J. Biochem. 2008;144(6)725–732
doi:10.1093/jb/mvn127

Characterization of the Peptidase Activity of Recombinant Porcine Pregnancy-associated Glycoprotein-2

Bhanu Prakash V.L. Telugu and Jonathan A. Green*
Division of Animal Sciences, University of Missouri, 163 ASRC, Columbia, MO 65211, USA

Received June 8, 2008; accepted September 15, 2008; published online October 3, 2008

The pregnancy-associated glycoproteins (PAGs) belong to the aspartic peptidase family. They are expressed exclusively in trophoblasts of even-toed ungulates such as swine, cattle, sheep, etc. In pigs, two distinct PAG transcripts (and some variants) have been described. One of the transcripts, porcine PAG-1 (poPAG-1) may not be capable of acting as a peptidase. The second transcript, poPAG-2, possesses a conserved catalytic centre and has been predicted, but not shown, to have proteolytic activity. The thrust of this work was to test such a possibility. PoPAG-2 was expressed as a recombinant protein with an amino-terminal 'FLAG-tag' in a Baculoviral expression system. The expressed proteins were affinity purified by using an anti-FLAG antibody. The purified preparations were then analysed for proteolytic activity against a fluorescent substrate. Porcine PAG-2 had optimal proteolytic activity around pH 3.5. Against this substrate, it had a k_{cat}/K_m of $1.2\,\mu M^{-1}\,s^{-1}$ and was inhibited by the aspartic peptidase inhibitor, pepstatin A, with a K_i of 12.5 nM. Since the proteolytic activity of PAGs in the pig has now been established, the search for putative substrates to gain insight into the physiological role of PAGs will likely be the focus of future investigations.

Key words: aspartic peptidases, placenta, porcine, pregnancy, trophoblast.

Abbreviations: PAG, pregnancy-associated glycoprotein; poPAG, porcine pregnancy-associated glycoprotein; pepF, pepsinogen F; AP, aspartic peptidase.

Aspartic peptidases (APs) are a class of proteolytic enzymes that have a wide distribution. They are found in retroviruses, fungi, plants and vertebrates (*1*). Examples of mammalian aspartic peptidases include pepsin, cathepsins D and E, napsin and renin (*1*). The principal defining characteristic of most APs is their near universal requirement for an acidic pH for maximal activity (one exception to this rule is renin). Another common characteristic is that most APs are inhibited by the statine-containing peptide, pepstatin A (*2*, *3*).

The eukaryotic aspartic peptidases are comprised of a two-domain or bi-lobed structure, probably resulting from a duplication event involving a single domain from an ancestral retroviral AP (*4*, *5*). The APs as their name suggests utilize two aspartic acid residues, Asp32 and Asp215 (porcine pepsin numbering), in the catalytic centre that participate in hydrolysis of peptide substrates (*2*, *6*). The generally accepted mechanism of action for APs is that of general acid–base catalysis (*1*, *2*, *5*). According to this model, a water molecule is coordinated between the two highly conserved aspartic acid residues (Asp32 and Asp 215) via a series of hydrogen bonds. One of the two aspartates, Asp215, acts as a general base extracting a proton from the water molecule, thereby activating it. The activated water molecule then engages in a nucleophilic attack upon the carbonyl carbon of the substrate scissile bond. The other aspartate, Asp32, behaves as a general acid donating a proton to the oxygen in the carbonyl moiety of the scissile bond. This results in a transient unstable tetrahedral intermediate. Rearrangement of this intermediate leads to protonation of the scissile amide and eventual hydrolysis of the substrate.

A unique multi-member family of proteins that are structurally related to aspartic peptidases, known as pregnancy-associated glycoproteins (PAGs), has been described in the placental trophoblasts of even-toed ungulates (swine, cattle, sheep, deer, etc.) (*7*–*9*). In ruminant ungulates (cattle and sheep) the PAG gene family is extensive and is comprised of dozens of expressed genes (*7*, *9*–*13*). In contrast, swine possess only two clearly distinct transcripts (*12*, *14*). In species outside the *Artiodactyla* order, proteins resembling PAGs, known as 'pepsinogen F (pepF)' have been identified in rabbits (*15*), rodents (*16*, *17*), cats and dogs (*18*) and horses (*19*). But unlike PAGs, the pepF locus did not undergo expansion and, therefore, only a single gene copy has been found in all these species. An additional differentiating characteristic of the pepF gene is that, unlike PAGs, it is not restricted in expression to trophoblasts. It is also found in the neonatal stomach mucosa and in other extraembryonic membranes, such as the yolk sac (*15*, *17*).

The two transcripts encoding porcine (po) PAGs, poPAG-1 and -2 were initially cloned from a d13–17 pig conceptus cDNA library (*12*). The transcripts share 79% identity in nucleotide and 64% in amino acid sequences. Recently, additional poPAG members have been reported that were classified as belonging to either the poPAG-2 subfamily (poPAGs-4, -6, -8 and -10) or poPAG-1 subfamily (poPAG-1, -3 and 5) (*14*, *20*). However, these transcripts seem to more

*To whom correspondence should be addressed. Tel: +573-884-1697, Fax: +573-882-6827, E-mail: greenjo@missouri.edu

appropriately be considered variants of poPAG-1 and -2, since they differ from these two proteins at only a few amino acid positions.

Of the two predominant PAGs in swine, poPAG-2 is the most abundant transcript in the conceptus and the poPAG-2 transcript has been identified as early as the 4-cell stage of embryo development (*12*, *21*). Mirroring the high abundance of message, copious amounts of poPAG-2 protein have been identified in explant cultures of pig trophoblast *in vitro* (*12*, *22*, *23*). In addition, a major secretory product of implanting pig embryos, known as porcine basic protein (pBP), was subsequently identified as poPAG-2 (*22*, *24–26*). Upon establishment of a mature placenta, profuse amounts of the poPAG-2 protein line the maternal fetal interface *in vivo* (*27*).

