

Original Research Article

Presence of multiple aspartic proteinases in porcine ovary

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. Aim of this study was to identify whether multiple aspartic proteinases are found in porcine ovaries.

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. AP was partially purified using anion exchange chromatography at pH 8.5 and gel permeation chromatography. AP activity (U/ml) and protein concentrations (mg/ ml) were measured to calculate the AP specific activities (U/mg) of the fractions. Different fractions obtained were analyzed using polyacrylamide gel electrophoresis conducted under denaturing (SDS-PAGE) and native conditions (PAGE) to detect the proteins and the AP activity respectively.

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 weight of 40 kDa. Specific activity of AP increased with the partial purification, and the highest of 160 U/ mg was observed with unbound AP fraction. PAGE showed the presence of at least three APs in porcine ovaries.

Conclusion: Results show evidence for the presence of multiple aspartic proteinases in porcine ovary.

Keywords: *Aspartic proteinases, porcine ovaries, PAGE*

1. INTRODUCTION

Proteolytic enzymes comprise of a large group of structurally and functionally diverse enzymes that catalyze the hydrolysis of peptide bonds. Proteinases 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 [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 scarce. Previously we have shown higher AP activity in porcine ovaries which have started cycling, compared to that of immature ovaries. Further, we observed a variation in AP activity during different stages of the ovarian cycle. Objective of the present study was to determine whether there are multiple APs present in porcine ovaries.

2. MATERIAL AND METHODS

2.1 Collection of ovaries and preparation of crude ovarian extract (OE)

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 Manager 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 (OE) 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 Partial purification

2.2.1 DEAE-52 anion exchange chromatography

OE 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.

2.3 Determination of AP specific activity

AP activity (U/ml) of the OE and the partially purified fractions was determined according to the method of Athauda *et al.*, [9] 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 μ l) and haemoglobin (pH 3) (400 μ l) and incubated at 37 °C for 45 minutes. Reactions were terminated by adding 5% (w/v) trichloroacetic acid (800 μ l). 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. Total protein concentrations (mg/ml) of the fractions were measured. AP specific activity was expressed in relation to the total protein concentration as U/ mg.

2.4 Measurement of total protein concentration

Total protein concentration of OE 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.

2.5 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 during partial purification. Electrophoresis was carried out according to the standard Laemmli method [11]. After separation at pH 8.6, protein bands were visualized by staining with Coomassie brilliant blue.

2.6 Polyacrylamide gel electrophoresis (PAGE) & detection of AP activity

OE and the fractions with peak AP activity obtained from chromatography were analyzed using PAGE with 7.5% gels [12]. 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

DEAE-52 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- first peak in red) in approximately 300 ml. This fraction was referred to as “unbound fraction” (U).

Elution of bound proteins (Fig 1-four peaks in blue) 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-second peak in red) in approximately 80 ml (Fig. 1). This fraction was referred to as “bound fraction” (B) and contained about 33% of the AP activity recovered. Most of the impurities were also eluted between 0.16 M and 0.51 M NaCl based on the absorbance at 280 nm (Fig. 1) and the results of total protein estimation.

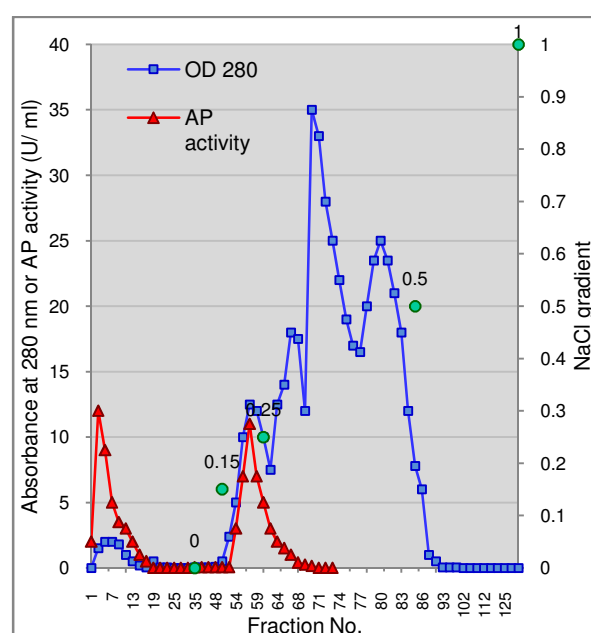
AP specific activity, percentage recovery of AP and approximate molecular weights of AP fractions obtained with DEAE-52 and Sephacryl S-200 chromatography are shown (Fig. 2). Specific activity of AP in OE, U and B fractions were 1, 38 and 4.5 U/ mg respectively (Fig. 2). Sephacryl S-200 chromatography of both U and B fractions resulted one AP fraction each

136 namely U-S and B-S respectively. AP recovered in both U-S and B-S had an approximate
137 molecular weight of 40 kDa. Specific activity of AP U-S and B-S were 160 and 25 U/ mg
138 respectively (Fig. 2).

140 SDS-PAGE was conducted to visualize the proteins present in ovarian fractions obtained
141 with partial purification (Fig. 3). Bound fraction contained more impurities (Fig. 3). Unbound
142 fraction was relatively free of impurities (Fig. 3).

