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Original articles

ISOLATION AND PURIFICATION OF PREGNANCY-ASSOCIATED GLYCOPROTEINS

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ABSTRACT

The aim of these studies was to develop a protocol for the isolation and purification of pregnancy-associated glycoproteins (PAG) from cotyledons from placenta of pregnant cows. As a result of this work, processes and parameters of the PAG isolation were determined, including: extraction in a buffer system, two-stage precipitation with ammonium sulfate (40% and 80% saturation) and purification of the target product using ion-exchange chromatography using Q Sepharose Fast Flow. The resulting product having a molecular weight of 55 kD was identified by MALDI-TOF mass-spectrometry analysis as «Pregnancy-associated glycoprotein 1». As a result of the immunoblotting, the presence of proteins with a molecular weight of 55 kDa in the obtained preparation, reacting with previously obtained monoclonal antibodies (MABs) specific to PAG1 was confirmed. To determine antigenic properties of the purified PAG, laboratory animals were immunized. It was found that immunization with the purified PAG efficiently elicits specific antibodies. These antibodies, after the proper purification procedure, can be used as components of immunological test systems for determining pregnancy in cows.

Key words: pregnancy diagnostics, pregnancy-associated glycoproteins, placenta, cotyledons.

INTRODUCTION

Currently, there exists a wide range of techniques used to detect pregnancy in cows, such as ultrasound, rectal examination, analysis of progesterone levels, and radiography [1, 2]. However, not all of the tests detect pregnancy sufficiently early. Ongoing intensification of animal husbandry requests developing new, simple, and accurate methods for the purpose of early pregnancy detection. Serological methods based on identifying the presence of specific antigens in cow's sera, such as pregnancy-associated glycoproteins (PAGs), are increasingly being used as a method of choice for diagnosing the pregnancy. Pregnancy-associated glycoproteins are a large family of catalytically-inactive aspartic proteases secreted exclusively by mono-and binuclear cells of the trophectoderm [3]. There have been published a large number of reports on the use of PAG as a biomarker for the early diagnosis of pregnancy in cattle [4, 5, 6], sheep [7] and goats [8, 9]. Molecular genetic studies have revealed that the ruminant genome contains more than 100 PAG-coding genes, most of which are expressed in the superficial layers of placenta. It was also possible to establish the possibility of selective expression of PAG genes at different stages of pregnancy in ruminants. At least 22 PAG species have been identified in cattle, which cluster into three groups according to their amino acid composition and phylogenetic systematics [10, 11]. The most convenient and standardized method for determining PAG is enzyme immunoassay (ELISA), using specific antibodies as the main detecting reagents [12, 13, 14].

To produce antibodies intended for the use as reagents in a test system, it is important to have a sufficient amount of purified antigen. With this regard, the purpose of this work was to determine the feasibility of isolating PAG and purifying this antigen from biological material.

MATERIALS AND METHODS

The studies were carried out at the Laboratory of Applied Genetics («National Center for Biotechnology») of the Ministry of Health of the Republic of Kazakhstan. Cow placenta was collected immediately after calving and delivered to the laboratory on ice. To obtain PAG preparations, a method described by T. E. Egen et al. [15] was used, with minor modifications. Briefly, cotyledones (approximately 50 g) were cut into pieces 3-5 mm in size, placed in in 450 ml of a buffer (20 mM Tris, pH 8.0; 50 mM NaCl; 2 mM EDTA; 0.2 mM PMSF; 0.02% (w/v) NaN3) and incubated on a shaker at 150 rpm at room temperature for 12 hours. Insoluble material was removed by centrifugation at 5000g for 30 min at 4C. The supernatant (12 ml) was taken and dialyzed (50,000 MWCO) against 100 volumes of a buffer (20 mM Tris, pH 7.0; 150 mM NaCl; 1 mM EDTA; 0.02% (w/v) NaN3; 20 µM PMSF; 1 mM 2-mercaptoethanol).

