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Source: Zoological Science, 12(1) : 87-90

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.87

ZOOLOGICAL SCIENCE 12: 87–90 (1995)

Occurrence of a Latent Serine Protease in the Follicular Fluid of Porcine Ovary

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ABSTRACT—Porcine ovary folliclular fluid contains a latent form of a protease which is activatable with trypsin. The active enzyme hydrolyzed peptide 4-methylcoumaryl-7-amide (MCA) substrates with a preference for the Arg-MCA bond. The enzyme was strongly inhibited by diisopropylfluorophosphate, aprotinin, leupeptin and antipain, but not by soybean trypsin inhibitor. The apparent molecular weight of the enzyme was approximately 630,000 as estimated by gel filtration. No significant difference in molecular size was seen between the inactive precursor and trypsin-activated enzyme. The results suggest that the present enzyme is a novel type of serine protease.

INTRODUCTION

Mammalian ovaries contain within their cortex numerous follicles of widely ranging sizes representing various stages of their development. Each follicle accumulates a fluid in the intrafollicular cavity called the antrum. The fluid is composed of many protein species derived from either follicular tissues or plasma [8]. Among the proteins, proteolytic enzymes such as plasmin and collagenase are of particular interest in terms of the ovulatory process, because of their ability to decrease the tensile strength of the follicle wall and degrade the basement membrane [1, 2, 4, 5, 11].

Recently, we have discovered a new endopeptidase from the follicular fluid of porcine ovaries [7, 13]. Purification and characterization studies demonstrated that this enzyme, follipsin, belongs to the group of serine proteinases and preferentially hydrolyzes Arg-X bonds of the synthetic substrates. While conducting functional studies with follipsin, including searching for possible physiological substrates, we have found another serine protease present in the follicular fluid. Interestingly, no activity was shown in untreated follicular fluid, while exposure to trypsin resulted in marked elevation of its enzyme activity. The present paper shows that the enzyme is distinct from any other proteases so far reported from the follicular fluid of mammalian ovaries.

MATERIALS AND METHODS

Materials

Trypsin, α-chymotrypsin, endoproteinase Lys-C, plasmin, diisopropylfluorophosphate (DFP), and soybean trypsin inhibitor (SBTI) were purchased from Sigma (St. Louis, MO). Endoproteinase Arg-C and antipain were obtained from Boehringer Mannheim (FRG). Aprotinin, leupeptin and synthetic peptide substrates containing 4-methylcoumaryl-7-amide (MCA) were from Peptide Institute (Osaka, Japan). All other chemicals were reagent grade.

Follicular fluid preparation

Porcine ovaries were obtained from a local slaughterhouse. Follicular fluids from follicles at various stages were prepared as described previously [13].

Enzyme assay

Activities were assayed at 37°C in 0.1 M Tris-HCl (pH 8.0) containing 0.1 mM substrate. After a 15-min incubation, the reaction was terminated by the addition of 2.5 ml of 30 mM sodium acetate (pH 4.3) containing 100 mM monochloroacetic acid. The release of fluorophore, 7-amino-4-methylcoumarin (AMC), was measured by spectrofluorometry using an excitation wavelength of 370 nm and an emission wavelength of 460 nm.

Protein assay

Protein was determined either by the method of Smith *et al.* [12] using BCA reagent (Pierce, Rockford, IL) or from UV-absorbance assuming OD (1%/280 nm)=10.

Gel filtration experiment

Porcine ovary follicular fluid (10 μl, approximately 600 μg protein) was mixed with 90 μl of 0.1 M Tris-HCl (pH 8.0) containing 0.5 μg trypsin and the mixture was incubated at 37°C for 15 min. The reaction was terminated with 10 μg of SBTI. The supernatant of the mixture was fractionated on a Diol-300 column (Shimadzu, 7.9×500 mm) with 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl using a Gilson HPLC apparatus. The column was eluted at a flow rate of 1 ml/min and fractions of 0.5 ml were collected.

