Partial Purification and Some Properties of a Neutral Proteinase in Rat Ovary

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ABSTRACT. The ovary of rat possessed a neutral proteinase which had an optimum pH at around 8.5 in the presence of 0.5 M NaCl. The proteinase was soluble only in media of high ionic strength such as 2 M NaCl. In order to prevent the reprecipitation in media of low ionic strength of the enzyme solubilized, it is necessary to add protamine sulfate to the solubilizing medium. When the solubilized enzyme was applied to a Sephadex column, the proteinase activity was eluted at the same position as bovine α -chymotrypsinogen. The results using some protease inhibitors showed that the proteinase was a chymotrypsin-like serine enzyme. When rats were treated with compound 48/80, the proteinase activity almost completely disappeared, suggesting that the enzyme is of mast cell origin.—**KEY WORDS:** neutral proteinase, partial purification, rat ovary.

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Although the role of proteolytic enzymes in the functional control of mammalian ovaries has been of great interest, the proteolytic system in the tissue remained to be clarified. Two specific enzymes active at neutral pH have been described. One is plasminogen activator [4, 14, 16, 20, 22, 27], and the other “collagenolytic” enzymes [6, 7]. Although a plasminogen/plasmin system is now believed to be a factor which induces the rupture of the Graafian follicle, several non-specific neutral proteinases have also been described [3, 17, 20, 25]. The possibility of the participation of the non-specific proteinases in the function of mammalian ovaries may be discussed with reference to the following points: i) the injection of small quantities of a high concentration of proteolytic enzymes such as nagase and pronase directly into the antrum of the rabbit Graafian follicle can cause morphological changes similar to normal swelling, stigma formation and rupture of the Graafian follicle [8], ii) some proteolytic enzymes such as trypsin and α -chymotrypsin can reduce the tensile strength of the follicle wall [5], and iii) rat ovarian membrane preparation stimulates adenylate cyclase activity [18] and serine protease inhibitors decreased stimulation by human chorionic gonadotropin of ovarian adenylate cyclase [19]. However, as hardly any of the non-specific proteinases found in rat ovary are purified, their physiological significance is still equivocal.

Although we reported proteinase activity at pH around 8.5 in the homogenized precipitate prepared from the ovary of immature rats pretreated with gonadotropins [25], details of the properties of the proteinase are not yet known. Since we recently found a proteinase similar to the above one also in adult rat ovary, we partially purified the enzyme from ovaries of adult rats and examined its properties. In this paper, we will describe these results.

Protease inhibitors from actinomycete and E-64 were obtained from Peptide Institute Inc. Other protease inhibitors and compound 48/80 were from Sigma Chemical Co., casein was from Merck, and Sephadex G-75 was from Pharmacia. Normal female adult rats of the Sprague-Dawley strain were used. They were kept in a room with 12–12 hr light-dark cycle and killed by decapitation without examining for the sexual cycle, and the ovaries were removed. All steps for purifying the proteinase were carried out at 4°C and all centrifugations done at 10,000

rpm for 20 min. About 5 g of ovaries obtained from about 60 rats were homogenized twice with 0.1% (v/v) Triton X-100 in an ice bath, and each homogenate was then centrifuged. The precipitate was washed twice in water, and at each washing the precipitate was recovered by centrifugation. The final precipitate was suspended in water (crude enzyme source). The proteinase in the crude enzyme source was extracted in 2 M NaCl containing 0.2% (w/v) protamine sulfate with gentle stirring overnight. The extract was centrifuged, and to the supernate was added solid $(\text{NH}_4)_2\text{SO}_4$ to give 70% saturation. The mixture was centrifuged after being gently stirred for 1 hr. The precipitate was suspended in a small volume of 10 mM potassium phosphate buffer (pH 7.5; buffer A), and the suspension was dialyzed against buffer A. After insoluble materials were centrifuged off, the supernate was applied to a column of Sephadex G-75 (2×75 cm) equilibrated with buffer A containing 2 M NaCl. The column was eluted with the same buffer at a flow rate of 20 ml/hr, and the eluate was collected in a 5 ml fraction. Fractions containing the proteinase activity were combined to use as a partially purified enzyme source.

In one experiment (Table 3), rats were injected intraperitoneally with compound 48/80 dissolved in 0.85% NaCl by the method in [15] with a slight modification. Briefly, rats were injected with the drug at a dose of 100 μg per 100 g of body weight. After 2 days, they were re-injected daily for 5 days according to the following plan: One hundred μg per 100 g of body weight 2 days after the first injection, 200 on day 2, 300 on day 3, 400 on day 4, and 500 on day 5. They were killed on day 6 by decapitation. This modified injection schedule caused more marked disappearance of the present proteinase activity than the schedule described in [15].

