OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Obtaining and Use of the Recombinant Bovine Pregnancy-Associated Glycoprotein 1

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Abstract | A convenient biochemical marker of cow pregnancy is the bovine pregnancy-associated glycoprotein 1 (boPAG1) antigen. The aim of this work was to produce a recombinant boPAG1 antigen and obtain monoclonal antibodies (mAbs) against boPAG1. We have obtained the boPAG1 cDNA and are reporting its nucleotide sequence. Bacterial expression of a portion of the natural gene encoding a mature form of boPAG1 failed. But a fusion protein made up of *E. coli* thioredoxin and boPAG1 was efficiently expressed in *E. coli.* Using the recombinant protein as an antigen, mAbs were generated. One mAb reacted with a protein present in extracts from cow placenta. The described results are useful for creating immunological reagents to detect pregnancy in cows, which is of great practical importance.

Keywords | Bovine pregnancy-associated glycoprotein 1, Fusion protein, Recombinant antigen, Monoclonal antibodies, Placental antigens.

Received | July 15, 2022; **Accepted** | August 15, 2022; **Published** | September 15, 2022
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Citation | Ryskeldina A, Iskakova I, Sarina N, Shevtsov A, Syzdykova L, Shustov A, Ramankulov Y, Kuibagarov M (2022). Obtaining and use of the recombinant bovine pregnancy-associated glycoprotein 1. Adv. Anim. Vet. Sci. 10(10): 2148-2159.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.10.2148.2159
ISSN (Online) | 2307-8316

INTRODUCTION

Pregnancy-associated glycoproteins (PAGs) are proteins produced in ruminants by the extra-fetal tissues of the embryo (trophoblast) and placental epithelium (Green et al., 1998; Xie et al., 1997; Santos et al., 2018; Wooding et al., 2005, Zoli et al., 1991). During pregnancy, PAGs are found in the maternal systemic circulation. Thus, detection of PAGs can be used for an early diagnosis of pregnancy in artiodactyl animals such as cows. The early detection of pregnant cows in a herd is of an economic importance for high-intensity animal husbandry, as it allows reducing time-interval between calving. Also, the widespread usage of artificial insemination technology requires early detection of pregnant cows for timely adjustment of keeping conditions and feeding, prompt repetition of artificial insemination in case of conception failure, diagnosing infertile cows and performing the control of the normal course of pregnancy (Fricke et al, 2002).

PAGs are products of a multigene family of approximately 100 genes in the bovine genome (Xie et al., 1997). According to the spatial fold of protein structures, PAGs belong to the superfamily of aspartic proteases (Xie et al., 1991, Jerome A J, 2011). However, for example PAG1 do not exhibit the proteolytic activity due to the absence of essential amino acid residues in the catalytic site (Green et al., 2000). Like cell-secreted aspartic proteases, PAGs are synthesized as prepropeptides which have an export signal peptide, and a pro-peptide (a short stretch of residues downstream the signal peptide) that is cleaved during maturation and not present in a mature protein (Jerome et

al., 2011). A member in the bovine PAGs family, pregnancy-associated glycoprotein 1 (boPAG1), is a useful marker of pregnancy because it is found throughout a longer part of the gestation period in cows. Maternal serum levels of boPAG1 increase beginning from the appearance of the embryonic trophoblast and peak just before delivery. The boPAG1 antigen is suitable for early detection of pregnancy because boPAG1 is detectable as early as 28 days after successful insemination (Green et al., 2005). Further, the boPAG1 antigen gradually disappears from the circulation in the event of embryonic death, thus boPAG1 can be used as a marker of embryo survival to control the course of pregnancy. It is also important for practical applications that the boPAG1 antigen is not synthesized in false pregnancies, uterine tumors, cysts, even when progesterone levels rise (Reese et al., 2019).

In this work, we pursued a goal to produce the recombinant boPAG1 antigen and to obtain monoclonal antibodies (mAbs) against boPAG1, and to check the reaction of the mAbs with a natural antigen derived from reproductive tissues of pregnant cows. The recombinant antigen boPAG1 was produced as a thioredoxin-fusion protein *E. coli,* however, attempts to obtain recombinant boPAG1 in the form of an individual protein were unsuccessful. The fusion protein is produced into inclusion bodies, from which the target protein has been converted into a soluble form by refolding. The recombinant protein was used as an antigen to generate mouse mAbs. In the collection of the obtained mAbs, an antibody was found which reacts with a natural antigen contained in an extract of a cow's placental tissue.

MATERIALS AND METHODS

Ethical Approval

This study was approved by the local ethics committee in the National Center for Biotechnology (NCB) located in Nur-Sultan, Kazakhstan. The local ethics committee is internationally registered (IRB00013497). The approval is in the Protocol No. 2 dated August 01, 2019. Animal works were performed in accordance with the ethical standards for the treatment of animals adopted by the European Convention for the Protection of Vertebrate Animals used for research and other scientific purposes.

mRNA amplification and sequencing of boPAG1 cDNA

Cows' placenta samples were provided by veterinarians working at the slaughterhouse. Placentas were collected immediately after slaughter and delivered to the laboratory on ice, the same day. In the laboratory, placentas (and embryos, if pregnancy was detected) were dissected and tissue samples (~100 mg) were taken. From veterinarians in livestock farms, placentas of cows were collected after calving. The largest part in the samples collection was pieces of the placenta containing the inner epithelial lining. These samples were immediately frozen in liquid nitrogen. To isolate RNA, ~100 mg of a tissue was taken, homogenized in a mortar cooled with liquid nitrogen by grinding with a pestle. Trizol reagent (ThermoFisher Scientific cat. 15596026) was added to the sample powder and the mixture was allowed to thaw with continuous mixing. RNA isolation was continued according to the manufacturer's instructions. Residual DNA was digested by treatment with RNase-free DNase. The amount of RNA was assessed using a NanoDrop 2000 UV-Vis spectrophotometer (ThermoFisher Scientific, USA).