To remove ballast proteins, dry ammonium sulfate ($(NH_4)_2SO_4$) was added to the dialyzed extract with constant stirring to 40% saturation and incubated at 4C overnight. The solution was centrifuged at 5000 g for 30 min. The supernatant was collected for further steps. To precipitate the target antigen, dry ammonium sulfate was added to the supernatant to 80% saturation and incubated at 4C overnight. Upon centrifugation at 5000 g for 30 min, the pellet was resuspended in 8 ml Tris-HCl buffer (10 mM, pH 7.6) and dialyzed against 100 volumes of Tris-HCl buffer (10 mM, pH 7.6). To inhibit proteases, PMSF was added to a concentration of 0.2 mM and the protein solution was stored in aliquots at -20C.

The SDS-PAGE electrophoresis was carried out in 10% polyacrylamide gel (PAAG) using the Mini-Protean equipment kit (Bio-Rad) according to the method of U. Laemmli [16].

Ion exchange chromatography was performed using Q Sepharose® Fast Flow (Sigma Aldrich, Q1126). The resin was pre-equilibrated with 10 mM Tris-HCl buffer, pH 7.6. The target glycoproteins were eluted in a linear salt gradient (0.1-0.8 M NaCl) after unbound proteins were separated.

To determine immunogenic proteins, Western blotting was performed according to the method of Towbin P. Etal [17]. Transfer was performed on Amersham™ Protran® Western blotting nitrocellulose membranes, 0.45 µm (Cytiva, GE10600002) using a Mini Trans-Blot transfer chamber (Bio-Rad). Protein-Free Blocking Buffer (G-Biosciences, 786-664)

was used to block free membrane sites. Primary antibodies used in this study were monoclonal antibodies (mAbs) specific for PAG1. As a secondary antibody, HRP conjugate of Protein G (Merck Millipore, 18-161) was used. 1-StepTM TMB-Blotting Substrate Solution (Thermo Fisher Scientific, 34018) was used as the substrate.

For MALDI-TOF mass spectrometry analysis, pieces of PAAG gels or blotting membranes were excised and transferred to vials and processed to LC-MS/MS. Samples were analyzed using reverse phase C18 liquid chromatography and tandem mass spectrometry (LC-MS/MS). Peptides were separated using a 75-minute multi-step ACN gradient. The resulting MS/MS peaks were analyzed using the Mascot 2.7 program using the SwissProt database and a local database.

The protein concentration was determined by the method of M. Bradford [18].

Balb/c mice were immunized intraperitoneally with 20 µg of antigen in 0.1 ml of complete Freund's adjuvant. On the 7th and 14th days, 20 µg of the antigen was injected intraperitoneally in 0.1 ml of incomplete Freund's adjuvant. On the 21st day of immunization, the animals were injected with 30 µg of antigen in sterile saline. The testing for the level of specific antibodies was carried out using an indirect ELISA on the 4th day after the last immunization.

RESULTS

The extraction and two-step precipitation with ammonium sulfate from cotyledon samples yielded a product with a total protein concentration of 8.1 mg/mL. For primary analysis, SDS-PAGE was performed in 10% PAAG (Figure 1).