Despite the apparent differences in the relative abundance of message and protein between the two porcine PAGs, there exists a key difference in respect to their putative proteolytic activity. poPAG-1 has been speculated to be proteolytically inactive due to mutations within the catalytic centre—the most prominent being the substitution of an alanine for a glycine in the nearly invariant DTG motif of the amino-terminal lobe of the protein (*12*, *28*, *29*). On the other hand, poPAG-2 has no such mutations within the catalytic centre and, therefore, was predicted to be capable of acting as a peptidase (*12*, *28*, *29*). However, no such activity has been demonstrated for poPAG-2 to date. In this report, we sought to determine if a PAG from the pig, specifically poPAG-2, is an active AP and to investigate its kinetic parameters.

MATERIALS AND METHODS

Evolutionary Relationships of poPAGs—The evolutionary relationship of poPAGs-1 and -2 with other ruminant PAGs, PAG-like molecules (pepF) and other mammalian APs was inferred from amino acid sequence alignments by using the minimum evolution method (*30*). The amino acid sequences were aligned by CLUSTALW and all positions containing gaps and missing data were eliminated from each pairwise comparison. The neighbor-joining algorithm (*31*) was used to generate the initial tree and a bootstrap analysis, with 1,000 replicates (*32*), was performed to build the consensus tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) was shown next to the branches (Fig. 1). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 447 positions in the final data set. Phylogenetic analyses were conducted by using the MEGA4 programme (*33*).

Cloning and Expression of Recombinant poPAG-2—Recombinant protein for poPAG-2 was expressed by using the BD BaculoGold™ Baculovirus insect cell expression system (BD biosciences Pharmingen, CA, USA). poPAG-2 was cloned into the pVL92 transfer vector by using the following oligonucleotides, sense: 5′ GAC AGT ***GCGGC CGC***ATG**GATTACAAGGACGATGACGATAAG**TTAGT CATGATCCCTCTC 3′ and antisense: 5′ GTCAGTCAGA GTCAGAGTCATGACTAGAG***TCTAGA***TGACTATTATGT GGCCTGAGCCAG 3′. A sequence encoding the FLAG peptide tag (DYKDDDDK) was incorporated into the forward primer (that sequence is shown in regular bold) allowing for the expression of the FLAG-epitope at the N-terminus of the recombinant poPAG-2 protein. Sequence recognition sites for restriction enzymes (bold italicized) Not-1 and Bgl-2, (New England Biolabs, MA, USA) were also incorporated into the sense and antisense oligonucleotides to permit directional cloning into the transfer plasmid. The integrity of the cloned sequence was verified by sequencing. The cloned transfer vector was then co-transfected with linearized Baculovirus DNA (BD BaculoGold) into Sf-9 insect cells, by using the BD baculogold transfection kit according to the manufacturer's recommendations. Following transfection, the recombinant viruses were extracted from the culture media and were amplified in two successive rounds of infection of fresh Sf-9 cells. Once the viruses reached an appropriate titre ($\sim 2 \times 10^7$ plaque forming units/ml), the recombinant viruses were used to infect fresh insect cells to generate recombinant proteins according to the methodologies described elsewhere (*34–36*). Following three days of culture, the infected cells were harvested, chilled on ice and centrifuged at 600*g* for 5 min at 4°C. This was followed by two wash cycles under similar conditions with cold 1× PBS (2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl and 8.10 mM Na_2HPO_4, pH 7.2). The final cell pellet was stored at −80°C until use.

Affinity Purification of Recombinant poPAG-2 on an Anti-flag Antibody Column—For purification of recombinant poPAG-2, the frozen insect cell pellets containing poPAG-2 were lysed on ice with I-Per insect cell protein extraction reagent (Pierce, IL, USA). A standard cocktail of protease inhibitors, which included recommended concentrations of various inhibitors 0.4 mM Pefabloc SC-AEBSF (Roche Applied Science, IN, USA), 5 μg/ml Aprotinin, 10 μM E-64, 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma, MO, USA) along with 1 mM dithiothreitol (DTT), was added to the lysis buffer just before use. The pellets were thoroughly mixed with the lysis buffer by pipetting or vortexing and were left to incubate on ice for 15 min. The lysate was then clarified by centrifugation at 15,100*g* for 30 min at 4°C, and dialysed overnight against a buffer containing 20 mM Tris–HCl, 250 mM NaCl pH 7.4 in 30 K MWCO dialysis tubing at 4°C. All the downstream purification procedures were performed in a refrigerated room at 4–6°C. Following dialysis, the lysate was fractionated on a sephadex-200 size exclusion column (1.5 cm × 106 cm) using 20 mM Tris–HCl, pH 7.4 and 150 mM NaCl as buffer. The fractions obtained from sizing column were analysed by dot blot with an anti-FLAG M2 antibody and all fractions that were determined to have FLAG peptide were pooled and subsequently affinity purified by using an anti-FLAG M2 agarose resin (Sigma, MO, USA). For affinity chromatography, the matrix was equilibrated with TBS buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.4), following which the pooled FLAG-containing protein sample, obtained from size fractionation, was loaded twice onto the column by gravitational flow at ~0.2 ml/min. The column was then subjected to subsequent washes with 20 column volumes of wash buffer (20 mM Tris–HCl, 150 mM NaCl, pH 7.4), 20 column volumes of

high-salt buffer (20 mM Tris–HCl, 500 mM NaCl, pH 7.4) and finally 20 column volumes of high-salt buffer supplemented with 0.1% Tween. The matrix was re-equilibrated with 10 column volumes of wash buffer, to remove residual detergent, and 10 column volumes of pre-elution buffer (10 mM phosphate buffer, pH 7.2). The column was eluted with 5 column volumes of 50 mM phosphate buffer, 2 M $MgCl_2$, pH 7.2 buffer. The eluted protein sample was desalted by dialysis in 20 mM Tris–HCl, 250 mM NaCl, pH 8.0 and concentrated on an Amicon-ultra-15 with ultra cell-30 membrane (Millipore, MA, USA). The concentrated protein samples were supplemented with the inhibitor cocktail described above and, for short term storage, cold (4°C) sterile glycerol (autoclaved) was added to a final concentration of 10% (v/v). For long-term storage, the protein sample was mixed with sterile glycerol to a final concentration of 50% and stored at −80°C, until use.