Synthesis of the first strand cDNA was carried out in a reverse transcription reaction using 2 µg of total RNA as a template, and the PAG1_AS oligonucleotide (5'-CAGC ACCATTAAGAGTGAAACCCTTTA-3') as a primer. Reverse transcriptase SuperScript II (ThermoFisher Scientific cat. 18064022) was used for cDNA synthesis, which was carried out according to the manufacturer's instructions.

The boPAG1 gene was amplified with primers PAG1_S (5'-GATGCTAAGAACCAAATCTTCCCTG-3') and PAG1_AS described above. PCR was performed in a volume of 25 µl of the reaction mixture containing 20 ng of cDNA, 1X Phusion Hot Start II High-Fidelity PCR Master Mix (ThermoFisher Scientific cat. F565L), 2 µM primers and 1 unit of Phusion Hot Start DNA Polymerase II (ThermoFisher Scientific cat. F549L). Amplification conditions were as follows: denaturation at 95°C for 2 min, 35 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min), final elongation at 72°C in within 10 min.

PCR products were analyzed in 1% agarose gel. Bands corresponding to the expected fragment size (1396 bp) were isolated from the gel, then used to add 3'-terminal adenyl residues, and cloned into the pGEM-T vector (Promega cat. A3600). The procedures used were in accordance with the recommendations of the kit's manufacturer Promega Corp. A ligation mixture was transformed into competent cells of the *E. coli* strain DH5a. Clones were selected by inoculation of transformants on a medium containing ampicillin. Plasmid DNA was isolated from propagated clones using the PureYield Plasmid Miniprep System (Promega cat. A1223). Inserts in three clones were completely sequenced in both directions.

Plasmids for bacterial expression

A part of the gene encoding a mature form of boPAG1 was amplified from bovine cDNA and inserted into the expression vector pRSET B (ThermoFisher Scientific cat. V35120). To do this, the target sequence was re-am-

plified with primers boPAG1_Nco (5'-CCACATGGTCATCATCATCACCATCACCACACCATCACTCGTCCGGTGCTTACAGTCTGTCCCAGAT-3') and boPAG1_Nhe (5'-CCACGCTAGCTTACACTGCCCGTGCCAGGCCAATC-3'). These primers were chosen to amplify the portion of the boPAG1 gene that codes for a mature form of the protein (i.e., without signal peptide and propeptide). The boPAG1_Nco primer carries the NcoI restriction site, and the boPAG1_Nhe primer carries the NheI site. The boPAG1_Nco primer also encodes a histidine tag for protein purification using metal affinity chromatography (IMAC). The PCR product was initially cloned into the pGEM-T vector (Promega cat. A3600) for sequencing and confirmation of the absence of errors. The insert was then excised by the NcoI and NheI sites and cloned into the pRSET B vector prepared by digestion with the same restrictases. The resulting expression construct was named pRSET/PAG1.

Further, when results of experiments on expression of the recombinant boPAG1 protein using the pRSET/PAG1 construct were available, it appeared necessary to improve the expression, for which purpose a new construct was devised. The latter was planned to encode a fusion protein consisting from *E. coli* thioredoxin (Trx) and boPAG1. A gene encoding Trx and a long linker at the C-terminus of Trx (linker with the sequence: SGSGHMGGGGSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDDKA) was amplified from an unrelated plasmid from the laboratory collection. For this amplification, a sense primer was used that included a ribosome binding site (TTAACTTTAAGAAGGAG). The sense primer carries the XbaI site, and the antisense primer carries the NcoI site. The resulting amplificate was cloned into pGEM-T, for sequencing-confirmation of the correctness. An expression construct to produce the fusion protein Trx-boPAG1 was obtained by cloning the gene for Trx (with the linker) into the previously described pRSET/PAG1 plasmid by the XbaI and NcoI sites. The resulting construct was dubbed pRSET/Trx-PAG1. As the result, a decahistidine tag (10xHis tag) which present in boPAG1, has been retained in Trx-boPAG1.

Complete sequences of the pRSET/PAG1 and pRSET/Trx-PAG1 expression constructs are available in the Supplementary Materials.

Expression of the recombinant antigen

Expression plasmids pRSET/PAG1 and pRSET/Trx-PAG1 were transformed into competent cells of the *E. coli* strain BL21(DE3) (Agilent Technologies cat. 200133). For protein expression, cultures of producer strains in Luria-Bertani (LB) medium were grown to an optical density of OD_{600} =0.6. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration 1 mM and the incubation continued. During initial experiments with pRSET/PAG1 and pRSET/Trx-PAG1, experimental conditions such as the IPTG concentration, time and temperature of incubation were varied to achieve the highest expression. During optimization experiments, 1 ml-aliquots of the induced culture were taken, the biomass was pelleted by centrifugation, the cells were lysed by ultrasound, and the lysates were separated into a soluble fraction (supernatant) and an insoluble fraction (precipitate). Protein contents of the supernatants and precipitates were studied by electrophoresis in 10% polyacrylamide gels under denaturing conditions (SDS-PAGE).

To obtain large amounts of the target antigen, only the pRSET/Trx-PAG1 construct was used. Cultures of the producer strain in a volume of 0.5 L of LB medium were grown and induced with 1 mM IPTG as described above. The production culture with the inducer was incubated overnight at 37°C with stirring (150 rpm). Bacterial biomass was collected by centrifugation, and then resuspended to 10% (w/v) in lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 5% glycerol). Lysozyme was added to a final concentration 1 mg/ml, DNase I (10 μg/ml), RNase A (100 μg/ml), and phenylmethanesulfonyl fluoride to 0.2 mM (PMSF, Sigma cat. P7626). Cells were destroyed by ultrasonic treatment (40 Hz, 10 pulses of 20 s at 200 W). Insoluble material containing inclusion bodies was precipitated by centrifugation (12000 rpm, 20 min, 4°C).