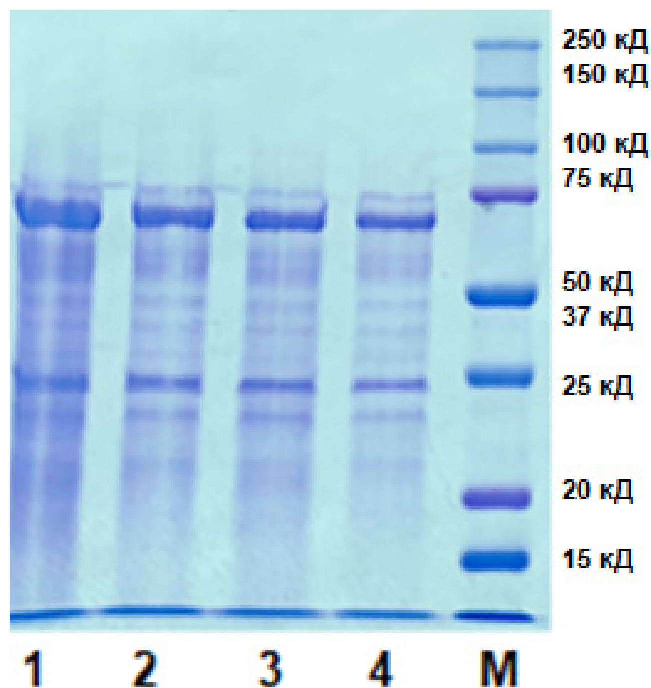

Figure 1 – SDS-PAGE of the proteins extracted from cotyledons: 1-2 - samples diluted 1/10; 3-4 - samples diluted 1/25; M - Precision Plus Protein™ Dual Color Standards Molecular Weight Marker (Bio-Rad, 1610394)

For further purification of the target antigen PAG, ion exchange chromatography was performed using Q Sepharose® Fast Flow (Sigma Aldrich, Q1126). The target proteins were eluted in a linear salt gradient after unbound proteins were removed. The protein concentration in the fractions was 0.8-0.9 mg/mL. The obtained fractions were analyzed in SDS-PAGE (Figure 2).

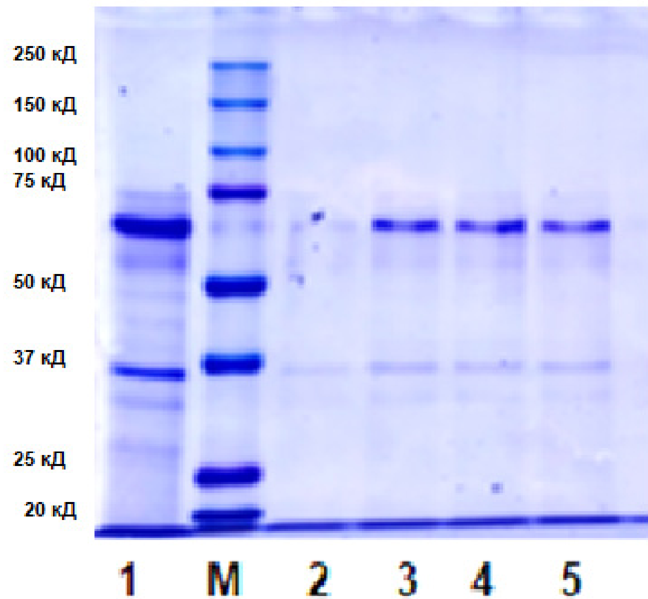

Figure 2 – SDS-PAGE of fractions collected during chromatography: 1 - sample in a dilution of 1/10 (preparation before RSI); 2-fraction 0.3M NaCl; 3-fraction 0.6M NaCl; 4-fraction 0.8M NaCl; 5-fraction 1M NaCl; M - Precision Plus Protein™ Dual Color Standards Molecular Weight Marker (Bio-Rad, 1610394)

Gel slices containing visible protein bands in fractions from a sample eluted with 0.6 M NaCl were excised from PAAG and used for MALDI-TOF analysis. As a result, «PAG1_BOVIN, Pregnancy-associated glycoprotein 1 OS=Bos taurus OX=9913 PE=1 SV=1» was identified in a sample with a molecular weight of 55 kDa, with a high confidence score – 1002. For immunological identification, immunoblotting (Western blotting) was carried out using mAbs specific for PAG1 obtained by the authors earlier [19] as primary antibodies. The culture liquid of mouse myeloma cells SP-2/0-Ag14 was taken as a negative control (Figure 3).