SDS–PAGE and Western Blot Analysis—For analysing the purified recombinant proteins, the protein samples were resolved on a 12% SDS–PAGE gel. One of the gels was stained with comassie blue stain (Sigma, MO, USA) and the other two gels were electrophoretically transferred on to an Immobilon PVDF-membrane (Millipore, MA, USA). Following overnight staining with comassie blue stain, the gels were destained with 10% acetic acid solution and visualized. Following transfer, the membranes were washed once with an excess of 1× TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween, pH 7.5) and non-specific protein binding was blocked with a buffer consisting of 3% bovine serum albumin and 3% non-fat dry milk (Sigma, MO, USA) in 1× TBST. The blots were subsequently incubated with either a 1:1,000 dilution of monoclonal anti-FLAG antibody (Sigma, MO, USA) or 1:2,000 polyclonal anti-poPAG-2 anti-serum in blocking buffer. The blots were then washed and incubated with a 1:2,000 dilution of anti-mouse (for anti-FLAG) or anti-rabbit IgG (for the poPAG-2 antisera) conjugated to an alkaline phosphatase for 45 min (Promega, WI, USA). The blots were finally washed and stained with a mixture of NBT and BCIP according to the manufacturer's instructions.

Determining Optimal pH for Activity Studies—To estimate the optimal pH for poPAG-2 activity, the recombinant PAG was incubated in various buffers with ionic strength adjusted to 100 mM with NaCl. All the pH activity experiments were conducted at 40°C. A synthetic FRET (fluorescence resonance energy transfer) substrate MOCAC[(7-methoxycoumarin-4-yl) acetyl]-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp) [2,4-dinitrophenyl]-D-Arg-NH2 (*37*) (FRET-cathepsin D/E substrate, Peptides International, KY, USA) was used to measure the activity of poPAG-2. The various buffers used for determination of an optimal pH for poPAG-2 activity were: 0.1 M glycine–HCl buffer for pH 2.5, 3.0 and 3.5; 0.1 M sodium citrate–citric acid buffer for pH 4.0 and 4.5; 0.1 M sodium acetate–acetic acid for pH 5 and 5.5; 0.1 M Bis-Tris–HCl buffer for pH 6 and 6.5; 0.1 M HEPES–sodium hydroxide for pH 7 and 0.1 M Tris–HCl for pH 7, 7.5 and 8.0 buffers. All the buffers were filter sterilized before use. A reaction mixture was prepared by mixing 10 μl of 200 μM substrate with each buffer. The final volume of the reaction mixture was adjusted to 80 μl with sterile double distilled water (autoclaved and filter sterilized to remove residual particles if any). The reactions were set up in individual wells of a costar black 96-well plate and allowed to pre-equilibrate to 40°C by incubating in a Synergy-HT plate reader. The reactions were then initiated by simultaneous transfer of 20 μl of fresh protein sample (~750 ng) with the help of a multi-well dispensing pipette. The reactions were thoroughly mixed and kinetic readings were obtained for 10 min by using a Synergy-HT fluorescent plate reader (Bio-tek, VT, USA) with 320/20 excitation and 380/20 emission filters. The initial readings were used to estimate the initial velocities which were displayed as relative fluorescent units (rfu)/min. All the reactions were performed in triplicate and the experiment was replicated multiple times. The initial velocities were then transformed to percent activity with the highest rate being 100%.

Determining the pH Stability of Recombinant poPAG-2 Preparation—To understand the stability of recombinant poPAG-2 in different pH conditions, 520 ng of total protein was incubated in a range of different buffers described above in a total volume of 90 μl and incubated in a water bath for 2 h at 40°C. Following the incubation, the peptidase assay was initiated by addition of 10 μl of 200 μM substrate. The concentration of buffer and salt concentration in the final reaction was maintained at 100 mM. Following the addition of substrate, the reactions were carried out for an additional 10 min in the water bath. The reactions were terminated by an addition of 100 μl of 10% trichloroacetic acid (TCA). The final solution was split into two wells of a 96-well plate described above and the end-point readings were obtained. The resultant reads were transformed to a percent activity with the highest value being 100%. The reactions were performed in duplicate to calculate experimental error in the reads.

Enzyme Assays for poPAG-2—The concentration of total protein in the purified poPAG-2 preparation was estimated by bicinchoninic acid (BCA) protein assay (Pierce Thermo Scientific, IL, USA). The amount of active enzyme within the protein preparation was obtained by titrating the active sites by using pepstatin A as described elsewhere (*38*, *39*). Briefly, eight different concentrations of pepstatin A were incubated with 100 nM of total protein at room temperature for 30 min. A 'reaction mixture' consisting of 20 μM substrate in 100 mM sodium citrate–citric acid buffer, pH 4.0 with 100 mM NaCl was prepared and dispensed into multiple wells in a costar black 96-well microtitre plate. The reactions were then incubated at 37°C for 5 min (pre–heated) in a Synergy-HT fluorescent plate reader (Bio-Tek, VT, USA) prior to the addition of the enzyme-pepstatin mixture. The kinetic readings for the first 10 min of the reaction were obtained in the plate reader by using 320/20 and 380/20 absorption and emission filters. The initial velocities from the reads were determined and the data were fitted by non-linear regression analysis to the following equation to obtain the K_i of pepstatin A for poPAG2 (*38*):

$$v = \left(\frac{v_o}{2E_o}\right)\left\{E_0 - I - K_i + \left[(I + K_i - E_0)^2 + 4K_iE_0\right]^{0.5}\right\},$$

where E_o and I_o are the intial concentration of enzyme and inhibitor respectively, v_o, is the initial rate of

the reaction without inhibitor and 'v' is the observed velocity.

For the determination of kinetic parameters, the reactions were performed in 100 mM sodium citrate–citric acid buffer, pH 4.0, 100 mM NaCl, at 37°C. The assays were performed with two different concentrations of active enzyme (7.5 nM and 10.0 nM) and six different concentrations of the cathepsin D/E—Fret substrate (2.5, 5, 7.5, 10, 20 and 40 μM). The substrate was thoroughly mixed with the reaction mixture (described above) and the reactions were set up in duplicate for each data point. The reaction mixtures were placed in a 96-well costar black round bottom microtitre plate and preheated to 37°C for 10 min, prior to the addition of enzyme (7.5 nM and 10 nM). The resulting fluorescence emitted was measured by the Synergy-HT plate reader by using 320/20 and 380/20 emission and excitation filters. The initial velocities were calculated from the first 10 min of the kinetic reads. The K_m and V_{max} values were evaluated from the intercepts and slopes of double-reciprocal plots of rate (μM/min) versus substrate concentration (μM), by using 'GOSA-fit' curve fitting software (Bio-log, France). The k_{cat} value was calculated from the equation

$$V_{max} = k_{cat}\,[E];$$

where, [E] corresponds to the amount of active site titrated enzyme (*37*).