Purification of inclusion bodies by gradient ultracentrifugation

The pellet of crude inclusion bodies was resuspended in 6 ml of buffer I (50 mM Tris, pH 8.5, 1 mM EDTA, 0.2 mM PMSF). Ultracentrifuge tubes with a filling volume 32.5 ml (Beckman Coulter cat. 253020) for SW28 rotor were filled with a stepwise gradient of sucrose solutions. For this purpose, 3 ml-aliquots of sucrose solutions with concentrations (w/w) of 80%, 72%, 70%, 68%, 66%, 64%, 62% were successively layered, and then 6 ml of a solution of 60% sucrose was layered on top. All sucrose solutions were prepared in buffer I. A suspension of inclusion bodies in buffer I was added on top of the 60% sucrose layer. The tubes were centrifuged in a SW28 rotor at 25,000 rpm for 6 hours at 4°C. After centrifugation, the gradient above the 80% sucrose layer was aspirated and discarded. Purified inclusion bodies were collected from the dense layer concentrated on the border of 80% and 72% sucrose. The purified inclusion bodies were resuspended in 20 ml of water and pelleted by low-speed centrifugation to remove sucrose.

Purification and refolding of recombinant boPAG1 protein

The recombinant boPAG1 and Trx-boPAG1 proteins described in this work carry decahistidine (10xHis) tags, allowing the use of IMAC for purification.

An aliquot of inclusion bodies (0.5 g wet weight) was resuspended in denaturing buffer (50 mM sodium phosphate, pH 8, 300 mM sodium chloride, 10 mM imidazole, 6 M guanidine hydrochloride (GuaHCl), 1 mM beta-mercaptoethanol) and the suspension was stirred to dissolve the protein for 1 hour at room temperature. Insoluble material was removed by centrifugation at 10,000 g for 25 min at 4°C. HisTrap Fast Flow columns with a bed volume 5 ml (Cytiva, Cat. 17-5255-01) were equilibrated with denaturing buffer. Purification was carried out in accordance with the manufacturer's recommendations under denaturing conditions. The protein solution was passed through the column. Loosely bound proteins were washed by passing through the columns 50 ml wash buffer (50 mM sodium phosphate, pH 8, 300 mM sodium chloride, 50 mM imidazole, 6 M GuaHCl). The Trx-boPAG1 antigen was eluted into elution buffer which is similar in composition to the wash buffer but contains 300 mM imidazole. The eluate was collected in 5 ml fractions. Fractions containing the recombinant protein as tested with Bradford 's reagent, were pooled.

Refolding was performed by the rapid dilution method. The protein solution (pooled fractions after purification), was slowly added to a large volume (100-fold excess) of refolding buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 10 mM reduced glutathione, 1 mM oxidized glutathione and 20% (w/v) glycerol) with continuous stirring at 4°C. The resulting mixture was allowed to stir overnight at 4°C. The next day, insoluble matter was removed by low-speed centrifugation. Next, the protein solution was subjected to intensive dialysis against 1X phosphate-buffered saline (PBS, Sigma P4417) at 4°C. The recombinant protein was concentrated by lyophilization. The lyophilized recombinant Trx-boPAG1 antigen was divided into portions. Prior to use as an antigen for immunization, aliquots of the lyophilisate were dissolved in water.

Immunization of mice

BALB/c mice aged 2 months (weight 21-24 g) were used in an immunization protocol. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME USA 04609) and were kept in the NCB animal house (Nur-Sultan, Kazakhstan). Mice were kept in a temperature-controlled room with a 12-hour light-dark cycle, unlimited access to standard chow (SSNIFF V1534-300, HTLab AG, Heideck, Germany) and drinking water.

Serum samples were collected from mice prior to immunization, for use as a background antibody control. On the first day of immunization, the lyophilized Trx-boPAG1 antigen was dissolved in water (0.1 ml), mixed with an equal volume of Freund's complete adjuvant (Sigma cat. F 5881) and administered to mice intraperitoneally at a dose of 50 µg of the antigen per mouse (n=10). On days 7 and 14, the mice were injected with 50 µg of the recombinant antigen mixed with incomplete Freund's adjuvant (Sigma, cat. F5506). On the 21 day, the mice were injected with the antigen at a dose of 50 µg dissolved in PBS (pH 7.2). On the 4 day after the last immunization, mice were sacrificed and used for blood sampling from the heart and collecting of the spleens.

Blood sera were used to determine antibodies to boPAG1. The spleens were immediately used to extract splenocytes.

Obtaining monoclonal antibodies

Hybridomas were obtained by fusing SP-2/0-Ag14 mouse myeloma cells with splenocytes from immunized mice (Oi and Herzenberg, 1980). The fusion was carried out using polyethylene glycol with a molecular weight (Mw) 1500 (PEG1500, Sigma cat. 10783641001). Cell fusion products were cultured in Opti-MEM (Gibco Cat. 22600134) in the presence of 1X Aminopterin Hybri-Max (Sigma cat. A5159) and 10% FBS (Sigma cat. F2442). After 14 days of incubation, cultures were used for clonal selection of hybridomas using limiting dilutions (Goding,1980).

mAb characterization and purification

Classes and subclasses of mAbs produced by propagated hybridoma clones were determined using the mouse Pro-Detect Rapid Antibody Isotyping Assay Kit (Thermo Scientific cat. A38550).

To obtain large amounts of mAbs, ascitic tumors in BALB/c mice were used. Mice (n=3 per hybridoma) were primed with 0.5 ml of incomplete Freund's adjuvant. Hybridoma cultures were injected into the abdominal cavity of a mouse at a dose of 1.3×10^6 cells.