Proteinase activity was assayed in 1.0 ml of incubation mixture containing 50 mM borate/HCl buffer (pH 8.5), 5 mg of casein, 0.5 M NaCl and enzyme. After incubation at 45°C for 2 hr, an equal volume of 0.4 M trichloroacetic acid was added. The mixture was allowed to stand for 30 min at room temperature, followed by filtration. The acid-soluble product was determined by the Lowry procedure [13], except that an alkaline copper reagent containing 0.15 N NaOH instead of 0.1 N was used. Blanks without incubation were always run in parallel. One unit

was defined as the activity which released a product equivalent to 1 mg of bovine serum albumin in the filtrate. If necessary, the units were expressed as units/mg of protein (specific activity) and milliunits/mg of the ovary.

Protein was assayed by our modification [9] of the Lowry procedure [13] with bovine serum albumin as a standard. Protein in the eluate in gel filtration was measured by the ultraviolet absorption method at 280 nm.

Although data are not shown, like the previous data obtained from rats of Wistar strain [25], the neutral proteinase activity in the crude enzyme source was insoluble in water and solubilized with 2 M NaCl. Moreover, the proteinase activity was greatly enhanced with increasing concentrations of NaCl in the incubation medium, reaching maximum activity at 0.5 M or above. The optimum pH of the caseinolytic activity was around 8.5 in the presence of 0.5 M NaCl. When the solubilized enzyme was dialyzed against buffer A, the enzyme was easily reprecipitated. However, the enzyme solubilized in 2 M NaCl containing protamine sulfate held the solubility also in buffer A. When the results showing that the proteinase activity in the crude enzyme source was activated by NaCl (see above), but that the activity of the partially purified enzyme was only slightly activated by

NaCl (see below) are considered, the activating effect of NaCl on the proteinase activity found in the crude enzyme source is due probably to the efficiency of the salt to dissociate the enzyme from an insoluble complex consisting of the enzyme and an acidic inhibitory substance(s) such as DNA [11], nucleotide-related compounds [23] or heparin [26]. Although data are not shown, a pretreatment with DNase failed to solubilize the proteinase activity in the crude enzyme source, showing that DNA seems not to be such an acidic compound as mentioned above in this case.

When the solubilized enzyme was applied to a column of Sephadex G-75, the activity was eluted as a single and symmetrical peak (Fig. 1). When bovine α -chymotrypsinogen was separately eluted under the same conditions, the standard protein was eluted in the same position as the present enzyme activity, showing that the molecular weight of the present proteinase is about 25,000. In the final step of preparation, the proteinase activity was recovered in yields of about 40% with an increase in specific activity of about 50-fold (Table 1). Although data are not shown, increasing concentrations of NaCl in the incubation mixture only slightly affected the activity of partially purified proteinase. Moreover, the optimum pH for the caseinolytic activity was at around 8.5 in the presence of 0.5 M NaCl and the highest activity was obtained at around 55°C.

Table 2 shows the effects of some modifiers on the activity of partially purified proteinase. The enzyme activity was almost completely inhibited by limabean trypsin inhibitor, chymostatin, and phenylmethylsulfonyl fluoride. High concentration of soybean trypsin inhibitor, tosyl-L-phenylalanine chloromethylketone, and rat serum also strongly inhibited the activity. Other modifiers used were inert to the proteinase activity under the present assay conditions. These results show that the present proteinase is a serine enzyme which has a chymotrypsin-like nature.

Table 3 shows the effects of *in vivo* injection of compound 48/80 on the proteinase activity in the crude enzyme source. With this treatment, the amount of protein in the ovaries was little affected, but the proteinase activity almost completely disappeared ($P < 0.001$), suggesting that the enzyme is of mast cell origin.

There have been some reports on proteinase activities at the physiological pH level in mammalian ovaries.

Fig. 1. A typical elution pattern of the proteinase activity from Sephadex G-75 column (2×75 cm) equilibrated with 10 mM potassium phosphate buffer (pH7.5). The column was eluted and the eluate was collected by the method described in the text. Absorbance at 280 nm (○) and proteinase activity (milliunits/ml of the eluate; ●) are shown in the figure.