RESULTS

Evolutionary Relationships of PoPAG Gene Family—The evolutionary relationships of poPAGs to ruminant PAGs, PAG-like proteins (pepF) and the APs, cathepsin D and pepsinogen A revealed an expected branching pattern, where the two poPAGs were clustered together as a small group separated from ruminant PAGs. The analysis also revealed that they are more closely related to PAGs than to the PAG-like proteins and other APs such as cathepsin D and pepsinogen A. In addition, even though the two poPAGs grouped together, there were relatively long-branch lengths in the poPAG-1 and -2 pairing, demonstrating that they are rather divergent from one another (Fig. 1). Based on this analysis, it is clear that even though the poPAGs are related to the ruminant PAGs, they constitute a rather unique grouping within the family.

Expression and Purification of Recombinant poPAG-2—The recombinant form of poPAG-2 was expressed in a Baculovirus insect cell expression system. The recombinant protein was expressed with a FLAG peptide at the N-terminus that allowed for affinity purification of fusion proteins by using an anti-FLAG-M2 antibody matrix. The bound proteins were eluted from the affinity column by using a high-salt buffer that yielded a full-length PAG protein. The expressed full-length proteins, following purification, were of the expected molecular weight. The expressed PAG was identified by western blot by using both an anti-FLAG monoclonal antibody (Fig. 2B) and a polyclonal anti-serum for poPAG-2 (Fig. 2C). Typically, the amount of recovered purified protein obtained from 4-5 T-75 culture flasks was in the range of 25–75 μg/ml in a total elution volume of 4–5 ml.

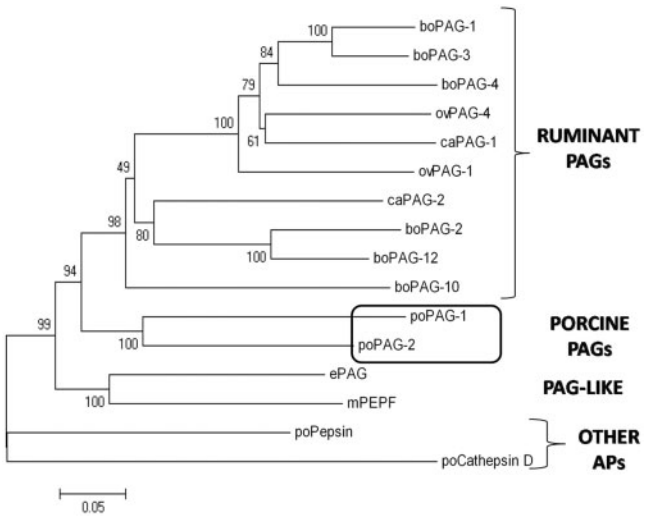

Fig. 1. **Evolutionary relationships of poPAGs, ruminant PAGs, PAG-like proteins and other APs: the phylogenetic relationships of poPAGs with other PAGs, PAG-like molecules and APs were computed based on amino acid sequence by 'minimal evolution' method and were displayed in this figure.** Notice the clear branching and separation of ruminant PAGs (bo, bovine; ca, caprine; ov, ovine) and PAG-like proteins (ePAG, equine PAG and mpepF, mouse pepsinogen F). Also notice that the two poPAGs are clustered together, and they are found branching from PAGs rather than the PAG-like proteins. APs, porcine pepsin (poPepsin) and cathepsin D (poCathepsinD), can be seen branching out as outliers.

Measuring the pH Activity Profile for poPAG2—The optimal pH for maximal activity for poPAG-2 was found to be around pH 3.5 (Fig. 3) based on the initial rates obtained from the experiment. Although it had maximal activity at pH 3.5, ~76% of the activity was retained at pH 3.0 and 65% at pH 4.0. It had very low activity at pH 2.5 (19%) and the activity was nearly undetectable at pH 6.0 (3%). The optimal pH of 3.5 for poPAG-2 was consistent with the acidic pH preferences of most other aspartic peptidases (*1–3*, *5*).

pH Stability of Recombinant poPAG-2—To investigate the stability of poPAG-2 under various pH conditions, the protein was incubated for an extended period of time (2 h) at 40°C in various buffers before the addition of substrate to measure the remaining peptidase activity, if any. In these experiments, the optimal pH for poPAG-2 shifted from 3.5 to 4.0, suggesting that poPAG-2 may be more stable at pH 4.0 than at pH 3.5 following extended periods of incubation (Fig. 4). It was also observed that the activity of poPAG-2 improved substantially in the buffers with pH ranging from 4.5–6.0 relative to the activity seen at pH 4.0, suggesting a possibility that much of the zymogen may have become activated within this timeframe.

Kinetic Parameters of Recombinant poPAG-2—The Michaelis–Menten kinetic parameters k_{cat}, K_m and k_{cat}/K_m and the K_i for pepstatin A were determined and listed in Table 1. The kinetic parameters revealed that poPAG-2 is a relatively robust enzyme with a k_{cat}/K_m of $1.24 \times 10^6\,M^{-1}\,s^{-1}$. It displayed a turnover rate of $9.49\,s^{-1}$