Ascitic fluids were collected on days 16-18 after hybridoma injection. The ascitic fluids were purified from cells by low-speed centrifugation and filtration. Purification of immunoglobulins from ascitic fluids was performed using spin columns NAb Protein A/G 1 ml (Thermo Scientific cat. 89958).

Obtaining natural cotyledon antigens

A method was used to obtain natural PAGs preparations from cotyledons, which method is described in (Egen et al., 2009). Dissected cotyledon tissues (~50 g sample weight) were homogenized in 450 ml of buffer A (20 mM

Tris, pH 8.0, 50 mM NaCl, 2 mM EDTA, 0.2 mM PMSF, 0.02% (w/v) NaN_3). Insoluble material was removed by centrifugation at 5000 g for 30 min at 4°C. A portion of 12 ml was taken from the supernatant and dialyzed against 100 volumes of buffer B (20 mM Tris, pH 7.0, 150 mM NaCl, 1 mM EDTA, 0.02% (w/v) NaN_3, 20 uM PMSF, 1 mM 2-mercaptoethanol). For dialysis, a bag made from a nitrocellulose membrane with an Mw cut-off value 50,000 MWCO was used.

Protein extracts from cotyledons were used for fractional precipitation with ammonium sulfate. Dry ammonium sulfate was added to the dialyzed extract to achieve 40% saturation at room temperature, and the solution was incubated at 4°C overnight. A fraction of unwanted admixtures was precipitated by centrifugation at 5,000 g at 4°C for 30 min. The supernatant was used for further purification. To precipitate proteins containing natural PAGs, dry ammonium sulfate was added to the supernatant until saturation was 80%, and the solution was incubated at 4°C overnight. Proteins were precipitated by centrifugation at 5000 g at 4°C for 30 min. The resulting pellet was resuspended in 8 ml of buffer C (10 mM Tris, pH 7.6) and dialyzed against 100 volumes of buffer C. PMSF was added to the protein solution to 0.2 mM and the preparation was stored in aliquots at -20°C.

Mass spectrometry analysis

Proteins were identified using an Impact II mass spectrometer (Bruker) connected to a Dionex high performance liquid chromatography system Ultimate 3000 RSLCnano. The analysis of peptides mass spectra was performed using the Mascot software (Matrix science). The obtained amino acid sequences were used to identify respective source proteins by searching the SwissProt and NCBI databases.

RESULTS

Obtaining the boPAG1 gene

cDNA was synthesized on a template of total mRNA isolated from a placenta of a pregnant cow. The boPAG1 coding sequence was amplified by PCR from the cDNA. We designed primers PAG1_S and PAG1_AS for this amplification. The primers target the ends of the prototype boPAG1 mRNA sequence published in Genbank (accession number BC134743). The PCR products were cloned, and three clones were sequenced. All three clones contained the same sequence shown in Figure 1. A search for sequences homologous to the cloned fragment using BLAST and the Genbank database allowed the confirmation that the cloned fragment is the PAG1 protein of *Bos taurus*, and the sequence BC134743 was the closest relative to our obtained sequence. However, in this work the sequence of the cloned fragment is not 100% identical to BC134743, as there is a difference in one encoded amino acid (the results are shown below).

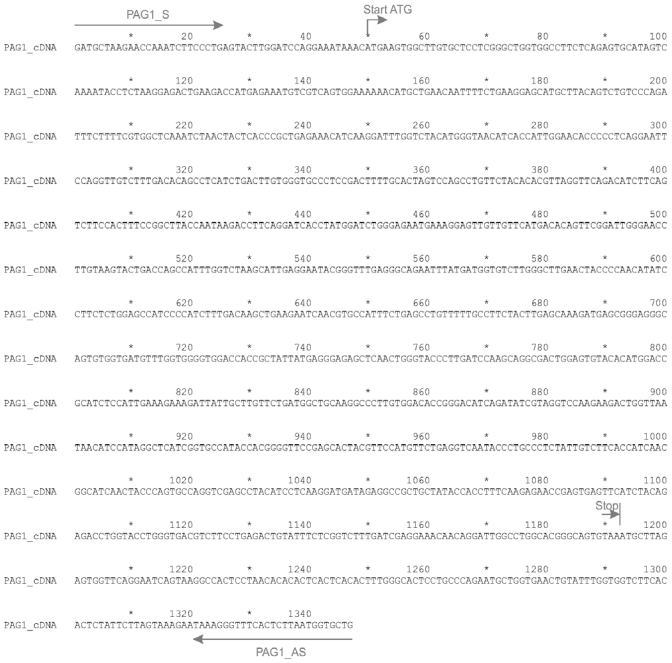

Figure 1: Sequence of a cloned DNA fragment encoding boPAG1. Three clones were sequenced and all three clones contained the same insert. The entire sequence of the insert is shown, including primers at the ends of the PCR product. The primers PAG1_S and PAG1_AS are indicated. The arrow above the ATG codon signifies the start codon for the reading frame. The arrow above the TAA codon shows the translational stop.

Plasmids for protein expression

To create a construct to express the recombinant antigen in *E. coli*, a clone shown in Figure 1 was used as a template to amplify a portion of the reading frame which encodes a mature form of boPAG1. A mature protein is without signal peptide and propeptide. The sequencing-confirmed mini-gene was cloned into the pRSET B expression vector as described in the Materials and Methods section. The genetic map of the resulting plasmid pRSET/PAG1 is shown in Figure 2a, and the nucleotide sequence of the plasmid pRSET/PAG1 is shown in Figure S1 in the Supplementary Materials.

Experiments on bacterial expression using the pRSET/PAG1 construct did not lead to the production of the recombinant protein, so there was a necessity to modify the pRSET/PAG1 construct. This was done by the addition of the *E. coli* thioredoxin (Trx) gene upstream the gene of interest, so that the new construct produces a fusion protein starting with Trx (Trx-boPAG1). The second expression plasmid was named pRSET/Trx-PAG1. The genetic map of pRSET/Trx-PAG1 is shown in Figure 2b, and the nucleotide sequence of pRSET/Trx-PAG1 is shown in

Figure S2 in the Supplementary Materials.

