPAG (Cruse and Lewis, 2002).

First immunization would trigger B-cell for proliferation and differentiation of antibody secretion cell and memory cell on producing primary immune response. Some of antibody cells would migrate and stay in bone narrow at long time. Second immunization or Booster I and third immunization or Booster II would produce secondary immune response, their concentration were higher than first immunization. While memory cell of B cell was activated to produce large number of antibody because of T cell stimulation (Goldsby *et al.*, 2000; Abbas *et al.*, 2007).

There was important result that control group produced low optical density because they were immunized only with NaCl 0.9% while other groups were immunized with ovPAG produced immune response. These indicated that rabbit could react to ovPAG as foreign agent or antigen.

Figure 1 presents the comparison of antibody produced by DN16 and DN32. Although ovPAG DN32 and DN16 had similar protein weight at 30.86; 33.64, and 71.87 kDa, the protein band of DN32 had a the better criteria related to its intensity. Also, the concentration of total protein DN32 measured with Protein Assay was higher than DN16, that was 86.00 ng/µl and 44.33 ng/µl, respectively. Of these, ovPAG DN32 was chosen as antigen source because it might produce a better immune response (rabbit anti-ovPAG DN32).

Determination of rabbit anti ovPAG

Western Blotting was applied to determine the ability rabbit anti ovPAG DN32 to bound ovPAG in cotyledon. Conjugated enzyme anti-Rabbit Alkaline Phosphatase has specific activity to lysis phosphate bounded in molecules. The number of phosphate bound could be read by added BICP and NBT substrates through nitrocellulose membrane. Protein band appeared in the membrane indicated there was specific reaction between rabbit anti ovPAG to ovPAG in the sample (Huebner, 2004).