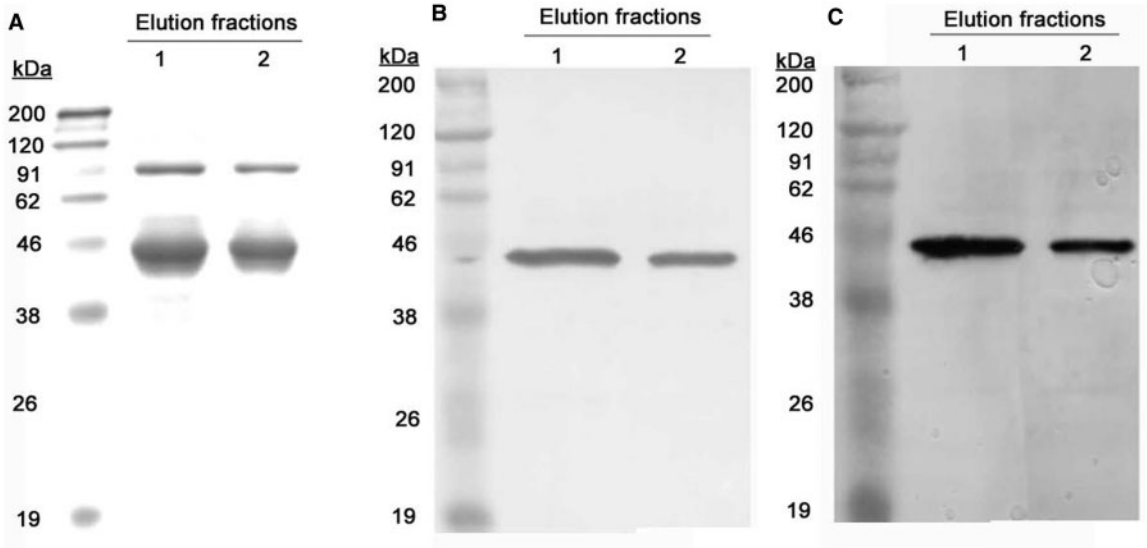

Fig. 2. **SDS–PAGE gels of purified recombinant poPAG-2 stained either with Coomassie blue or by western blotting: two elution fractions of purified poPAG-2 flag fusion proteins, 2 μg of each were resolved on SDS–PAGE gels.** SDS–PAGE gel stained with comassie blue stain. The transferred proteins from identical SDS–PAGE gels were immunoblotted with (B) anti-flag monoclonal and (C) anti-poPAG-2 polyclonal antibodies, respectively. In each gel, lanes 1 and 2 represent respective elution fractions of recombinant poPAG-2 from the anti-flag column in numerical order.

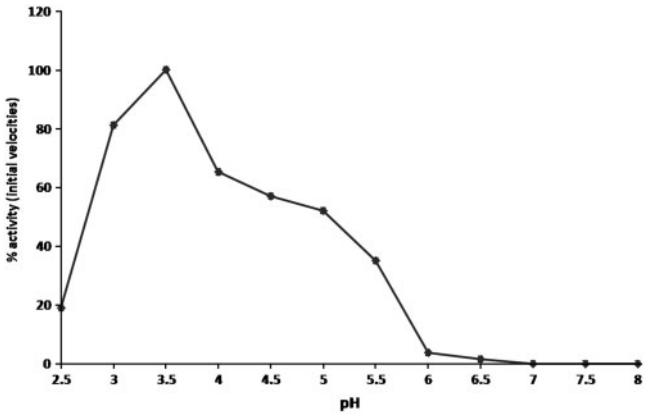

Fig. 3. **A graph showing the pH activity profile for recombinant poPAG-2.** The initial rates obtained from cleavage of the fluorescent substrate were transformed. The highest activity observed was set at 100% and remaining activities were adjusted relative to it. The percent activity was shown on the *y*-axis and the pH of respective buffers on *x*-axis. The error bars represent SD in results obtained from triplicate reads from a representative experiment.

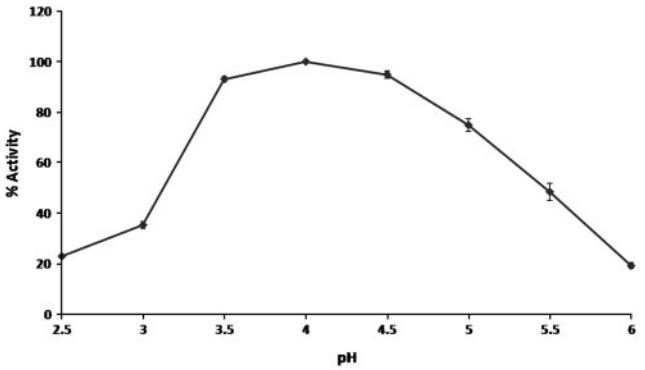

Fig. 4. **pH stability for recombinant poPAG-2: the graph represents the stability of recombinant poPAG-2 in different pH conditions and buffers (indicated in the picture).** The protein was incubated in different buffers representing a range of pH conditions, as indicated in the graph, for 2 h at 40°C. The substrate was then added to the incubated mixture and assayed at the same temperature. The values obtained were transformed into percent activity. The percent activity was depicted on the *y*-axis and the pH of the buffers was shown on the *x*-axis. The error bars represent the SD obtained from duplicate reads from a representative experiment.

and had a K_m of 7.66 μM (Fig. 5 and Table 1). The K_i for pepstatin A was calculated to be 12.5 nM (Table 1).

DISCUSSION

The PAGs in the placenta of the domestic pig make up a simple group, with only two clearly distinct PAG genes, poPAG-1 and -2 compared to the complex and heterogeneous grouping found in cattle and other ruminants. In those species, dozens of PAG genes have been identified and characterized in the placenta (*8*, *9*, *40*). Based on phylogenetic studies, the PAGs belong to the AP family of genes (*8*, *41–43*). Interestingly, several members of the family have been shown to have unusual residues in and around the catalytic site that are predicted to preclude enzymatic activity in these PAGs (*9*, *29*, *41*). Other PAGs have all the hallmarks of typical active APs. However, peptidase activity has yet to be shown for these potentially active peptidases. Therefore, the goal of this work was focused on determining if poPAG-2 was capable of acting as a peptidase and,

Table 1. **Steady state enzyme kinetic parameters for poPAG-2 obtained from global fitting of initial rates and substrate concentrations.**

Source	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	Pepstatin K_i (nM)
poPAG-2	7.66	9.49	1.24×10^6	12.5