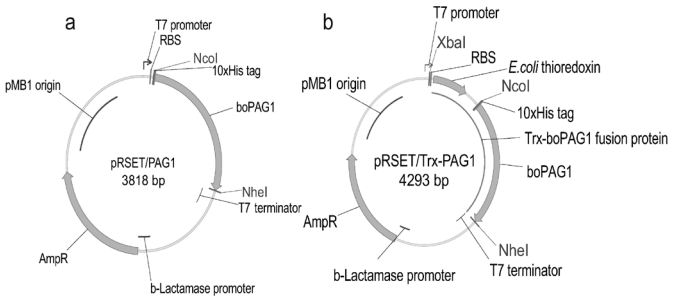

Figure 2: Genetic maps of plasmids for protein expression in *E.coli*. Panel a, plasmid pRSET/PAG1 encodes the recombinant protein boPAG1, with a decahistidine tag (10xHis tag) at the N-terminus. Panel b, plasmid pRSET/Trx-PAG1 encodes a fusion protein comprising *E. coli* thioredoxin (Trx) in the N-terminal part and boPAG1 in the C-terminal part. Genetic elements such as T7 promoter, ribosome binding site (RBS), T7 terminator, etc. are shown. Also shown are reading frames for the recombinant proteins boPAG1, Trx-boPAG1 and for a marker of antibiotic resistance (AmpR). Restriction sites used for cloning XbaI, NcoI, NheI are indicated.

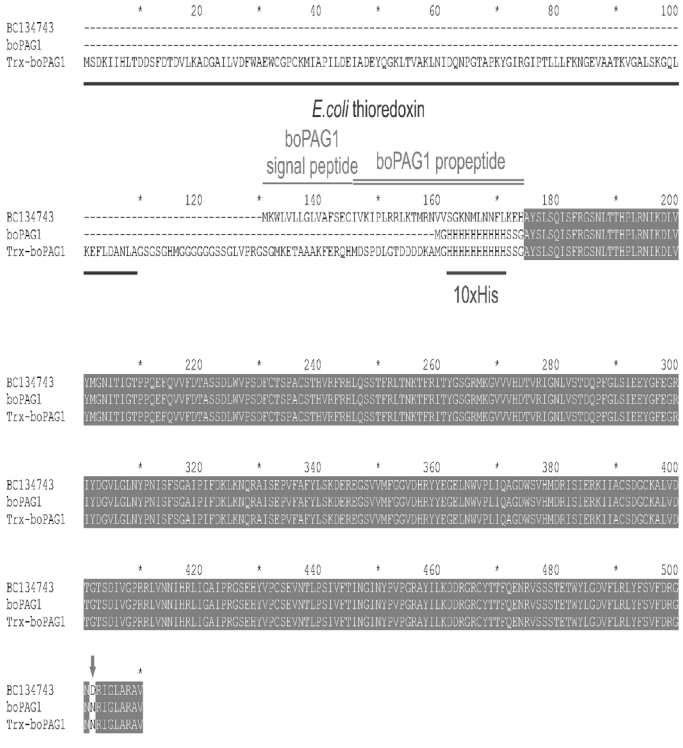

Figure 3: Amino acid sequences of the recombinant proteins boPAG1 and Trx-boPAG1 as compared to a prototype from Genbank (BC134743). Identical positions in the alignment are shown against a dark background. Missing sequences in non-homologous regions are indicated by dashes. For the Genbank sequence, the export signal (single horizontal line) and propeptide (double horizontal line) are marked. The export signal and propeptide are absent in the recombinant proteins. Thioredoxin is marked with a line under the alignment as well as a protein purification tag (10xHis). Aspartic acid-372 in BC134743 is replaced by asparagine in recombinant proteins because a mini-gene obtained in this work differs in this amino acid.

A comparison of amino acid sequences of the recombinant proteins boPAG1 and Trx-boPAG1 with the PAG1 prototype from Genbank, is shown in Figure 3. Sequences of the signal peptide, which serves to export PAG1 from the cell, and the pro-peptide, which is proteolytically cleaved off during protein maturation, have not been included in the expression constructs. In the recombinant proteins, the amino acid sequence of boPAG1 is identical to the Genbank prototype (accession number BC134743) except for one amino acid (Figure 3). The Asp-372 residue (numbered according to BC134743) is replaced by Asn in the gene obtained in this work.

Expression of recombinant proteins

Experiments were performed to obtain the recombinant boPAG1 protein using BL21(DE3) cells transformed with the plasmid pRSET/PAG1. A typical experiment is described in this paragraph, and its results are shown in Figure 4a. In this experiment, a culture of the expression strain BL21(DE3)/pRSET/PAG1 was grown to OD_{600} = 0.6. At this time point, IPTG was added to 1 mM. Samples of the culture by the 1 ml-volume were taken at 0, 1, 2, 4 and 6 hours after the addition of IPTG. Cell biomass was precipitated, destroyed by ultrasound and clarified by centrifugation. The supernatants and pellets were mixed with Laemmli loading buffer and examined by SDS-PAGE. In all experiments with the pRSET/PAG1 construct, no dominant bands with the expected Mw were found in the SDS-PAGE gls. For recombinant boPAG1, the expected Mw is 39.5 kDa. We performed several experiments, varying parameters such as the concentration of the inducer (IPTG), time after induction, incubation temperature after addition of IPTG. These efforts were unproductive. This failure to express the recombinant boPAG1 protein in *E. coli* requested a different solution for the production of the recombinant antigen.