Table 1. Purification of proteinase activity

Fraction ^{a)}	Total protein (mg)	Total activity (Units)	Recovery (%)	Specific activity (Units/mg of ovary)	Purification (-fold)
Crude enzyme source	283.50	163.50	100.0	0.58	1.00
NaCl extraction	87.54	139.79	85.5	1.60	2.76
Dialysis	20.03	63.96	39.1	3.19	5.50
Gel filtration	2.16	61.11	37.4	28.29	48.78

a) About 5 g of ovaries were used as starting material, and each fraction was obtained by the method described in the text.

Table 2. Effects of some modifiers on partially purified proteinase activity

Modifier	Concentration	Relative activity (%)
None	—	100.0
Phenylmethylsulfonyl fluoride	1.0 ^{mM}	0
Tosyl-L-phenylalanine chloromethylketone	1.0	24.2
Tosyl-L-lysine chloromethylketone	1.0	97.0
p-Chloromercuribenzoate	1.0	96.3
Antipain	10 ^{μg}	109.5
Chymostatin	10	14.7
Elastatinal	10	107.9
E-64	10	98.6
Leupeptin	10	104.7
Limabeen trypsin inhibitor	10	0
Pepstatin	10	101.1
Soybean trypsin inhibitor	100	33.0
Rat serum	1:2000 dil.	37.8

Table 3. Effects of *in vivo* injection with compound 48/80 on the proteinase activity of rat ovary

	Protein	Neutral proteinase ^{a)}	
	mg/g of ovary	mU/mg of ovary	U/mg of protein
Control	31.99 ± 1.97	40.93 ± 1.59	1.29 ± 0.04
Sample	31.09 ± 4.78	1.65 ± 0.60 ^{***b)}	0.06 ± 0.02 ^{***}

a) Proteinase activity was assayed by using a crude enzyme source.

b) Data are expressed as the mean ± S.E. (n=3 each). ***: $p \leq 0.001$ between control and sample analyzed by Student's *t*-test.

Reichert [17] reported an alkaline protease in rat ovaries. Although he described strong inhibition by p-hydroxymercuribenzoate of the enzyme activity, the present enzyme was not inhibited at all by such a modifier (Table 2). Espey and Landall [6], Espey and Coons [7] reported "collagenolytic" enzymes in the sow and/or rabbit ovarian follicles. These enzymes specifically hydrolyzed the glycyl-glycyl bond existing in a synthetic substrate or gels composed of reconstituted collagen. Another specific proteinase, plasminogen activator, has also been reported in rat ovaries and rat granulosa cells [4, 14, 16, 20, 22, 27]. In general, tissue plasminogen activator which activates plasminogen to plasmin has little general proteolytic activity, and has molecular weights of 42,000–75,000 [1]. Thus the present enzyme is different from the two specific enzymes mentioned above. Roche and Ryan [20] reported neutral caseinolytic activities in the luteinized ovary of rat on electrophoretic analysis. These proteinases have molecular weights of 26,000, 28,000 and 30,000. Although these molecular weights are similar to that of the present proteinase, the proteinases reported by Roche and Ryan are not inhibited at all by a chymotrypsin inhibitor, tosyl-L-phenylalanine chloromethylketone. As the present enzyme is strongly inhibited by this inhibitor (Table 2), it seems to be different from them also. Bicsak *et al.* [3] reported a trypsin-like protease produced by rat granulosa cells, and Takahashi *et al.* [24] reported a plasma kallikrein-like serine protease in

the follicular fluid of swine ovary. As the present proteinase is a chymotrypsin-like enzyme, it differs clearly from both these enzymes.

When the effect of a histamine releaser, compound 48/80, on the proteinase activity was tested, the enzyme activity almost completely disappeared (Table 3), suggesting that the present proteinase is of mast cell origin. These enzymatic properties (the insolubility in media at low ionic strength, the solubility in high concentrated salt media, the molecular weight of 25,000, the chymotrypsin-like nature, and the mast cell origin) suggest that the present proteinase is chymase I. On the participation of mast cells in ovulation, Szego and Gitin reported that histamine may play a role in the acute hyperemic response to luteinizing hormone in immature rats [21]. On the other hand, Lipner reported that histamine may play a role in the acute increase in ovarian weight of gonadotropin-treated immature rats [12]. In the same paper, he also reported that the administration of compound 48/80 cannot inhibit ovulation, but that the number of ova released was largely decreased with the inhibition of an increase in the ovarian histamine concentration [12]. As chymase is involved in the process of degranulation of mast cells [10], the present chymase-like proteinase may participate in ovarian function through the function of histamine.

As the physiological significance of the present proteinase is yet unknown, further purification and characterization of the proteinase and the effect of *in vivo* injection

of gonadotropins on the enzyme activity in the immature female rats are under way in our laboratory.

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