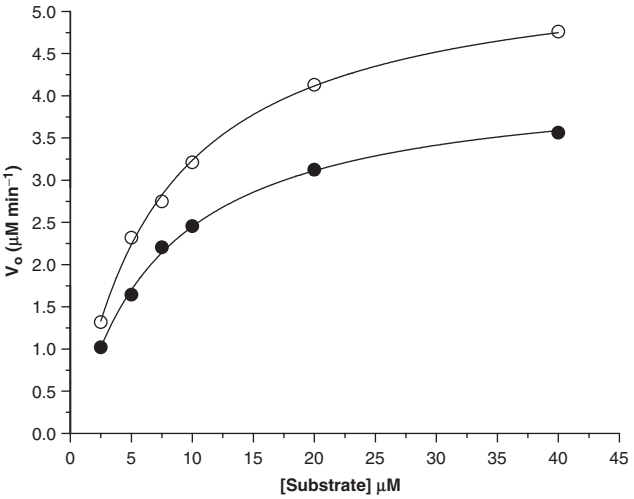

Fig. 5. **Estimation of kinetic parameters for poPAG-2 (k_{cat}, K_m and k_{cat}/K_m) by global fitting of data.** The concentrations of substrate (μM) used in the experiments are shown on the *x*-axis, the relative rates obtained (μM/min) are displayed on the *y*-axis. Two different concentrations of the enzyme 7.5 nM (filled circle) and 10 nM (open circle) and six different concentrations of the cathepsin-D/E Fret substrate (2.5 μM, 5 μM, 7.5 μM, 10 μM, 20 μM and 40 μM) were used in the assay. The initial velocities (V_0) resulting from the lysis of the substrate from respective concentrations of the enzyme are shown in the figure.

if so, to perform an analysis of its kinetic characteristics. Since poPAG-2 is the only candidate that possesses characteristics that are typical of an active peptidase, we chose to express it as a recombinant protein and investigate its activity.

A baculovirus insect cell expression system was used to produce recombinant poPAG-2. This system was selected because it has proven its ability to express soluble, functional PAGs. The protein was expressed as a fusion protein with an N-terminal 'FLAG-tag'. The presence of the FLAG-peptide allowed for monitoring the expression of proteins as well as permitting the purification of poPAG-2 to homogeneity by using an anti-FLAG antibody. The purified proteins of poPAG-2 were found to be proteolytically active against a synthetic fluorescent substrate, MOCAC-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2. This peptide was originally designed as a substrate for cathepsins D and E (*37*). Presumably, the activity against this substrate could be explained by the fact that the core sequence for this substrate was derived from a general substrate consensus sequence for APs [Lys-Pro-Ile-Gln-Phe*Nph-Arg-Leu (Nph: nitro-phenylalanine)] described elsewhere (*44*). Against this substrate, poPAG-2 had maximal activity at around pH 3.5. This pH activity profile was not surprising given that most APs have acidic pH optima. However, following incubation in various buffers for 2 h prior to analysis, we noticed that poPAG-2 activity was more prominent at pH 4.0. When the kinetic parameters for poPAG-2 in pH 4.0 buffer at 37°C were calculated against this substrate, it was apparent that poPAG-2 is a relatively robust enzyme with a k_{cat} of 9.49 s^{-1} and k_{cat}/K_m of ~1.24×10^6 $M^{-1}s^{-1}$. In addition, the estimated K_i for pepstatin was found to be 12.5 nM. These kinetic parameters, when compared to the activity of cathepsin D against the same substrate, revealed that cathepsin D had approximately an order of magnitude higher activity than poPAG-2 with a k_{cat}/K_m of 15.6×10^6 $M^{-1}s^{-1}$ and a turn-over rate of 57.8 s^{-1} and K_m of 3.3 μM (note: 50 mM sodium citrate buffer, pH 4.0; at 40°C were the reaction conditions for those studies) (*37*).

The placenta in swine is a simple epitheliochorial type, where the trophectoderm is simply apposed and attached to the uterus with no invasion into maternal uterine tissues (*45*). However, in cattle, there are focal points of fusion of an invasive cell type of trophoblast called 'binucleate cells' with the epithelium of the maternal endometrium (*46–48*). Since the binucleate cells are the source of many PAGs, the fusion of these cells with those of the maternal uterus results in the eventual accumulation of PAGs in the maternal circulation (*49*, *50*). The PAGs in cattle are, therefore, positioned to perform a potentially systemic role. In swine, since there are no invasive cell types in the placenta and no reports of invasion into the maternal uterus, it is unlikely that the poPAGs enter maternal blood. However, poPAGs, especially poPAG-2, accumulate at the maternal–fetal interface (i.e. at the microvillar junction between trophoblasts and the uterine epithelia) and this location is likely to be where poPAGs function (*27*, *40*).

A multifaceted role for poPAG-2 proteolytic activity could be envisioned either within the secretory pathway of trophoblasts or at the maternal–fetal interface. During the transit via the secretory pathway, poPAG-2 is likely exposed to a pH as low as 5.2 (*51*). This pH might allow for maturation of the poPAG-2 zymogen by an intra- or intermolecular mechanism. Indeed, many APs are capable of removing their own propeptide (or that of a neighbouring AP), under acidic conditions, thereby exposing the peptide-binding cleft for interaction with substrates (*2*, *5*, *52*, *53*). Alternatively, poPAG-2 may become activated by an as yet unknown proprotein convertase in a manner similar to other proteins (*54*, *55*). The activated PAG-2 could subsequently cleave propeptides from other poPAGs or may become involved in the processing of other protein substrates.

Once activated, poPAG-2 has been proposed to be involved in degradation of the microvillar surface ('thinning of microvilli') on the endometrium, allowing greater surface area for apposition and attachment by the placenta (*14*). Alternatively, poPAG-2 might increase the bioavailability of trophic factors by cleaving their binding partners/inhibitors, thereby promoting such diverse mechanisms as angiogenesis, growth of the placenta, etc. A discrete plausible alternative is that, poPAG-2 may be functioning as a degradative enzyme. The poPAG-2 may become rerouted during secretion to endosomes or it may

be actively endocytosed back into the trophoblast along with the uterotroph, which is the principal source of nutrition for the growing fetus (*56*). The PAGs in the endo-lysosomal pathway could then function in proteolytically digesting the nutrients. Clearly, there is a good deal that is not understood about the function of this gene family. Since there are only a few closely related proteolytically active PAGs (the poPAG-2 subfamily) in swine, poPAG-2 might be an ideal candidate to help dissect PAG peptidase function during ungulate pregnancy.