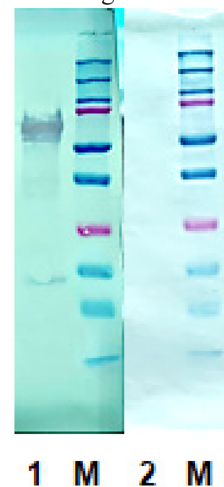

Figure 3 – Results of Western blotting: 1 – sample with mAbs to PAG1; 2 - negative control;

M - Precision Plus Protein™ Dual Color Standards Molecular Weight Marker (Bio-rad, 1610394)

As a result of the immunoblotting, epitope targeting to proteins with a molecular weight of 55, 37, and 20 kD was determined. A slice of the membrane with a visible stained band on it was excised from the membrane and used for MALDI-TOF mass spectrometry analysis. As a result, "PAG1_BOVIN, Pregnancy-associated glycoprotein 1 OS=Bos taurus OX=9913 PE=1 SV=1" was identified in the protein group in the sample with a molecular weight of 55 kDa, confidence score – 431.

In order to evaluate the antigenic properties of the obtained PAG, Balb/c mice were immunized. High titers of specific antibodies were produced as the result of the immunization, in the range 1:12800-1:25600.

DISCUSSION

Pregnancy-associated glycoproteins (PAG) are produced from the first day of early placental formation, present in the circulation throughout cow pregnancy, and rapidly eliminated from blood after calving. PAG usage as markers makes it possible to achieve high sensitivity and specificity (reaching 99%) of enzyme immunoassay (ELISA) [6, 20, 21]. These are important parameters for the test systems to exclude false positive and false negative results.

As a part of this work, the parameters for the isolation and purification of PAG from bovine placental cotyledons were tested. The used protocol included tissue homogenization, two-stage ammonium sulfate precipitation, and purification of the target product using ion-exchange chromatography (Figure 4).

The resulting purified product was identified by MALDI-TOF analysis as the bovine glycoprotein PAG1 «PAG1_BOVIN, Pregnancy-associated glycoprotein 1 OS=Bos taurus OX=9913 PE=1 SV=1. The molecular weight of PAG was 55 kDa, which corresponds to similar studies carried out earlier [22, 23]. Immunization of laboratory animals was used to demonstrate that the obtained PAG preparation is an efficacious antigen and its use stimulates the immune system of laboratory animals to generate high titers of specific antibodies.

CONCLUSION

Intensification of animal husbandry requests more sensitive and specific means for detecting pregnancy in cows. Modern immunochemical methods for diagnosing pregnancy of cows set a task of producing pregnancy-specific antigens. The protocol described herein for obtaining pregnancy-associated glycoproteins (PAG) can be used during development of serological test systems for cow pregnancy testing.

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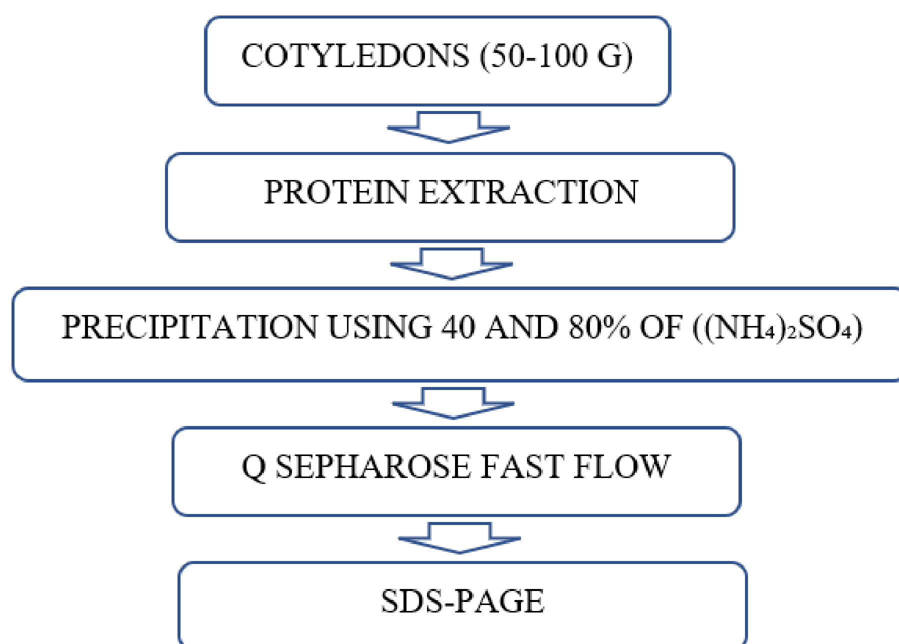

