

Zymographic detection of aspartic proteinase activities in porcine ovarian extracts

ABSTRACT

Aims: Tightly regulated proteolytic activity is essential in mammalian ovary to maintain follicular and luteal functions. Studies conducted on ovarian aspartic proteinases (APs) are limited. Previously we have shown that the AP activity increases towards the later part of luteal phase. Aim of this study was to isolate AP from porcine ovarian extract and to identify whether multiple AP activities are found in the extracts.

Place and Duration of Study: Department of Biochemistry, between December 2009 and February 2012.

Methodology: Porcine ovaries (n= 100) were collected and ovarian extracts were prepared. APs were fractionated, using anion exchange chromatography at pH 8.5, gel permeation chromatography and affinity chromatography. AP activity (U/ml) of the fractions were measured in presence and absence of Pepstatin A. AP specific activities (U/mg) were calculated after measuring total protein concentrations (mg/ ml) of the fractions. Fractions were analyzed using polyacrylamide gel electrophoresis conducted under denaturing (SDS-PAGE) and native conditions (PAGE) followed by zymography.

Results: With anion exchange chromatography, AP was recovered during column washing as an unbound fraction (~67%) and during elution as a bound fraction (~33%). Bound AP was recovered around 0.23 M NaCl with 0-1 M NaCl gradient used during elution. Both AP fractions had an approximate molecular mass of 40 kDa. AP activity was completely inhibited in presence of 1 μ M Pepstatin. Specific activity of AP increased with fractionation from 1 in the ovarian extract to 747 and 511 U/ mg with unbound and bound AP respectively. SDS-PAGE also showed elimination of impurities with the progress of fractionation. PAGE and zymography showed the presence of at least three AP activities in porcine ovaries.

Conclusion: Proteinases fractionated using three chromatography procedures were APs. Results showed the presence of multiple AP activities in porcine ovary.

Keywords: Aspartic proteinases, porcine ovaries, pepstatin, zymography

1. INTRODUCTION

Proteolytic enzymes comprise of a large group of structurally and functionally diverse enzymes that catalyze the hydrolysis of peptide bonds. Proteinases (proteases or peptidases) are classified in to subfamilies based on their mechanism of catalysis. Proteinases occur in all forms of life, making proteolysis one of the most important post-translational modifications of proteins. Proteinases are vital in many physiological processes, including digestion, protein degradation and in regulating diverse biological functions [1]. Limited and specific processing of bioactive molecules mediates profound alterations in the cell behavior, according to the needs of the body. For example, proteinases are required to remodel extracellular matrix proteins, modulate bioavailability of growth factors, regulate the level of receptor proteins and regulate apoptosis. Therefore, proteinases are critical determinants of homeostasis. Thus, proteinases are targets for drug discovery to selectively control a metabolic process for therapeutic needs [2]. There are five major sub families of proteinases namely, aspartate, cysteine, threonine, serine and metallo proteinases [1].

Aspartic proteinases (APs) (also known as acid proteinases) are a subfamily of proteinases that have been isolated from diverse sources from viruses to higher animals. APs have an optimum acidic pH which lies between 1.5 and 5 which is different from pH optima of other classes of proteinases [3].

Female reproductive system undergoes rapid and extensive tissue remodeling throughout each estrus cycle [4]. Tightly regulated proteolytic activity is essential in mammalian ovary to maintain follicular growth, ovulation and formation of corpora lutea and to have regular estrous cycles. Most of the studies conducted on ovarian proteinases were focusing on matrix metalloproteinases (MMPs). MMPs and their tissue inhibitors were postulated to play a key role in the remodeling of the extracellular matrix during the ovarian cycle [4,5,6]. Presence of insulin-like growth factors (IGFs) [7,8] and IGF binding proteins (IGFBPs) [7] have been revealed in the ovary of many species, including the pig. Even though these studies revealed the importance of serine, cysteine and matrix metalloproteinases on ovarian functions, investigations carried out on APs are limited. Hence we have initiated studies on AP activity in ovarian extracts. Previously we have shown higher AP activity in porcine ovaries which have started cycling, compared to that of immature ovaries [9]. Further, we observed a variation in AP activity during different stages of the ovarian cycle [9]. Objective of the present study was to isolate AP from porcine ovarian extract and to identify whether multiple AP activities are found in the extracts.