FUNDING

Animal Reproductive Biology Group of the University of Missouri, Food for the Twenty-first Century Programme.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Szecsi, P.B. (1992) The aspartic proteases. *Scand. J. Clin. Lab. Invest. Suppl.* **210**, 5–22
2. Davies, D.R. (1990) The structure and function of the aspartic proteinases. *Annu. Rev. Biophys. Biophys. Chem.* **19**, 189–215
3. Kay, J. and Dunn, B.M. (1992) Substrate specificity and inhibitors of aspartic proteinases. *Scand. J. Clin. Lab. Invest. Suppl.* **210**, 23–30
4. Tang, J. and Wong, R.N. (1987) Evolution in the structure and function of aspartic proteases. *J. Cell. Biochem.* **33**, 53–63
5. Dunn, B.M. (2002) Structure and mechanism of the pepsin-like family of aspartic peptidases. *Chem. Rev.* **102**, 4431–4458
6. James, M.N. and Sielecki, A.R. (1986) Molecular structure of an aspartic proteinase zymogen, porcine pepsinogen, at 1.8 A resolution. *Nature* **319**, 33–38
7. Garbayo, J.M., Green, J.A., Mannekin, M., Beckers, J.-F., Kiesling, D.O., Ealy, A.D., and Roberts, R.M. (2000) Caprine pregnancy-associated glycoproteins (PAG): their cloning, expression and evolutionary relationship to other PAG. *Mol. Reprod. Devel.* **57**, 311–322
8. Green, J.A., Xie, S., and Roberts, R.M. (1998) Pepsin-related molecules secreted by trophoblast. *Rev. Reprod.* **3**, 62–69
9. Green, J.A., Xie, S., Quan, X., Bao, B., Gan, X., Mathialagan, N., Beckers, J.-F., and Roberts, R.M. (2000) Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. *Biol. Reprod.* **62**, 1624–1631
10. Garbayo, J.M., Remy, B., Alabart, J.L., Folch, J., Wattiez, R., Falmagne, P., and Beckers, J.F. (1998) Isolation and partial characterization of a pregnancy-associated glycoprotein family from the goat placenta. *Biol. Reprod.* **58**, 109–115
11. Xie, S., Green, J., Bao, B., Beckers, J.-F., Valdez, K., Hakami, L., and Roberts, R. (1997) Multiple pregnancy-associated glycoproteins are secreted by day 100 ovine placental tissue. *Biol. Reprod.* **57**, 1384–1393
12. Szafranska, B., Xie, S., Green, J., and Roberts, R.M. (1995) Porcine pregnancy-associated glycoproteins: new members of the aspartic proteinase gene family expressed in trophectoderm. *Biol. Reprod.* **53**, 21–28
13. Brandt, G.A., Parks, T.E., Killian, G., Ealy, A.D., and Green, J.A. (2007) A cloning and expression analysis of pregnancy-associated glycoproteins expressed in trophoblasts of the white-tail deer placenta. *Mol. Reprod. Dev.* **74**, 1355–1362
14. Szafranska, B. and Panasiewicz, G. (2002) The placental expression of the porcine pregnancy-associated glycoprotein (pPAG) gene family examined in situ and in vitro. *Anim. Reprod. Sci.* **72**, 95–113
15. Kageyama, T., Tanabe, K., and Koiwai, O. (1990) Structure and development of rabbit pepsinogens. Stage-specific zymogens, nucleotide sequences of cDNAs, molecular evolution, and gene expression during development. *J. Biol. Chem.* **265**, 17031–17038
16. Kageyama, T., Ichinose, M., Tsukada-Kato, S., Omata, M., Narita, Y., Moriyama, A., and Yonezawa, S. (2000) Molecular cloning of neonate/infant-specific pepsinogens from rat stomach mucosa and their expressional change during development. *Biochem. Biophys. Res. Commun.* **267**, 806–812
17. Chen, X., Rosenfeld, C.S., Roberts, R.M., and Green, J.A. (2001) An aspartic proteinase expressed in the yolk sac and neonatal stomach of the mouse. *Biol. Reprod.* **65**, 1092–1101
18. Gan, X., Xie, S., Green, J.A., and Roberts, R.M. (1997) Identification of transcripts for pregnancy-associated glycoprotein (PAG) in Carnivora and Perissodactyla. *Biol. Reprod.* **60** (Suppl. 1), 431
19. Green, J.A., Xie, S., Gan, X., and Roberts, R.M. (1998) An aspartic proteinase expressed in the equine placenta. *Adv. Exp. Med. Biol.* **436**, 163–167
20. Panasiewicz, G., Majewska, M., and Szafranska, B. (2004) Trophoblastic cDNA cloning of porcine pregnancy-associated glycoprotein genes (pPAG) and in silico analysis of coded polypeptide precursors. *Reprod. Biol.* **4**, 131–141
21. Do, H., Kim, J., Abeydeera, L., Han, Y., Green, J., Roberts, R., Matteri, R., Day, B., and Prather, R. (2001) Expression of pregnancy-associated glycoprotein 1 and 2 genes in in vitro-, in vivo- and parthenogenetically-derived preimplantation pig embryos. *Zygote* **9**, 245–250
22. Dore, J.J. Jr, Kattesh, H.G., and Godkin, J.D. (1996) Isolation and identification of porcine embryonic basic protein as a fragment of pregnancy-associated glycoprotein-2. *Int. J. Biochem. Cell Biol.* **28**, 1249–1255
23. Szafranska, B., Panasiewicz, G., Majewska, M., and Beckers, J.F. (2003) Chorionic expression of heterogeneous products of the PAG (Pregnancy-Associated Glycoprotein) gene family secreted in vitro throughout embryonic and foetal development in the pig. *Reprod. Nutr. Dev.* **43**, 497–516
24. Godkin, J.D., Bazer, F.W., Geisert, R.D., Lewis, G.S., and Roberts, R.M. (1982) Synthesis and release of polypeptides by pig conceptuses during the period of blastocyst elongation and attachment. *Biol. Reprod.* **27**, 977–987
25. Godkin, J.D., Bazer, F.W., and Roberts, R.M. (1985) Protein production by cultures established from day 14-16 sheep and pig conceptuses. *J. Reprod. Fert.* **74**, 377–382
26. Baumbach, G., Bartley, N., and Kattesh, H. (1988) Immunocytochemical localization of conceptus and uterine secretory proteins in peri-implantation pig blastocysts. *Biol. Reprod.* **38**, 190
27. Majewska, M., Panasiewicz, G., Majewski, M., and Szafrańska, B. (2006) Localization of chorionic pregnancy-associated glycoprotein family in the pig. *Reprod. Biol.* **6**, 205–230
28. Szafranska, B., Miura, R., Ghosh, D., Ezashi, T., Xie, S., Roberts, R.M., and Green, J.A. (2001) The gene for porcine pregnancy-associated glycoprotein 2 (poPAG2): its structural organization and analysis of its promoter. *Mol. Reprod. Dev.* **66**, 137–146
29. Guruprasad, K., Blundell, T.L., Xie, S., Green, J., Szafranska, B., Nagel, R.J., McDowell, K., Baker, C.B., and Roberts, R.M. (1996) Comparative modelling and analysis of amino acid substitutions suggests that the family of pregnancy-associated glycoproteins includes both