The follicular fluid (10 μl) mixed with 90 μl of 0.1 M Tris-HCl(pH 8.0) without trypsin was also directly applied to the column. The resulting fractions were assayed for enzyme activity before and after trypsin treatment. The conditions for trypsin treatment were the same as described above.

RESULTS

We have found that proteolytic enzyme activities of the follicular fluid of porcine ovary towards a synthetic, arginine-containing peptide amide substrate (Boc-Gln-Gly-Arg-MCA) are increased dramatically with trypsin treatment, in a dose-

Accepted November 21, 1994
Received August 26, 1994
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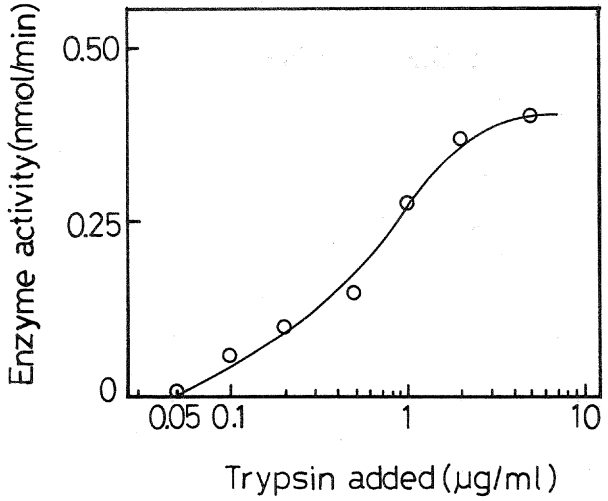

FIG. 1. Follicular fluid enzyme activation with trypsin. Follicular fluid was obtained from ovarian follicles with a diameter of 5–8 mm. Two microliters of the fluid was mixed with 98 μl of 0.1 M Tris-HCl (pH 8.0) containing various amounts of trypsin, and the mixtures were preincubated at 37°C for 15 min. The reactions were terminated with 10 μl of SBTI (1 mg/ml). Each mixture was then adjusted to a total volume of 0.5 ml with 0.1 M Tris-HCl (pH 8.0), and assayed for enzyme activity using Boc-Gln-Gly-Arg-MCA as substrate. The activities with 2 μl of the fluid are shown.

dependent manner (Fig. 1). The activation was accelerated with increasing amounts of added trypsin, reaching a plateau at a final trypsin concentration of 5 μg/ml. This activation was completely inhibited in the presence of SBTI, indicating that the activation occurs through the proteolytic action of trypsin. Effects of endoproteinase Arg-C, endoproteinase Lys-C, plasmin and α-chymotrypsin on the fluid enzyme activation were also examined, but these enzymes were without effect. Treatment of the fluid with sodium dodecyl sulfate was not effective for the activation either.

Experiments were conducted to gain insight into the mechanism by which trypsin activates Boc-Gln-Gly-Arg-MCA-hydrolyzing enzyme(s) present in the fluid. The follicular fluid, which had been treated with trypsin, was mixed with SBTI and was subjected to the gel filtration chromatography. As shown in Figure 2, a single active peak was eluted at a position corresponding to 630 kDa. In a separate experiment, the intact fluid was first fractionated on the gel filtration column, and the resulting fractions were treated with trypsin. The activated enzyme was located in a position with the same molecular weight. The results indicate that the activation is not accompanied by significant alteration of molecular weight.

Several synthetic MCA substrates containing Arg or Lys were tested with the activated enzyme (Table I). The enzyme hydrolyzed Boc-Gln-Arg-Arg-MCA and Boc-Gln-Gly-Arg-MCA effectively. Boc-Val-Pro-Arg-MCA was also hydrolyzed fairly well by the enzyme, but a dipeptide MCA substrate Z-Phe-Arg-MCA was not. Boc-Glu-Lys-Lys-MCA was found to be a substrate for the enzyme, although

TABLE 1. Substrate specificity of the 630 kDa protease toward various MCA substrates