We decided to create a modified expression construct which encodes a fusion protein consisting of Trx and boPAG1 coupled in that order via a linker. Thus, the plasmid pRSET/Trx-PAG1 was created and transformed into cells of the BL21(DE3) strain. The resulting strain was used in protein expression experiments which appeared successful. Results of one experiment are shown in Figure 4b. To obtain the gel shown in Figure 4b, a culture of the producer strain was induced with 1 mM IPTG. Samples were collected at 0, 1, 2 and 4 hours after the start of the induction, and after overnight incubation. Bacterial cells were collected from the samples, the cells were sonicated, and the

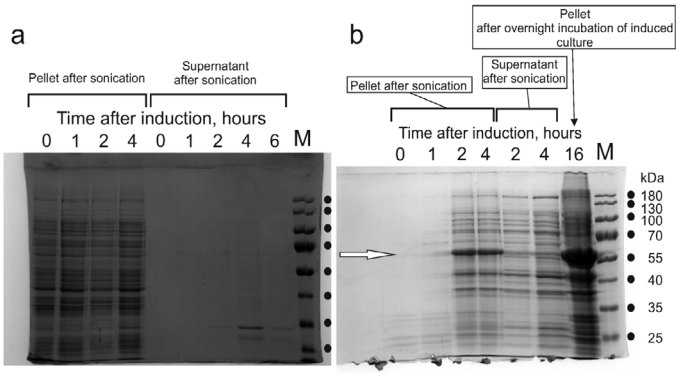

Figure 4: Results of experiments on the recombinant expression of boPAG1 (Panel a) and Trx-boPAG1 (Panel b). Samples of bacterial cultures were collected at time points indicated in the figures. Cell biomass was disrupted and separated into a soluble supernatant and insoluble precipitate. Protein contents in supernatants and precipitates were analyzed by SDS-PAGE. Photographs present stained SDS-PAGE gels. In Panel b, the white arrow points to the band of 56.2 kDa compatible with the Trx-boPAG1 protein. Lanes "M", loaded with PageRuler Mw marker, 10-180 kDa (ThermoScientific 26616). The molecular masses of the marker bands are indicated.

soluble and insoluble fractions of the lysates were analyzed in SDS-PAGE. A band of the expected Mw (56.2 kDa for Trx-boPAG1) is present indicating the presence of the recombinant protein. The desired product accumulates in the insoluble fractions. The accumulation (of the recombinant protein) becomes visible starting from 2 hours after induction, however the amount of the recombinant protein increases significantly upon overnight incubation.

Gel pieces containing the protein with the Mw ~56.2 kDa (bands indicated by an arrow in Figure 4b) were excised from a gel slab and used for mass-spectrometric identification of the protein. The results confirmed that this protein is "pregnancy-associated glycoprotein 1, *Bos taurus*" (Figure S3 in the Supplementary Material).

Obtaining monoclonal antibodies

The recombinant Trx-boPAG1 antigen was purified using IMAC and utilized to immunize BALB/c mice as described in Materials and Methods.

As a result of the immunization, antibodies were induced in mice that reacted with the same antigen used for immunization. The antibodies were present in blood serum in high titers (1:6400-1:12800) (data not shown). Mouse spleens were used as a source of immune splenocytes for hybridization with myeloma cells. Following hybridization, cell fusion products were seeded at a multiplicity of 1 cell per well into four 96-well plates. The growth of hybrid cells in the selective medium was observed in 175 wells. Hybridoma clones were expanded and starting from the moment when the confluence in the well was at least 30%, the culture liquids were tested for the presence of mAbs reactive with the antigen used for immunization. In this assay, 29 hybridomas were selected which produced mAbs reactive with Trx-boPAG1. Titers of the culture fluids in ELISA against Trx-boPAG1 ranged from 1:3200 to 1:6400 (data not shown).

Six hybridomas were selected to obtain preparative amounts of the mAbs from ascitic fluids. Then, the purified mAbs were tested for reactivity against the antigen used for immunization. All six mAbs reacted with Trx-boPAG1 in ELISA as illustrated in Figure 5a.

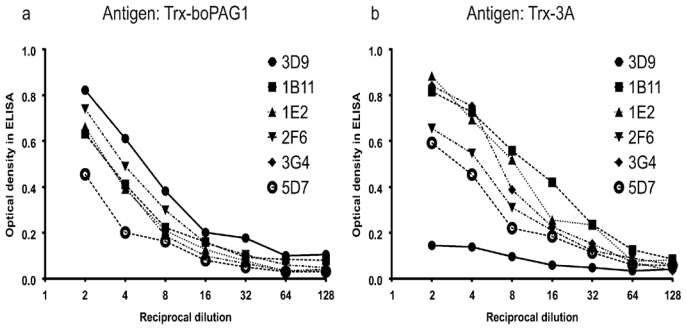

Figure 5: Panel a, a panel of six purified monoclonal antibodies react with the antigen Trx-boPAG1 which was used for immunization. Panel b, the same six mAbs were tested for reactivity against an irrelevant Trx-containing antigen. The mAb 3D9 reacts with the antigen used for immunization (Panel a) but not with the irrelevant antigen (Panel b).

At the next step, a work was conducted to search in the obtained mAbs collection for antibodies reactive with a bovine antigen of interest. Initially, culture fluids from 29 hybridomas and six purified mAbs were tested in ELISA for reactivity with an irrelevant antigen that contains an amino acid sequence of *E. coli* Trx (the used antigen from a laboratory collection is a fusion protein of Trx with the 3A protein of foot-and-mouth disease virus). Of the 29 hybridomas, 28 (96.6%) were found to be positive (i.e. reactive) with an irrelevant Trx-containing antigen. Only one hybridoma 3D9, which initially showed high reactivity with Trx-boPAG1, did not react in ELISA with the irrelevant antigen (Figure 5b). Determination of a class and subclass of the mAb 3D9 using the Pro-Detect Rapid Antibody Isotyping Assay Kit showed that 3D9 is IgG of the subclass G1 and has a kappa-type light chain.

