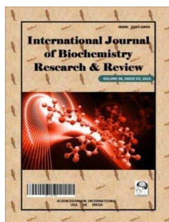

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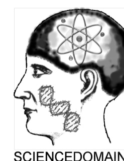

Zymographic Detection of Aspartic Proteinase Activities in Porcine Ovarian Extracts

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Authors' contributions

This work was carried out in collaboration between all authors. Author HKIP concept of the study, collected ovaries, carried out all the experiments, prepared the manuscript and revised the manuscript according to the comments of the reviewers and editors, Author PHPF Collaborator and revision of manuscript, Author SBPA Designed the study and collaborator. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Tightly regulated proteolytic activity is essential in the mammalian ovary to maintain follicular and luteal functions. Studies conducted on ovarian aspartic proteinases (APs) are limited. Previously it has been noted that the AP activity increases towards the latter part of luteal phase. The 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 the presence and absence of Pepstatin A. AP specific activities (U/mg) were calculated after

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measuring total protein concentrations (mg/ml) of the fractions. Fractions were analyzed using polyacrylamide gel electrophoresis conducted under denaturing (SDS-PAGE) and native (PAGE) conditions. PAGE was 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 apparent molecular mass of 40 kDa. AP activity was completely inhibited in the presence of 1 µM 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 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 a large group of structurally and functionally diverse enzymes that catalyze the hydrolysis of peptide bonds. Proteinases (proteases or peptidases) are classified into 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 the regulation of 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. Proteinases are therefore critical determinants of homeostasis. Consequently, 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 estrous cycle [4]. Tightly regulated proteolytic activity is essential in the 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 have focused 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 [4-6]. 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, cystein and matrix metalloproteinases on ovarian functions, investigations carried out on APs are limited. Hence the current study on AP activity in ovarian extracts was initiated. Previously we have shown higher AP activity in porcine ovaries which have started cycling, when compared with immature ovaries [9]. Further, we observed a variation in AP activity during different stages of the ovarian cycle [9]. The objective of the present study was to isolate AP from porcine ovarian extract and to investigate whether multiple AP activities are found in the extracts.

2. MATERIALS AND METHODS

2.1 Collection of Ovaries and Preparation of Crude Ovarian Extract

Ovaries from pigs of approximately 6-8 months in 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 extract was 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

Three chromatography procedures were used [10] to fractionate the ovarian extract as described below.

2.2.1 DEAE-52 anion exchange chromate-graphy

Ovarian extract was dialyzed in 20 mM Tris/HCl (pH 8.5) overnight and applied on to a 100 ml DEAE-52 cellulose column (4 x 8 cm) equilibrated with 20 mM Tris/HCl (pH 8.5). The 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 chromategraphy

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 (3.3 x 100 cm) equilibrated with 20 mM phosphate buffer (pH 7.5) containing 0.2 M NaCl. Fractions were collected by passing 800 ml of the same buffer (pH 7.5). Absorbance at 280 nm and the AP activity of the fractions 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 fractions were collected to determine the approximate molecular mass of the eluted AP.

2.2.3 Pepstatin sepharose affinity chromate-graphy

Fractions with AP activity obtained after Sephacryl S-200 step were dialyzed for 2½ hrs with 5 L of 20 mM 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 20 mM acetate buffer (pH 4.0). The column was washed with 300 ml of 20 mM acetate buffer (pH 4.0). Bound proteins were eluted with 40 mM Tris/HCl (pH 8.2) containing 1M NaCl. AP activity of the fractions collected were measured immediately after fractionation and the pooled fraction with peak AP activity was dialyzed in 20 mM phosphate buffer (pH 7.0) overnight to minimize the 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 [11] with bovine serum albumin as the standard. Triplicate measurements were taken for each fraction.

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. [10]. Denatured bovine haemoglobin was used as the substrate. Tests were prepared with extract or fractions (50 µl), 1 M formic buffer (pH 3.0) (50 µl) and haemoglobin (pH 3.0) (400 µl). Reaction mixtures were 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. After 10 minutes the reaction mixtures were centrifuged. Absorbances of the supernatants were 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 in each fraction, in relation to the total protein concentration was expressed as U/mg.

2.5 Determination of AP Specific Activity in Presence of Inhibitors

Ovarian extract and 1 M formic buffer (pH 3.0) were pre incubated with or 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. AP specific activity was measured as described in 2.4.