Substrate	630 kDa protease (%)	plasmin[a] (%)	follipsin[b] (%)
Boc-Gln-Arg-Arg-MCA	100.0	100.0	100.0
Boc-Gln-Gly-Arg-MCA	116.0	9.8	98.1
Boc-Val-Pro-Arg-MCA	44.0	65.9	19.6
Boc-Glu-Lys-Lys-MCA	25.6	91.2	2.5
Z-Phe-Arg-MCA	4.0	—	101.0

a, cited from [13]; b, cited from [7]
The follicular fluid enzyme was partially purified by gel filtration column chromatography as described in MATERIALS AND METHODS. The relative activity values are shown. The substrate specificities of porcine plasmin and follipsin are also included for comparison.

the activity was one-fourth of that seen using Boc-Gln-Arg-Arg-MCA. The substrate specificity is apparently different from those of plasmin and follipsin. The enzyme showed no activity toward Boc-Leu-Leu-Val-Tyr-MCA, a substrate for chymotrypsin-like endoproteases.

DFP (10 mM), aprotinin (0.1 mg/ml), leupeptin (0.1 mM) and antipain (0.1 mM) strongly inhibited the 630 kDa enzyme activity, indicating that the enzyme is a trypsin-like

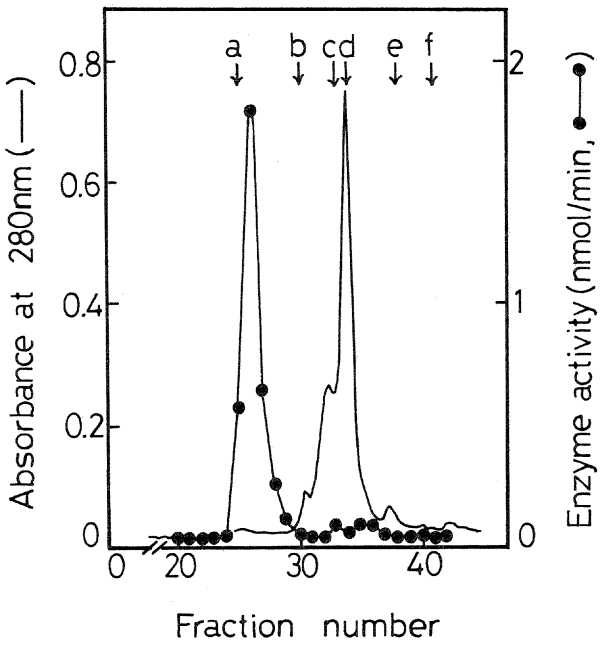

FIG. 2. Gel filtration of the follicular fluid treated with trypsin. Ten microliters of the fluid was incubated at 37°C for 15 min with 0.5 μg of trypsin in a volume of 100 μl, as described in MATERIALS AND METHODS. The reaction was terminated with 10 μg of SBTI, and centrifuged at 4,000 rpm for 3 min. The supernatant was applied to a gel filtration column. Fractions were assayed for enzyme activity using Boc-Gln-Gly-Arg-MCA, and the activities per fraction are shown. Arrows with a figure indicate the elution positions of molecular weight markers. a, thyroglobulin (669 kDa); b, apoferritin (440 kDa); c, aldolase (158 kDa); d, bovine serum albumin (67 kDa); e, ovalbumin (45 kDa) and f, lysozyme (14 kDa).

serine protease. However, the enzyme was not inhibited by SBTI at all.

The activities of the trypsin-treated fluid derived from various sizes of follicles were assayed toward Boc-Gln-Gly-Arg-MCA in the presence of SBTI. This assay was judged to be suitable for determining exclusively the 630 kDa protease activity because the gel filtration of each aliquot of the treated fluids gave rise to a single activity peak as in Figure 2. As summarized in Figure 3, the enzyme is present in a latent form in all follicles. The activated level appears to be the same irrespective of their sizes.