Monoclonal antibody 3D9 reacts with an antigen from bovine placenta

An extract of placental antigens was prepared as described in the Materials and Methods. The extract was subjected to SDS-PAGE and then immunoblotting was conducted

using either the mAb 3D9 or a different unreactive mAb as primary antibodies. Results of these immunoblots are shown in Figure 6.

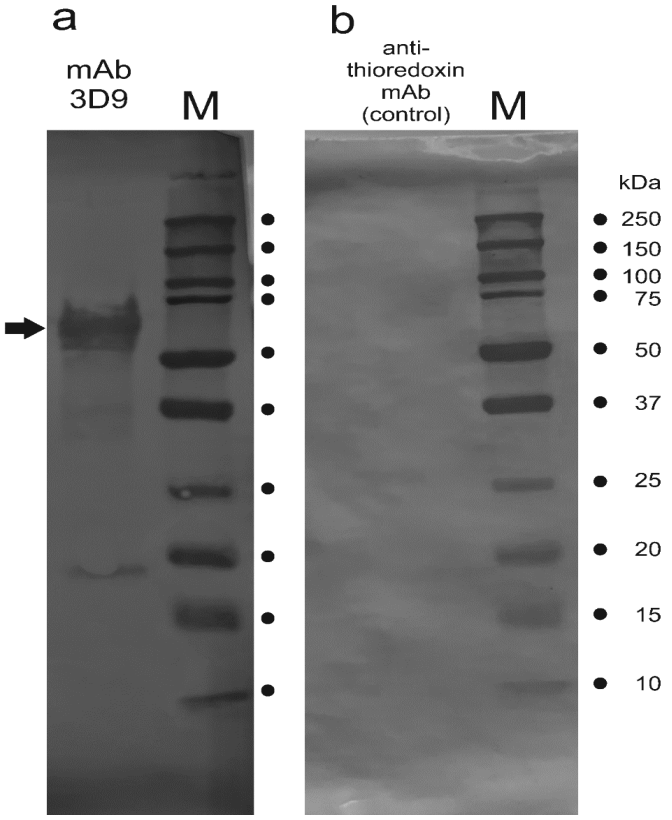

Figure 6: The mAb 3D9 reacts with a protein extracted from cow's placenta. Results of an immunoblot are shown. Placenta-extracted proteins were separated in SDS-PAGE and blotted with the 3D9 mAb (Panel a) or a different control antibody as described in the text (Panel b). In Panel a, the arrow points to the band which was cut out from the membrane and used for the confirmation of native PAG1 in the immune complex using mass spectrometry. Lanes "M", marker Precision Plus Protein Dual Color Standards (Biorad Cat. 1610394). The molecular masses of the marker bands are indicated.

The mAb 3D9 reacts with a protein having an estimated Mw ~60 kDa (Figure 6a). The antigen with the Mw ~60 kDa is present in the placenta extract and is probably a glycosylated form of boPAG1. In addition to the major band, there are weaker minor bans of ~25 kDa and ~40 kDa on the membrane processed with 3D9. The minor bands probably have been produced by proteins immunologically related to boPAG1, or presumably products of proteolytic degradation of boPAG1.

A different mAb which reacts with Trx-containing antigens does not react with the placenta extract (Figure 6b).

After producing the stained immunoblot membrane (Figure 6a), a small nitrocellulose stripe holding the major band (~60 kDa) was excised from the membrane. The stripe was used for trypsin digestion of protein complexes followed by the mass-spectrometry identification of tryptic peptides. Upon comparison to the SwissProt database, the bovine pregnancy glycoprotein boPAG1 was found among the proteins on the stripe (results of the protein identification are given in Figure S4 in the Supplementary Materials).

DISCUSSION

At present, physical transrectal and ultrasound examination are the predominant methods for diagnosing of pregnancy in cows worldwide. However, an interest has been growing among livestock breeders in the use of biochemical and protein markers both for the early detection of pregnancy and monitoring the normal course of pregnancy (Geertruida et al., 2009; Samsonova et al., 2017; Gábor et al., 2007). This is because the intensification of animal husbandry and widening use of the artificial insemination technology. The PAGs proteins are widely considered to be the markers of choice (Green et al., 2005; Community et al., 2016; Oliveira Filho et al., 2020; Shephard and Morton, 2018; Szenci, 2021; Karen et al., 2015). In this article, we present a sequence of a gene encoding the boPAG1, recombinant expression of the boPAG1 protein, and obtaining of a mAb which reacts with the recombinant protein and with an antigen present in a cow's placenta.

Using primers targeting a prototypic PAG1 sequence from Genbank (entry no. BC134743), a cDNA was amplified from a total RNA sample. Three sequenced clones contained the same insert, and the obtained minigene is almost identical in the nucleotide sequence to the prototypic boPAG1. The deduced boPAG1 amino acid sequence in this study differs from the prototypic sequence by one amino acid (Asp372->Asn).

One initially planned expression construct coding for a mature form of the boPAG1 protein failed to produce the recombinant protein in *E. coli*. Another expression construct was devised to express a fusion protein consisting of *E. coli* thioredoxin (Trx) in the N-terminal part and boPAG1 in the C-terminal part. With the latter expression construct, the recombinant fusion protein was successfully produced standard expression conditions such as the BL21(DE3) host strain, induction with 1 mM IPTG and all incubations at 37°C.

The recombinant protein Trx-boPAG1 was purified using IMAC and used as an antigen to immunize mice. A collection of 29 mAbs-producing hybridomas was developed from splenocytes of the immunized mice. It was shown that the majority of hybridomas synthesized mAbs which

reacted with Trx-containing antigens. However, one mAb numbered 3D9, reacted with the antigen used for the immunization, and did not react with the irrelevant Trx-containing antigens.