2. MATERIAL AND METHODS

2.1 Collection of ovaries and preparation of crude ovarian extract

Ovaries from pigs of around 6-8 months of age were obtained from a private abattoir, during routine operations with the kind cooperation of the Factory Manger and his team. Ovaries (n= 100) were cleaned and brought to the laboratory in dry ice. Extraction and fractionation were conducted at 4 °C. Crude ovarian extracts were obtained by homogenizing the sliced ovaries in 50 mM phosphate buffered saline (pH 7.5). Extract was centrifuged to remove debris and the supernatant was used for further analysis.

2.2 Fractionation procedure

2.2.1 DEAE-52 anion exchange chromatography

Ovarian extract was dialyzed in 20 mM Tris/HCl (pH 8.5) overnight and applied on to a 100 ml DEAE-52 cellulose column (4x8 cm) equilibrated with 20 mM Tris/HCl (pH 8.5). Column was washed with the same buffer (1 L) and fractions were collected during the initial washing. Proteins bound to the column were eluted with a 0-1 M linear NaCl gradient (600 ml) and fractions were collected. Absorbance at 280 nm and the AP activity of the fractions collected during washing and the elution were measured. Fractions with high AP activity were pooled.

2.2.2 Sephacryl S-200 gel permeation chromatography

Pooled fractions with high AP activity were concentrated using 70% ammonium sulphate and dialyzed in 20 mM phosphate buffer (pH 7.5) overnight. Concentrated pooled fraction was loaded on to Sephacryl S-200 column (3.3x100 cm) and fractions were collected by passing 800 ml of 20 mM phosphate buffer (pH 7.5). Absorbance at 280 nm and the AP activity of the fractions collected were measured. Fractions with high AP activity were pooled.

Subsequently a mixture of molecular weight markers namely, bovine serum albumin (66 kDa), ovalbumin (45 kDa) and sperm whale myoglobin (16.8 kDa) were applied to the same

column and collected fractions in the same manner to determine the approximate molecular mass of the eluted AP.

2.2.3 Pepstatin Sepharose affinity chromatography

Fractions with AP activity obtained after Sephacryl S-200 step were dialyzed separately for 2½ hrs with 5 L of 0.02 M acetate buffer (pH 4.0). About 2/3 volume of the same buffer was added to the dialyzed sample and applied to Pepstatin Sepharose 0.8 x 2 cm column equilibrated with 0.02 M acetate buffer (pH 4.0). The column was washed with 300 ml of 0.02 M acetate buffer (pH 4.0). Bound proteins were eluted with 0.04 M Tris/ HCl (pH 8.2) containing 1M NaCl. AP activity of the fractions collected were measured immediately after fractionation and pooled fraction with peak AP activity was dialyzed in 0.02M phosphate buffer (pH 7.0) overnight to minimize auto catalysis of AP at pH 4.0.

2.3 Measurement of total protein concentration

Total protein concentration of ovarian extract and partially purified fractions was determined by the standard Bradford method [10] with bovine serum albumin as the standard. For each extract, three assays were conducted.

2.4 Determination of AP specific activity

AP activity (U/ml) of the ovarian extract and the partially purified fractions was determined according to the method of Athauda *et al.*, [11] using 2% denatured bovine haemoglobin as the substrate at pH 3.0. Tests were prepared with extract or fractions (50 µl), 1 M formic buffer (pH 3.0) (50 µl) and haemoglobin (pH 3) (400 µl) and incubated at 37 °C for 45 minutes. Reactions were terminated by adding 5% (w/v) trichloroacetic acid (800 µl). Controls were prepared by adding the extract/ fraction after terminating the AP reaction. The reaction mixture was then centrifuged and the absorbance of the supernatant was measured at 280 nm. An increase in absorbance of 1.0 at 280 nm per hour per ml of sample was defined as one unit (U) of proteolytic activity. AP specific activity was expressed in relation to the total protein concentration as U/ mg.