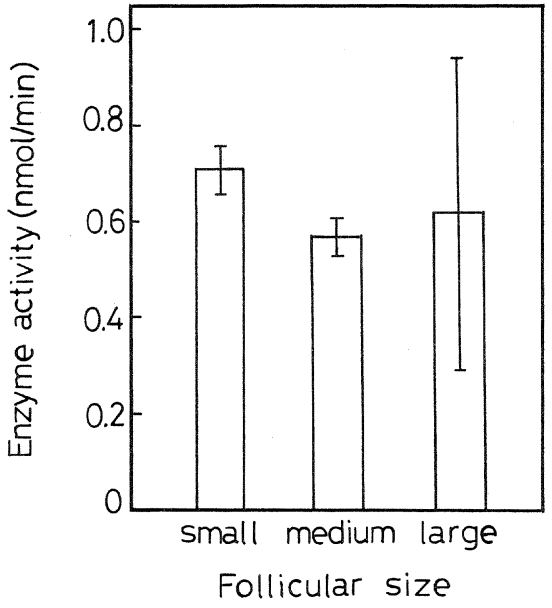

FIG. 3. Enzyme activity during follicular development. Follicular fluids were obtained from 5 each of small (diameter<3 mm), medium (3~6 mm), and large (>6 mm) follicles of porcine ovaries. Two microliters each of the fluids was separately treated with 500 ng of trypsin as in Figure 2. Enzyme activities of the treated fluids were assayed toward Boc-Gln-Gly-Arg-MCA in the presence of SBTI. The activities with 2 μl fluid are shown (the mean ± SD, 5 assays).

DISCUSSION

We observed that trypsin treatment of the follicular fluid of porcine ovaries resulted in a dramatic increase in the enzyme activity toward the synthetic protease substrate Boc-Gln-Arg-Arg-MCA. The enzyme responsible for the activity was demonstrated to be a serine protease based on its sensitivity to DFP. Thus, it is interesting to compare its properties with those of other serine proteinases such as plasmin [1,2] and follipsin [7], both of which are known to occur in the fluid.

Several lines of evidence serve to differentiate the present enzyme from plasmin and follipsin. First, the molecular weight of the enzyme is approximately 630,000 as estimated by gel filtration. This value is much greater than those for plasmin (80,000) and follipsin (80,000). Second, the 630 kDa proteinase shows a substrate specificity distinct from those of the above two enzymes. For example, Boc-Gln-Gly-Arg-MCA, a poor substrate for plasmin, is well hydrolyzed by the present enzyme. On the other hand, the dipeptide MCA substrate Z-Phe-Arg-MCA is hydrolyzed poorly by the present enzyme, but is a good substrate for follipsin. Third, the behavior of the enzymes toward SBTI is different. Plasmin and follipsin are completely inhibited by the inhibitor, but the 630 kDa protease is not affected at all even at a higher inhibitor concentration.

Several authors also have found kallikrein-like [6], trypsin-like [3] and tissue-type plasminogen activator [9] activities in the follicular fluid of mammalian ovaries. Although the kallikrein-like activity was similarly insensitive to SBTI as in the 630 kDa protease, the activity was easily detectable without any treatment for activation. On the other hand, the latter two enzyme activities were reported to be resistant to aprotinin, in contrast to the aprotinin-sensitive nature of the current enzyme. The apparent molecular weight of the enzyme is close to those reported for the multicatalytic protease complexes, proteasomes [10, 14]. However, the lack of chymotrypsin-like protease activity of the enzyme and its occurrence in the extracellular spaces (follicular fluid) are not consistent with the established criteria for proteasomes. Indeed, treatment of the crude follicular fluid with SDS, a known activator of proteosomes, did not enhance the trypsin-like and chymotrypsin-like activities, suggesting that intrinsic proteasomes are not present in the fluid of porcine ovaries. From these considerations, we tentatively suggest that the 630 kDa protease is a novel type of serine endopeptidase distinct from any other proteases hitherto described.

Since the enzyme is present in the follicular fluid in a latent state, it needs to be activated by a protease(s) with a cleavage specificity similar to trypsin. A search for a physiological activator is in progress. In addition, further studies on this 630 kDa protease including the complete purification and characterization, and identification of *in vivo* substrate(s) are necessary to clarify its functional role.

ACKNOWLEDGMENTS

We would like to thank Dr. Ronald T. MacFarland for critical reading of this manuscript. This study was supported in part by a research grant from Takeda Science Foundation.

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