144 Native PAGE (without SDS) was conducted under native conditions to visualize the APs
145 present in fractions obtained with partial purification (Fig. 4). Arrows indicate the bands
146 corresponding to different APs (Fig. 4). Native PAGE separated APs into several bands and
147 observed as clear areas in a bluish black background after the detection procedure, due to
148 hydrolysis of haemoglobin bound to the gel. At least three clear areas of AP activity were
149 detected in PAGE, indicating the presence of multiple aspartic proteinases (Fig. 4).

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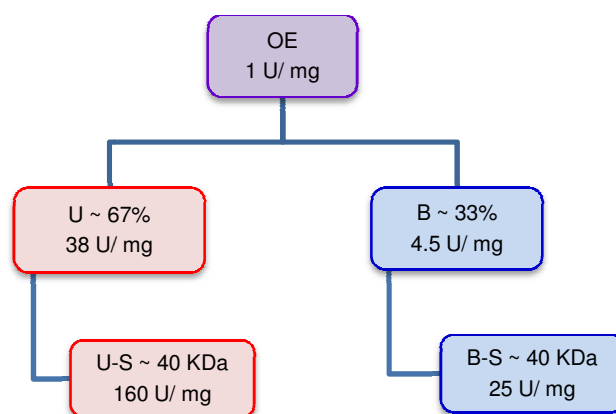
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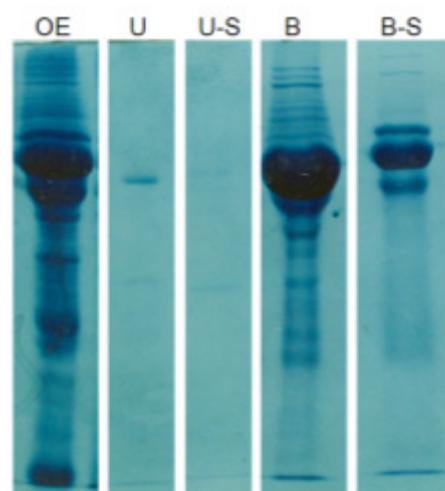
Fig. 1. Proteins recovered during DEAE-52 anion exchange chromatography



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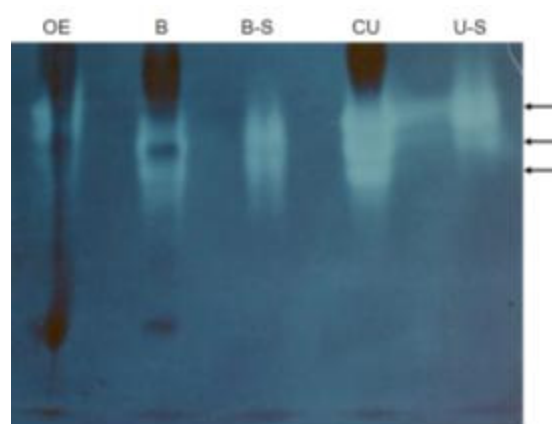
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Fig. 2. Specific activity of fractions obtained with partial purification of ovarian APs
OE: Ovarian extract, U: DEAE unbound fraction, B: DEAE bound fraction, U-S & B-S: AP fractions obtained after Sephacryl S-200 chromatography with U and B respectively.



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Fig. 3. Detection of proteins present in ovarian fractions
B: DEAE bound fraction, U: DEAE unbound fraction, U-S & B-S: Proteinase fractions obtained after Sephacryl S-200 chromatography with U and B respectively.



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Fig. 4. Detection of ovarian AP activity in PAGE
OE: Ovarian extract, B: DEAE bound fraction, U: DEAE unbound fraction, CU: concentrated U fraction, U-S & B-S: AP fractions obtained after Sephacryl S-200 chromatography with U and B respectively.

3.2 Discussion

Previously we have revealed that porcine ovarian aspartic proteinases show an optimum pH of 3.0 for bovine haemoglobin. 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 [13]. Hypovascularity, hypoxia

and anaerobic glycolysis were observed in the ovary, during late folliculogenesis and early development of corpora lutea [14]. In early events of luteal regression, vasoconstriction, detachment of vascular endothelial cells and occlusion of blood vessels occur [15] resulting in a diminished oxygen supply to the luteal parenchyma [16]. Active H⁺-pumps are present in endothelial cells [17] and macrophages [18] which are the predominant cell types of corpus luteum.

Based on the recovery of AP during DEAE-52 chromatography at pH 8.5, there should be at least 2 APs 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. 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 isozymes 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 [19]. 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 [13]. Prolactin was shown to stimulate progesterone production in porcine early corpora lutea [20]. Expression of prolactin and their receptors in bovine CL was demonstrated [21]. 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 [22]. In another study, role of cathepsin D in apoptosis via regulating caspase-3 was demonstrated [23]. Together, these studies indicate that there is an AP-prolactin mediated mechanism governing the luteal regression. Whether the APs identified are cathepsin D isozymes or different enzymes, need to be investigated. Further studies are necessary to identify the role of AP in porcine ovary.

4. CONCLUSION

Results show evidence for the presence of multiple aspartic proteinases 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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