2.5 Determination of AP activity in presence of inhibitors

AP activity was measured after pre incubating the ovarian extract and 1 M formic buffer (pH 3.0) with and without the inhibitor for 15 min. Inhibitors used were 1 µM Pepstatin A, 1 mM Phenylmethylsulphonyl fluoride and 1 µM Soy bean trypsin inhibitor. Denatured bovine haemoglobin was added to the reaction mixtures at the end of pre incubation.

2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using 12% gels, to analyze the gross pattern of proteins in the fractions obtained before and after the Pepstatin Sepharose chromatography. Electrophoresis was carried out according to the standard Laemmli method [12]. After separation at pH 8.6, protein bands were visualized by staining with Coomassie brilliant blue with the fractions obtained before Pepstatin Sepharose chromatography to identify the extent of impurities. Silver stain was used for the fractions obtained after Pepstatin Sepharose chromatography.

2.7 Polyacrylamide gel electrophoresis (PAGE) & detection of AP activity using zymography

Ovarian extract and the fractions with peak AP activity obtained from chromatography were analyzed using PAGE with 7.5% gels [13]. In order to detect AP activity, native conformation of the proteins was maintained until the staining step. At the end of electrophoresis, the gel was washed gently and equilibrated with formic acid buffer at pH 3. Gel was incubated with 1% haemoglobin at pH 3.0 for 15 min. Subsequently the gel was washed gently with formic acid buffer (pH 3) and incubated for 1 h at 37 °C in a humid chamber. Finally the gel was stained with Amido black for 30 min and destained overnight.

3. RESULTS AND DISCUSSION

3.1 Results

Ovarian extract was fractionated using DEAE-52 cellulose, Sephacryl S-200 and Pepstatin Sepharose chromatography in the respective order. DEAE-52 cellulose anion exchange chromatography was conducted at pH 8.5. Fraction volumes collected during washing and elution were approximately 20 and 6 ml respectively. Majority of the AP (approximately 67%) was recovered during early part of column washing (Fig.1- fractions 1-15) in approximately 300 ml. This fraction was referred to as "unbound" fraction.

Elution of bound proteins was carried out with 0-1 M NaCl gradient (Fig. 1- fraction 35 onwards). AP bound to the column was eluted with a peak activity around 0.23 M NaCl (Fig 1- fractions 54-67) in approximately 80 ml (Fig. 1). This fraction was referred to as "bound" fraction and contained about 33% of the AP activity recovered. Most of the impurities were also eluted between 0.16 and 0.51 M NaCl based on the absorbance at 280 nm (Fig 1- four peaks between fractions 48 to 93) and the results of total protein estimation.

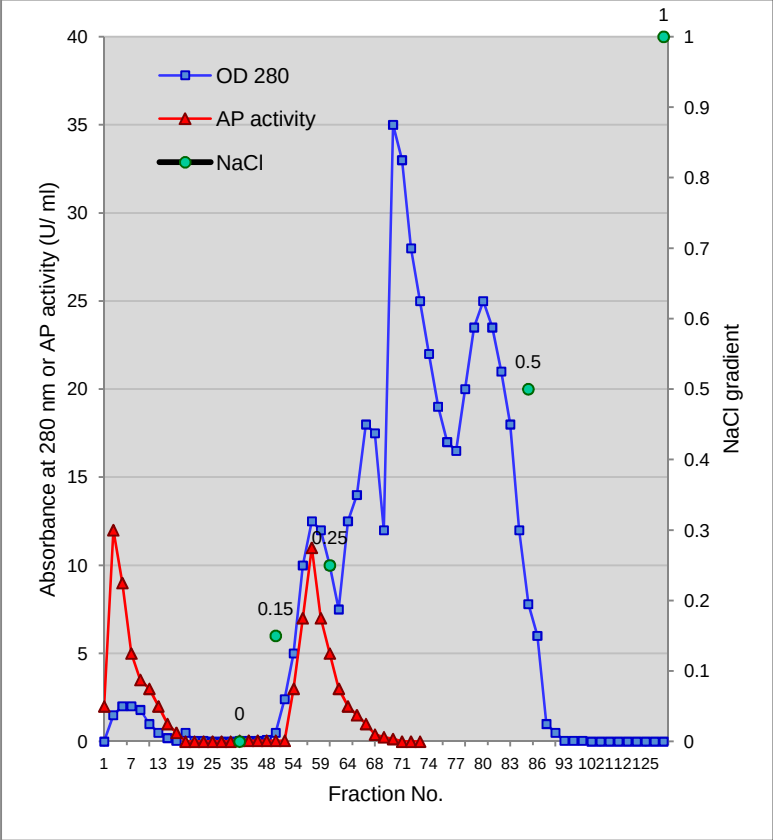
AP specific activity and protein concentrations of fractions obtained with DEAE-52 cellulose chromatography, Sephacryl S-200 chromatography and Pepstatin Sepharose chromatography are shown (Table 1). AP specific activities of ovarian extract, unbound and bound AP fractions were 1, 38 and 4.5 U/ mg respectively (Table 1). Sephacryl S-200 chromatography of both unbound and bound AP fractions resulted one AP fraction each with an approximate molecular mass of 40 kDa. AP specific activities of unbound and bound AP fractions after Sephacryl S-200 step were 160 and 25 U/ mg respectively (Table 1).