Figure 4 – PAG isolation and purification scheme

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ВЫДЕЛЕНИЕ И ОЧИСТКА ГЛИКОПРОТЕИНОВ АССОЦИИРОВАННЫХ СО СТЕЛЬНОСТЬЮ

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АБСТРАКТ

Целью данных исследований была отработка протокола выделения и очистки гликопротеинов, ассоциированных со стельностью (pregnancy associated glycoproteins PAG) из котиледонов планценты стельных коров. В результате проведенной работы были определены этапы и параметры выделения PAG, включающие: экстракцию в буферной системе, двухэтапную преципитацию сульфатом аммония (до 40 и 80% насыщения) и очистку целевого продукта с помощью ионообменной хроматографии с использованием Q Sepharose Fast Flow. Полученный в результате продукт с молекулярной массой 55 кД был идентифицирован с помощью MALDI-TOF масс-спектрометрического анализа как «Pregnancy-associated glycoprotein 1». В результате проведенного иммуноблоттинга было определено наличие в полученном препарате белков с молекулярной массой 55 кД реагирующих с моноклональными антителами (МКА) специфичными PAG1, полученных ранее. Для определения антигенных свойств полученного препарата PAG, была проведена иммунизация лабораторных животных. Было определено, что использованный в качестве иммуногена очищенный препарат PAG, может быть применен для получения специфических антител. Данные антитела после необходимой процедуры очистки, могут быть использованы в качестве компонентов иммунологических тест-систем для определения стельности коров.

Ключевые слова: диагностика стельности, гликопротеины ассоциированные со стельностью, плацента, котиледоны.

БУАЗДЫҚПЕН ҚАУЫМДАСТЫРЫЛҒАН ГЛИКОПРОТЕИНДЕРДІ БӨЛІП АЛУ ЖӘНЕ ТАЗАРТУ

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ТҮЙІН

Бұл зерттеулердің мақсаты буаз сиырлардың плацентасының котиледондарынан буаздықпен байланысты гликопротеидтерді (PAG) бөліп алу және тазарту хаттамасын әзірлеу болып табылады. Жұмыс нәтижесінде PAG бөліп алу кезеңдері мен параметрлері анықталды, оның ішінде: буферлік жүйедегі экстракция, аммоний сульфатымен екі кезенді преципитация (40 және 80% дейін қанықтыру) және ион- Q Sepharose® Fast Flow көмегімен алмасу хроматографиясы. Нәтиже барысында алынған молекулалық салмағы 55 кД өнім MALDI-TOF масс-спектрометриялық анализімен «Pregnancy-associated glycoprotein 1»ретінде анықталды. Иммуноблоттау нәтижесінде алынған препаратта бұрын алынған PAG1 спецификалық моноклоналды антиденелермен (МКА) әрекеттесетін молекулалық салмағы 55 кД ақуыздардың болуы анықталды. Алынған PAG препаратының антигендік қасиеттерін анықтау үшін зертханалық жануарларға иммундау жүргізілді. Иммуноген ретінде қолданылатын тазартылған PAG препаратын арнайы антиденелерді алу үшін қолдануға болатыны анықталды. Бұл антиденелер қажетті тазарту процедурасынан кейін сиырлардың буаздығын анықтауға арналған иммунологиялық сынақ жүйелерінің құрамдас бөлігі ретінде пайдаланылуы мүмкін.

Кілт сөздер: буаздықты диагностикалау, буаздықпен қауымдасқан гликопротеин, плацента, котиледондар.