The mAb 3D9 was tested for the ability to react with antigens present in placentas of cows after calving. In immunoblot, mAb 3D9 was found to react with a placenta-derived antigen having an apparent Mw of ~60 kDa. The immune complexes upon the immunoblot were subjected to mass-spectrometry which identified the presence of bovine PAG1 in a protein complex stained as the 60 kDa-band.

The results of this study would be useful for the creation of test systems for diagnosing pregnancy in cows. The prospects of this work are due to the early appearance of PAG1 in the blood of pregnant cows and this antigen's presence throughout the entire normal pregnancy.

CONCLUSION

A convenient biochemical marker of bovine pregnancy is the boPAG1 antigen. We disclose expression constructs and conditions for the bacterial expression of the recombinant boPAG1 antigen. Also, we report producing a monoclonal antibody which is capable of recognizing a protein present extracts from cow's placenta. The results would help creating immunochemical reagents for detecting and monitoring pregnancy in cows, the task of great practical importance.

ACKNOWLEDGMENTS

A grant of the Ministry of Education and Science of the Republic of Kazakhstan (Grant No. AP 08052441) supported this study.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

NOVELTY STATEMENT

For the first time among published protocols, the bovine pregnancy glycoprotein PAG1 was expressed in E.coli as a full-length product. This was made possible by fusing to a partner thioredoxin which facilitates the expression.

AUTHORS CONTRIBUTION

This manuscript's materials preparation, data analysis, and text writing were all done by all of the authors.

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Xie S, Low BG, Nagel RJ, Kramer KK, Anthony RV, Zoli AP, Beckers JF, Roberts RM (1991). Identification of the major pregnancy-specific antigens of cattle and sheep as inactive members of the aspartic proteinase family. Proceed. National Acad. Sci. 88(22):10247-51. Https://doi.org/10.1073/pnas.88.22.10247

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Supplementary Figures

S1: Sequence of the plasmid pRSET/PAG1

Figure S1. Sequence of the plasmid pRSET/PAG1

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S2: Sequence of the plasmid pRSET/Trx-PAG1

Figure S2. Sequence of the plasmid pRSET/Trx-PAG1

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2022
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aagactggtt
     1321 aataacatcc ataggctcat cggtgccata ccacggggtt ccgagcacta
cgttccatgt
     1381 tctgaggtca ataccctgcc ctctattgtc ttcaccatca acggcatcaa
ctacccagtg
     1441 ccaggtcgag cctacatcct caaggatgat agaggccgct gctataccac
ctttcaagag
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gagactgtat
     1561 ttctcggtct ttgatcgagg aaacaacagg attggcctgg cacgggcagt
gtaagctagc
     1621 aagcttgatc cggctgctaa caaagcccga aaggaagctg agttggctgc
tgccaccgct
     1681 gagcaataac tagcataacc ccttggggcc tctaaacggg tcttgagggg
tttttttgctg

     1741 aaaggaggaa ctatatccgg atctggcgta atagcgaaga ggcccgcacc
gatcgccctt
     1801 cccaacagtt gcgcagcctg aatggcgaat gggacgcgcc ctgtagcggc
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     1861 cggcgggtgt ggtggttacg cgcagcgtga ccgctacact tgccagcgcc
ctagcgcccg
     1921 ctcctttcgc tttcttccct tcctttctcg ccacgttcgc cggctttccc
cgtcaagctc
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gtttttcgcc
     2101 ctttgacgtt ggagtccacg ttctttaata gtggactctt gttccaaact
ggaacaacac
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tcggcctatt
     2221 ggttaaaaaa tgagctgatt taacaaaaat ttaacgcgaa ttttaacaaa
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gtttattttt
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     2701 ctattctcag aatgacttgg ttgagtactc accagtcaca gaaaagcatc
ttacggatgg
     2761 catgacagta agagaattat gcagtgctgc cataaccatg agtgataaca
ctgcggccaa
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     2881 ggatcatgta actcgccttg atcgttggga accggagctg aatgaagcca
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tggccttttg
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ataaccgtat
     4141 taccgccttt gagtgagctg ataccgctcg ccgcagccga acgaccgagc
gcagcgagtc
     4201 agtgagcgag gaagcggaag agcgcccaat acgcaaaccg cctctccccg
cgcgttggcc
     4261 gattcattaa tgcaggatct cgatcccgcg aaa
//
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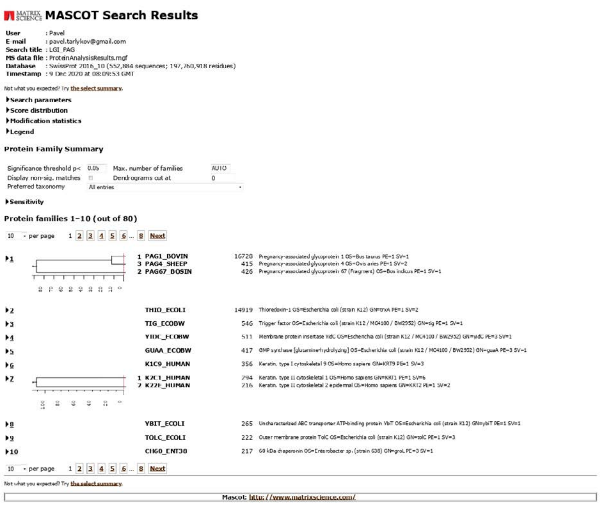
S3: Results of mass-spectroscopic (MASCOT) identification of the recombinant protein extracted from a gel slice

LGI_PAG (Mascot Search Results) http://mascot-server/mascot/cgi/master_results_2.pl?file=../data/20201...



S4: Results of mass-spectroscopic (MASCOT) identification of the recombinant protein present in a band on a blotting membrane which reacts with an anti-PAG1 antibody

1_WB (Mascot Search Results) http://mascot-server/mascot/cgi/master_results_2.pl?file=..%2Fdata%..