Approximately 60% AP activity was recovered during elution at Pepstatin Sepharose chromatography with both unbound and bound AP fractions. Specific activity of pooled fraction with peak AP activity was around 747 U/ mg with unbound AP fraction and 511 U/mg with bound AP fraction (Table 1).

AP activity was inhibited 100% after pre incubating the AP fractions and 1 M formic buffer (pH 3.0) with 1 µM Pepstatin A for 15 min. There was no inhibition of AP activity when pre incubated AP fractions with 1 mM Phenylmethylsulphonyl fluoride and 1 µM Soy bean trypsin inhibitor.

According to the proteins stained with Coomassie blue after SDS-PAGE (Fig. 2A), bound AP fraction contained impurities (Fig. 2A- Lane 4). Unbound AP fraction was relatively free of impurities (Fig. 2A- Lane 2). Similar findings were seen with total protein estimation (Table 1) and measurement of absorbance at 280 nm (Fig. 1) of those fractions. Proteins in the fractions collected after Pepstatin Sepharose step were visualized with silver stain and showed bands corresponding to approximately 64, 54, 32 kDa and a 30 kDa (Fig. 2B).

183 Native PAGE (without SDS) was conducted under native conditions to visualize the APs
184 present in fractions obtained before **Pepstatin Sepharose step** (Fig. 3). Arrows indicate the
185 bands corresponding to different APs (Fig. 3). Native PAGE separated APs into several
186 bands and observed as clear areas in a bluish black background after the detection
187 procedure, due to hydrolysis of haemoglobin bound to the gel. **Overall,** at least three clear
188 areas of AP activity were detected in PAGE, **suggesting** the presence of multiple aspartic
189 proteinases or three **isozymes** or three forms resulted due to different post translational
190 **modifications** (Fig. 3).
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196 **Fig. 1. Proteins recovered during DEAE-52 cellulose chromatography**
197 **DEAE-52 cellulose chromatography was conducted at pH 8.5. Fractions 1 to 35 represent**
198 **column washing. Elution of bound proteins was carried out with 0-1 M NaCl gradient starting**
199 **from fraction 35 onwards. Absorbance at 280 nm and the AP activity was measured in**
200 **fractions. Most of bound proteins were eluted between 0.16 and 0.51 M NaCl.**
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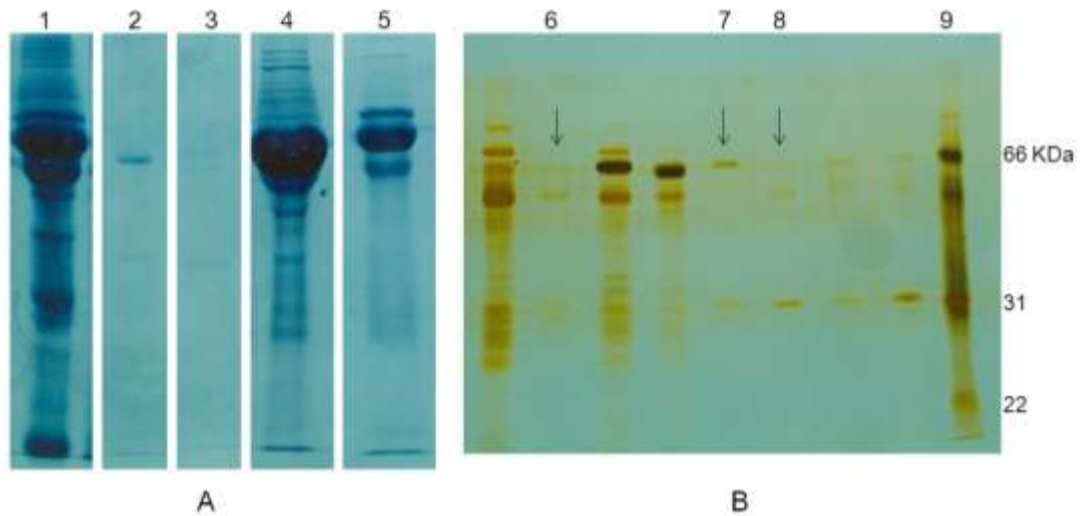
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Table 1. Specific activity of fractions obtained during fractionation of ovarian APs