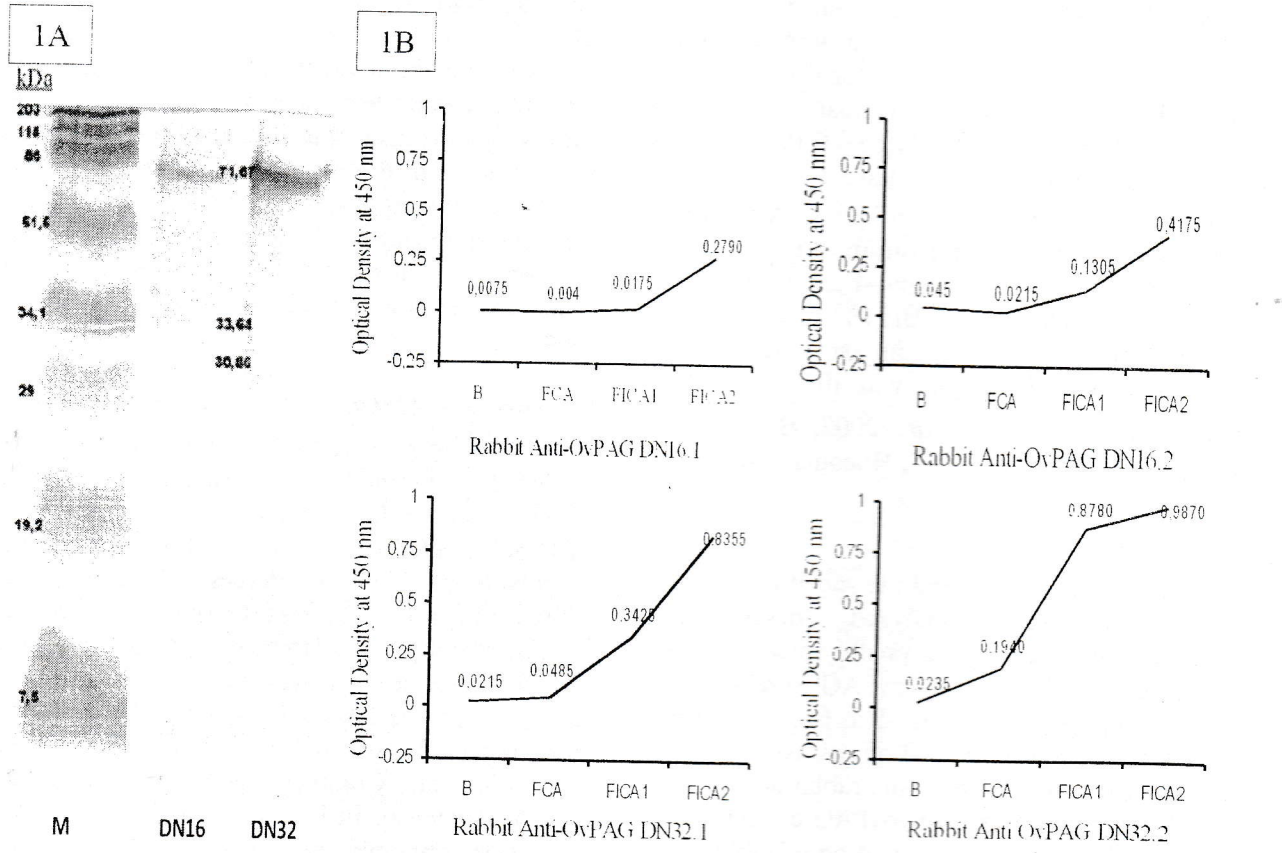

Figure 1. Comparison between DN16 and DN32 as Rabbit Anti-ovPAG. 1A= molecular weight; 1B = immune response. B: baseline antibody collected from blood plasma before first immunization. FCA: antibody at the first immunization , FCA1: antibody of the first booster; FCA2: antibody of the second booster.

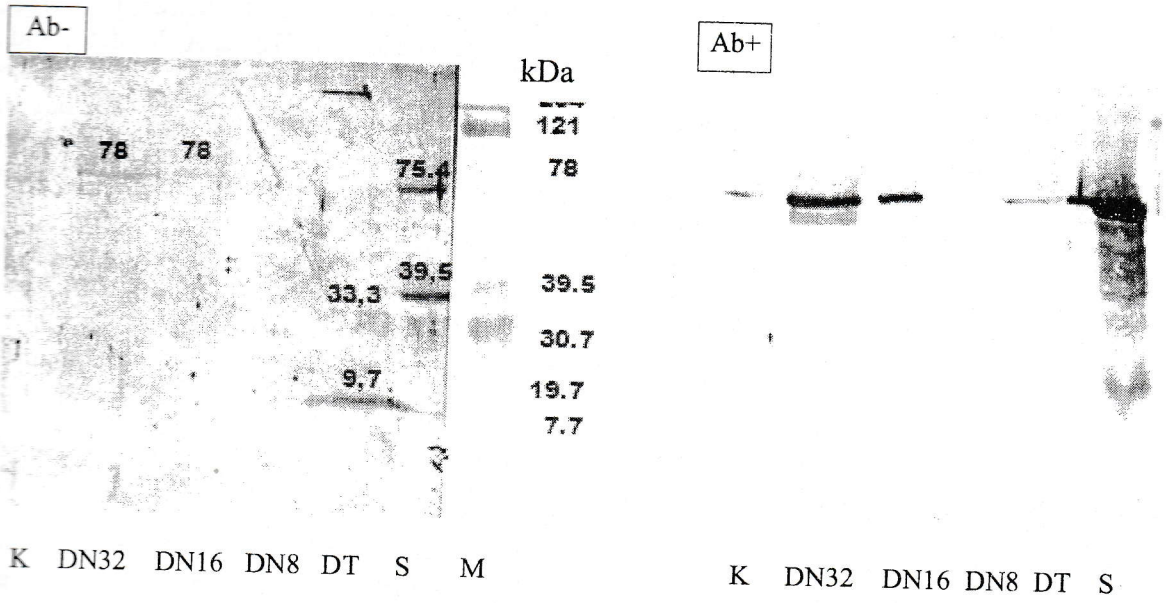

Figure 2. Determination of negatif (Ab -) and positif (Ab +) rabbit anti-ovPAG DN32 to ovPAG source (K; DN32; DN16; DN8; DT; S). Rabbit's blood has spesific protein at 78 kDa.