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. Electrophoresis was carried out

according to the standard Laemmli method [12]. After SDS-PAGE, AP fractions obtained before Pepstatin Sepharose chromatography were visualized by staining with Coomassie brilliant blue 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 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 3.0. The gel was incubated with 1% haemoglobin at pH 3.0 for 15 min. Subsequently it was washed gently with formic acid buffer (pH 3.0) and incubated for 1 h at 37°C in a humid chamber. At the end 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 the “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 the “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 in 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 in one AP fraction each with an apparent 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 746.7 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 with 1 μM Pepstatin A. There was no inhibition of AP activity when AP fractions were pre incubated with 1 mM Phenylmethylsulphonyl fluoride or 1 μM Soy bean trypsin inhibitor.

Bound AP fraction contained impurities according to the findings of the SDS-PAGE gel stained with Coomassie blue (Fig. 2A- Lane 5). 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. Protein bands observed had apparent molecular masses of 64, 54, 32 kDa and a 30 kDa (Fig. 2B).

PAGE (without SDS) was conducted under native conditions to visualize the AP activities present in fractions obtained before Pepstatin Sepharose step (Fig. 3). Arrows indicate the bands corresponding to different AP activities (Fig. 3). During zymography, haemoglobin bound to the gel was hydrolyzed at the positions where AP activity was found. As a result APs were observed as clear areas against a bluish black background after staining with Amido black. At least three different clear areas of AP activity were detected in PAGE when all the lanes were compared (Fig. 3). This finding suggests the presence of three APs or three isozymes or products of post translational modification of a single protein (Fig. 3).

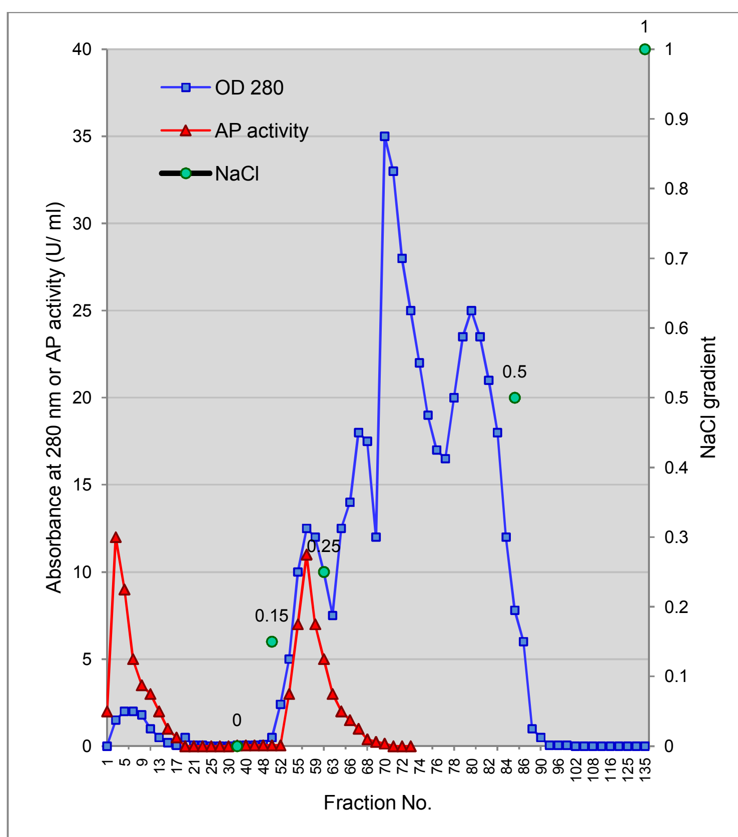

Fig. 1. Proteins recovered during DEAE-52 cellulose chromatography

DEAE-52 cellulose chromatography was conducted at pH 8.5. Fractions 1 to 34 represent column washing. Elution of bound proteins was carried out with 0-1 M NaCl gradient starting from fraction 35 onwards. Absorbance at 280 nm and the AP activity was measured in fractions. Most of bound proteins were eluted between 0.16 and 0.51 M NaCl

Table 1. Specific activities 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

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. Latter two chromatography steps were conducted separately with bound and unbound AP fractions

3.2 Discussion

Previously we have optimized the assay conditions to detect AP activity in porcine ovarian extracts. AP activity was measured at pH 3.0 as it was the optimum pH for bovine haemoglobin [9]. These APs did not have a significant activity below pH 2.0 and above pH 5.0. Several mechanisms contributing to an acidic microenvironment were identified in tissues including the 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.