Fractionation	Fraction	Total Protein (mg/ml)	Specific activity (U/ mg)
Crude extract	Ovarian Extract	23.04	1
DEAE-52 cellulose	Unbound AP ~ 67%	0.216	38
	Bound AP ~ 33%	6.31	4.5
Sephacryl S-200	Unbound AP ~ 40 KDa	0.099	160
	Bound AP ~ 40 KDa	0.399	25
Pepstatin Sepharose	Unbound AP	0.006	746.7
	Bound AP	0.009	511

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Ovarian extract was fractionated using DEAE-52 cellulose, Sephacryl S-200 and Pepstatin Sepharose chromatography in the respective order. DEAE-52 cellulose step resulted two AP fractions, bound and unbound. Later two chromatography steps were conducted separately with bound and unbound AP fractions.



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Fig. 2. Detection of proteins present in ovarian fractions

Panel A: with Coomassie blue stain. A volume of 25 µl/ lane were loaded with a protein quantity varying from 2.5 to 60 µg. Proteins shown are from two gels. Major band seen is around 66 KDa.

Panel B: With Silver stain. Proteins shown are from one gel. Minimum protein quantity loaded was approximately 0.15 µg.

1: Ovarian extract 2: DEAE unbound AP fraction, 3: Unbound AP fraction after Sephacryl S-200 chromatography, 4: DEAE bound AP fraction, 5: Bound AP fraction after Sephacryl S-200 chromatography. 6: Bound AP after Pepstatin Sepharose chromatography, 7: Unbound AP fraction after Sephacryl S-200 chromatography, 8: Unbound AP fraction after Pepstatin Sepharose chromatography, 9: Molecular weight markers (66, 31 and 22 KDa).

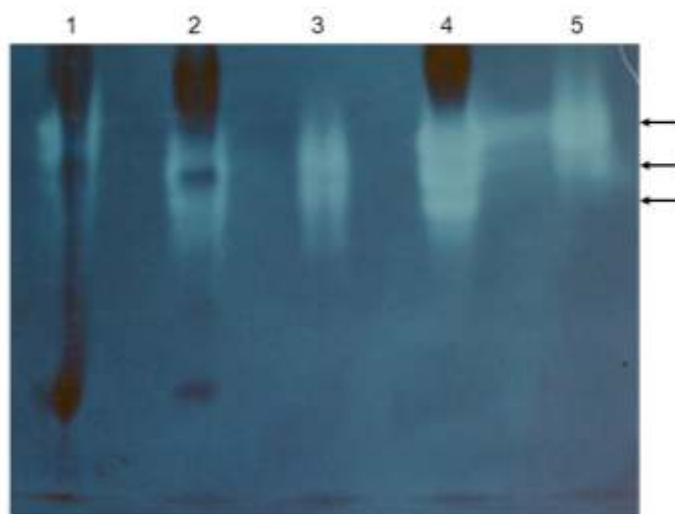


Fig. 3. Detection of ovarian AP activities in PAGE

Proteins shown are from one gel. 1: Ovarian extract, 2: DEAE bound AP fraction, 3: Bound AP fraction after Sephacryl S-200 chromatography 4: Concentrated unbound fraction before Sephacryl S-200 chromatography, 5: Unbound AP fraction after Sephacryl S-200 chromatography