active and inactive aspartic proteinases. *Protein Eng.* **9**, 849–856
30. Rzhetsky, A. and Nei, M. (1992) A simple method for estimating and testing minimum evolution trees. *Mol. Biol. Evol.* **9**, 945–967
31. Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425
32. Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791
33. Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596–1599
34. BD Biosciences. (1999) *Baculovirus Expression Vector System Manual*, 6th edn, Pharmingen, San Diego
35. Smith, G.E., Ju, G., Ericson, B.L., Moschera, J., Lahm, H.-W., Chizzonite, R., and Summers, M.D. (1985) Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. *Proc. Natl Acad. Sci. USA* **82**, 8404–8408
36. O'Reilly, D.R., Miller, A.K., and Luckow, V.A. (1992) *Baculovirus Expression Vectors. A Laboratory Manual* W. H. Freeman and Company, New York
37. Yasuda, Y., Kageyama, T., Akamine, A., Shibata, M., Kominami, E., Uchiyama, Y., and Yamamoto, K. (1999) Characterization of new fluorogenic substrates for the rapid and sensitive assay of cathepsin E and cathepsin D. *J. Biochem.* **125**, 1137–1143
38. Knight, C.G. (1995) Active-site titration of peptidases. *Meth. Enz.* **248**, 85–101
39. Copeland, R.A. (2000) *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*, 2nd edn, Wiley-VCH, Inc., New York
40. Wooding, F.B.P., Roberts, R.M., and Green, J.A. (2005) Light and electron microscope immunocytochemical studies of the distribution of Pregnancy-Associated Glycoproteins (PAGs) throughout pregnancy in the cow: possible functional implications. *Placenta* **26**, 807–827
41. Xie, S., Green, J., Bixby, J.B., Szafranska, B., DeMartini, J.C., Hecht, S., and Roberts, R.M. (1997) The diversity and evolutionary relationships of the pregnancy-associated glycoproteins, an aspartic proteinase subfamily consisting of many trophoblast-expressed genes. *Proc. Natl Acad. Sci. USA* **94**, 12809–12816
42. Xie, S.C., Low, B.G., Nagel, R.J., Kramer, K.K., Anthony, R.V., Zoli, A.P., Beckers, J.F., and Roberts, R.M. (1991) Identification of the major pregnancy-specific antigens of cattle and sheep as inactive members of the aspartic proteinase family. *Proc. Natl Acad. Sci. USA* **88**, 10247–10251
43. Hughes, A.L., Green, J.A., Piontkivska, H., and Roberts, R.M. (2003) Aspartic proteinase phylogeny and the origin of pregnancy-associated glycoproteins. *Mol. Biol. Evol.* **20**, 1940–1945
44. Beyer, B.B., Johnson, J.V., Chung, A.Y., Li, T., Madabushi, A., Agbandje-McKenna, M., McKenna, R., Dame, J.B., and Dunn, B.M. (2005) Active-site specificity of digestive aspartic peptidases from the four species of plasmodium that infect humans using chromogenic combinatorial peptide libraries. *Biochemistry* **44**, 1768–1779
45. King, G.J. (1993) Comparative placentation in Ungulates. *J. Exp. Zool.* **266**, 588–602
46. Wooding, F.B.P. (1982) Structure and function of placental binucleate (giant) cells. *Bibl. Anat.* **22**, 134–139
47. Wooding, F.B.P. (1982) The role of binucleate cell in ruminant placental structure. Reprod. Fertil. Suppl. **31**, 31–39
48. Wooding, F.B.P. (1992) Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta* **13**, 101–113
49. Green, J.A., Parks, T.E., Avalle, M.P., Telugu, B.P., McLain, A.L., Peterson, A.J., McMillan, W., Mathialagan, N., Xie, S., Hook, R.R., and Roberts, R.M. (2005) The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. *Theriogenology* **63**, 1481–1503
50. Lee, C.S., Wooding, F.B.P., and Brandon, M.R. (1986) Light and electron microscope immunocytochemical studies on the role of binucleate cells in villus growth in goat placentomes. *J. Submicrosc. Cytol.* **18**, 661–672
51. Paroutis, P., Touret, N., and Grinstein, S. (2004) The pH of the secretory pathway: measurement, determinants, and regulation. *Physiology* **19**, 207–215
52. Beyer, B.M. and Dunn, B.M. (1996) Self-activation of recombinant human lysosomal procathepsin D at a newly engineered cleavage junction, "short" pseudocathepsin D. *J. Biol. Chem.* **271**, 15590–15596
53. Koelsch, G., Mares, M., Metcalf, P., and Fusek, M. (1994) Multiple functions of pro-parts of aspartic proteinase zymogens. *FEBS Lett.* **343**, 6–10
54. Taylor, N.A., Van De Ven, W.J.M., and Creemers, J.W.M. (2003) Curbing activation: proprotein convertases in homeostasis and pathology. *FASEB J.* **17**, 1215–1227
55. Seidah, N.G. and Prat, A. (2002) Precursor convertases in the secretory pathway, cytosol and extracellular milieu. *Essays Biochem.* **38**, 79–94
56. Bazer, F., Mejia, A., Clark, W., and Vallet, J. (1984) Protein, electrolytes and glucose in uterine flushings of pregnant and nonpregnant gilts. *Biol. Reprod.* **30**, 176