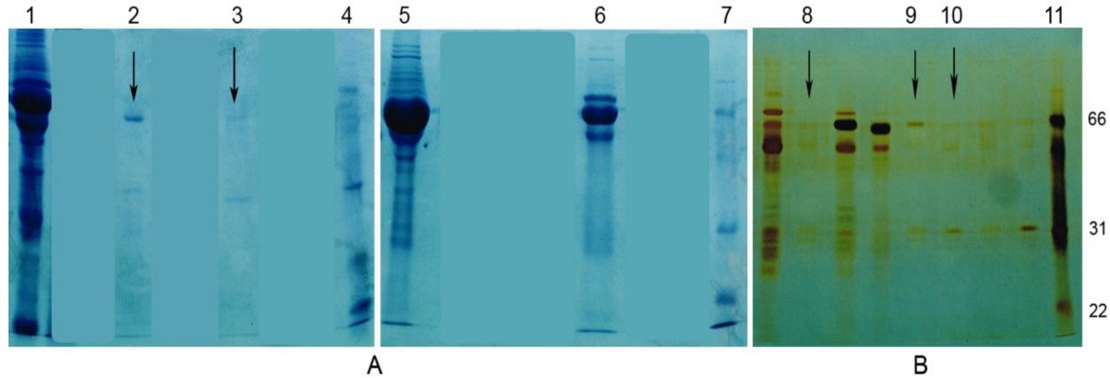

Fig. 2. Detection of proteins present in ovarian fractions

Panel A: With commassie 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, 5: DEAE bound AP fraction, 6: Bound AP fraction after sephacryl S-200 chromatography, 8: Bound AP after pepstatin sepharose chromatography, 9: Unbound AP fraction after sephacryl S-200 chromatography, 10: Unbound AP fraction after pepstatin sepharose chromatography, 4,7,11: Molecular weight markers (66, 31 and 22 KDa)

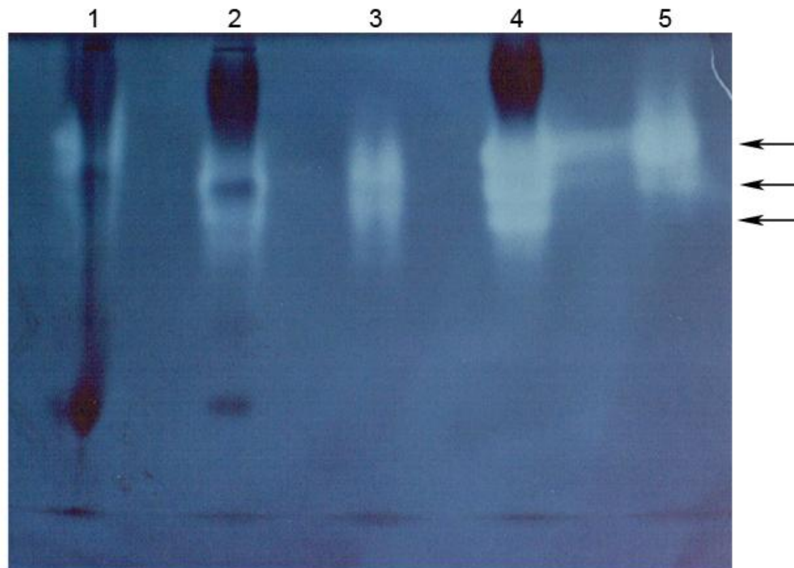

Fig. 3. Detection of ovarian AP activities with zymography

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

Based on the recovery of AP during DEAE-52 cellulose chromatography at pH 8.5, there should be at least 2 AP activities in the ovarian extract. Bound AP fraction seems to bear a net negative charge at pH 8.5, which was eluted around 0.23 M NaCl. The 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. Mature form of the well known aspartic proteinase cathepsin D is formed after several proteolytic processing steps. Procathepsin D originates after the first proteolytic cleavage of pre-pro-enzyme is a 52 kDa protein. 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 bands with approximate molecular masses of 64, 54, 32 and 30 kDa. Further studies are necessary to identify whether those bands correspond with cathepsin D.

Clear areas observed with zymography correspond with AP bands with different net charges. According to the findings, there should be at least three different APs or isoforms or products of post translational modifications in the porcine ovary.

Further studies are required to identify the role of these APs. Previously it has been observed that AP activity increases when the ovaries show cycling features (presence of corpora lutea) [9]. In the same study it was found that, early phase of corpus albicans had the highest AP activity, when different stages of follicles and corpora lutea were tested [9]. These findings indicate a likely function of AP during the luteal phase. However, in the current study the ovarian extract was prepared from the whole ovary which includes AP coming from all the structures of the 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 was demonstrated in bovine CL [22]. Erdmann et al. [23] 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. Johansson et al. [24] identified the role of cathepsin D in apoptosis. Together, these studies suggest that there is an AP-prolactin mediated mechanism governing the luteal regression even though our study does not provide evidence to support. Whether the APs identified are cathepsin D isoforms or different enzymes, need to be investigated. Further studies are required to identify the role of AP in the porcine ovary.

4. CONCLUSION

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

ETHICAL APPROVAL

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

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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