3.2 Discussion

Previously we have optimized the assay conditions to detect AP activity in porcine ovarian extracts and used pH 3.0 for the assay as it was the optimum pH for bovine haemoglobin [9]. These APs did not have a significant activity beyond the pH range of 2.0-5.0. Several mechanisms contributing to an acidic microenvironment were identified in tissues including ovary. These include the Na^+/H^+ exchanger and H^+/ATPase which mediate local acidification [14]. Hypovascularity, hypoxia and anaerobic glycolysis were observed in the ovary, during late folliculogenesis and early development of corpora lutea [15]. In early events of luteal regression, vasoconstriction, detachment of vascular endothelial cells and occlusion of blood vessels occur [16] resulting in a diminished oxygen supply to the luteal parenchyma [17]. Active H^+ -pumps are present in endothelial cells [18] and macrophages [19] which are the predominant cell types of corpus luteum.

Based on the recovery of AP during DEAE-52 cellulose chromatography at pH 8.5, there should be at least 2 AP activities in porcine ovary. Bound AP fraction seems to bear a net negative charge at pH 8.5, which was eluted around 0.23 M NaCl. Unbound fraction had almost no affinity to the positively charged DEAE-52 column. Most of the ovarian proteins were bound to the ion exchange resin at pH 8.5.

According to the outcome at gel filtration, both bound and unbound APs were similar in their molecular mass, which was approximately 40 kDa. Well known AP cathepsin D mature proteinase is formed after several proteolytic changes. Procathepsin D originates after the first proteolytic cleavage of pre-pro-enzyme is 52 kDa. Procathepsin D undergoes further proteolytic processing in the acidic milieu forming single chain cathepsin D with an apparent mass of 44 kDa. Cathepsin D would also consist of a double chain form with 31 and 14 kDa peptides [20]. In the present study, fractions collected after Pepstatin Sepharose

chromatography showed 64, 54, 32 and 30 kDa bands. Further studies are necessary to identify whether those bands correspond with cathepsin D.

With the PAGE, haemoglobin bound to the gel, during the detection procedure was hydrolyzed by AP, at the positions corresponding to AP bands and remained as protein free areas against a background bound with intact haemoglobin. Based on the band positions observed with PAGE, there should be at least three different APs or isoforms or post translational modifications with different net charges at pH 8.8 (pH of the resolving gel), that are present in porcine ovary.

Further studies are necessary to identify the role of these APs. Previously we have observed that AP activity increases when the ovaries show cycling features with presence of corpora lutea and this increase was mainly coming from corpora lutea, more specifically from the early phase of corpus albicans, indicating a likely function of AP during the luteal phase [9]. However, in the current study the ovarian extract was prepared from the whole ovary which includes AP coming from all the structures of ovary. A previous study revealed the role of cathepsin D, a well known AP, in releasing antiangiogenic fragments by processing prolactin in mice [14]. Prolactin was shown to stimulate progesterone production in porcine early corpora lutea [21]. Expression of prolactin and their receptors in bovine CL was demonstrated [22]. Erdmann *et al.*, demonstrated a concomitant increase in cathepsin D levels between early and late luteal stages in the bovine CL and also found that the product of prolactin cleavage by cathepsin D, inhibited the growth of CL-derived endothelial cells [23]. In another study, role of cathepsin D in apoptosis via regulating caspase-3 was demonstrated [24]. Together, these studies suggest that there is an AP-prolactin mediated mechanism governing the luteal regression even though our study does not provide evidence. Whether the APs identified are cathepsin D isoforms or different enzymes, need to be investigated. Further studies are necessary to identify the role of AP in porcine ovary.

4. CONCLUSION

Proteinases fractionated using three chromatography procedures were APs. Results showed the presence of multiple AP activities in porcine ovary.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

Not applicable. Porcine ovaries were collected at the time of routine factory operations with the permission of the factory manager.

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