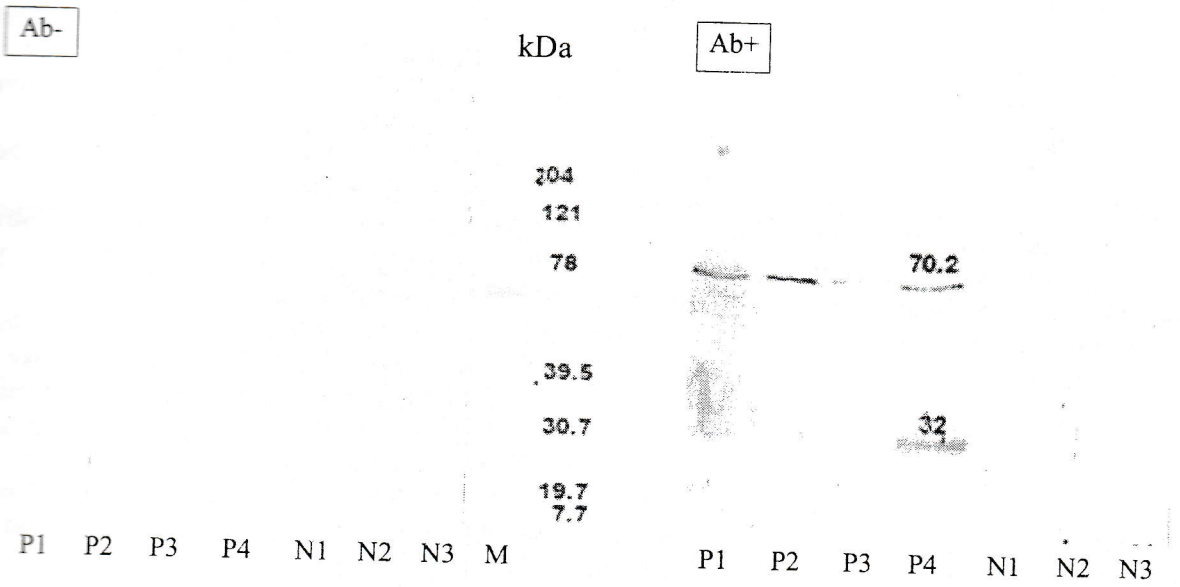

Figure 3. Determination of negative and positive rabbit anti-ovPAG DN32 to ovPAG in the pregnant and non pregnant urine of garut sheep (P = pregnant, N = non pregnant. Ab - , No band appears or no response; Ab +, Bands appear)

Negative rabbit anti-ovPAG was baseline antibody (B) collected from blood plasma before first immunization (Figure 1). There was no protein band and immune response in K group. This indicated that there was no immune response to NaCl 0.9%. Isolate DN32 and DN16 had one band at 71 kDa whereas DN8 did not have band although had high immune response. Moreover ovPAG DT had two bands at 31 and 14 kDa also ovPAG S had three bands at 71, 34, and 14 kDa.

Positive Rabbit anti-ovPAG DN32 had been responded to all source of ovPAG at 71 kDa with different intensity. This might occur because there were two antigens that had the same unidentical epitop, therefore one epitop could bound stronger than another (Huebner, 2004). Isolate ovPAG DN32 and DN16 had similar protein bands at 71 and 62 kDa, moreover ovPAG DN32 also had other band at 14 kDa. Isolate ovPAG S had five protein bands at 71, 62, 53, 34, and 14 kDa

(Figure 2). Based on these results, ovPAG DT, S, and DN8 could not be used as rabbit anti ovPAG sources because they had cross reaction. Protein at molecular weight 71 kDa became general protein belong to rabbit because this protein could react to negative and positive rabbit anti ovPAG DN32.

Continuing determination of rabbit anti ovPAG DN32 was applied to ovPAG in pregnant and non pregnant urine of garut sheep. There was no reaction between negative rabbit anti ovPAG DN32 to ovPAG in the urine of pregnant and non pregnant ewes. Moreover, there were obvious band as the result of anti ovPAG to ovPAG in the urine. Both pregnant and nonpregnant group had similar protein band at 71 kDa. This indicated that this protein has belonged to the rabbit. An important result shown that ovPAG pregnant urine only bounded to rabbit anti ovPAG at 31 kDa and in nonpregnant urine there was no reaction at all (Figure 3). Of this, protein with molecular weight at 31 kDa could be used as pregnancy marker for garut sheep. A similar result reported by Setiatin *et al.* (2009) that protein resulted from purification and isolation of PAG from garut sheep cotyledon is 30.86 kDa.

Specific reaction between rabbit anti ovPAG DN32 to ovPAG in pregnant and nonpregnant urine could be used to differentiate pregnancy status of garut sheep. While, polyclonal antibody could be produced successfully from isolate ovPAG DN32, this study should be continued to produce more applicative pregnancy detection tools using modified ELISA Ttechnique.

CONCLUSION

Ovine Pregnancy-associated glycoprotein (ovPAG) DN32 is a specific source of rabbit anti-ovPAG production. Protein of ovPAG at molecular weight 31 kDa is a pregnancy protein marker of garut sheep and could be developed as a major protein for producing antibodi.

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