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# A MICROASSAY FOR THE DIRECT DEMONSTRATION OF COLLAGENOLYTIC ACTIVITY IN GRAAFIAN FOLLICLES OF THE RAT

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## Summary

1. A microassay for tissue collagenase (EC 3.4.24.3) activity has been developed based upon the method of Ryan and Woessner (Biochem. Biophys. Res. Commun. (1971) 44, 144—149). The insoluble fraction of homogenized tissue is incubated in buffer at 37°C and collagenase activity is estimated by the release of soluble hydroxyproline-containing fragments. The assay requires only 0.15—2.5 mg of tissue. To validate the microassay, rat uterine collagenase has been measured and the activity shown to be linear with time of incubation and equivalent to that detected by the original assay. The digestion of follicular collagen by partially purified rat uterus collagenase is shown to be linear with respect to enzyme concentration using the microassay.

2. Collagenolytic activity is detected in homogenates of Graafian follicles of immature rats stimulated with pregnant mare's serum gonadotropin.

3. The follicular enzyme has the properties of a true collagenase. It is localized in the insoluble tissue fraction, it attacks collagen under physiological conditions of pH and temperature and it is inhibited by EDTA and activated by $Ca^{2+}$. Extracts of the follicles cleave the synthetic peptide substrate for mammalian collagenase, dinitrophenol-Pro-Leu-Gly-Ile-Ala-Gly-D-Arg at the appropriate glycine-isoleucine bond; hydrolysis of this peptide is blocked by EDTA. The follicular collagenolytic enzyme exists in a latent form which can be revealed by enzymic (trypsin) or chemical (aminophenylmercuric acetate) treatment.

## Introduction

The Graafian follicle is a vesicular structure supported within the ovary by a framework of collagen. During the hours preceding ovulation this collagenous tissue weakens [1,2] and electron microscopy shows an apparent dissolution of

the collagen bundles into thinner fibrils [3,4]. In the apical region about to undergo rupture there is a decrease in the total number of fibrils per unit area of follicular wall [4]. Coons and Espey [5] cultured strips of rabbit follicles and after 40 h of culture they were able to show an enzyme diffusing out of the tissue which lysed collagen gels. This enzyme acted at neutral pH and was inhibited by EDTA and serum. The authors concluded that although the enzyme resembled collagenase, further proof of its identity is needed. The present studies were undertaken to quantitate directly the amount of enzyme present in the follicle in vivo and to extend the characterization of the follicular enzyme by comparison of its properties to those of the known collagenases (EC 3.4.24.3). For this purpose we developed a microassay capable of measuring collagenolytic activity in 1—2 follicles of the rat.

## Methods

*Tissue sources.* Immature Sprague-Dawley female rats were obtained from Charles River Breeding Laboratories at 22 days of age. They were kept in air-conditioned quarters, with light from 0500 h to 1900 h. Rat chow and water were provided at libitum. On day 28 of life, at 0600 h, rats were injected subcutaneously with 20 I.U. of pregnant mare's serum gonadotropin in 0.4 ml 0.154 M NaCl. For the preparation of uterine collagenase, pregnant rats were obtained several days before parturition from Sprague-Dawley, Madison, Wisc. [6].

*Tissue collection.* Immature rats were injected with pregnant mare's serum gonadotropin and then killed after 54 h by a blow to the head. Their ovaries were excised and placed in ice-cold assay buffer (0.05 M Tris · HCl buffer, pH 7.6/0.01 M $CaCl_2$/0.154 M NaCl/0.25% Triton X-100). Follicles were dissected on a chilled plate under a microscope; the largest, most vascularized follicles were selected from each ovary. Batches of 30 follicles (4 rats) were collected in 200 µl assay buffer and stored frozen (—20° C). Involuting uteri were collected from rats 1 day after parturition [6].

*Collagenase assays.* The assay is based on the principle that collagenase will digest endogenous collagen when homogenates are incubated at 37° C. Insoluble collagen is separated from solubilized digestion products by centrifugation and the two fractions are quantitated by their content of hydroxyproline.

(1) Tissue preparation and activation treatments: The method of Ryan and Woessner [6] was modified as follows: Batches of 30 follicles were homogenized in 450 µl assay buffer (4° C) using a hand-driven glass homogenizer (Duall No. 20, Kontex Glass Co., Vineland, N.J.). Follicular pellets were prepared by centrifugation at 6000 × *g* for 20 min at 4° C and resuspended in the original volume of Tris buffer. For activation of the follicular collagenase, trypsin (0.5 mg/ml, Worthington Biochemicals, Freehold, N.J.) was added to aliquots of the homogenate (3 µl per 100 µl homogenate). The samples were incubated at 37° C for 3 min, then cooled in an ice bath. 3 µl soybean trypsin inhibitor (Worthington Biochemicals, Type I S, 1.8 mg/ml) was added to block trypsin. Alternatively, for activation of collagenase, 10 µl 10 mM aminophenylmercuric acetate was added per 100 µl follicular homogenate or pellet suspension (4° C); this activator was present throughout the incubation described

in the next section. Rat uterine pellets were prepared for collagenase assay as described previously [7]; 25-μl aliquots were assayed without activation as described below.

(2) Incubations: Blanks were prepared by adding EDTA to a final concentration of 0.02 M. Each sample and blank were homogenized again (5 passes) immediately before transfer to the capillary tubes to ensure accurate distribution of the sample; Kimax capillary tubes (1.6—1.8 × 100 mm) calibrated with a mark at 25 μl were used. The 25-μl aliquots used for each incubation corresponded to 1—2 Graafian follicles (each weighing approx. 100 μg and containing 400—450 ng hydroxyproline) or 2.5 mg wet weight of uterine tissue. The capillaries were sealed and placed in a screw-capped tube filled with water and immersed in a 37° C water bath.

Following overnight (18 h) incubation, the samples were centrifuged in an International microcapillary centrifuge (Model MB; 4 min, room temperature). The supernatant was transferred to a 1 ml Microflex tube (Kontex Glass Co.) with a 50 μl Hamilton syringe. The pellet was resuspended in 20 μl distilled water, dispersed with the needle of the syringe and transferred into a separate Microflex tube. The syringe and capillary tube were rinsed three times with 10 μl distilled water and the washings added to the pellet. Concentrated HCl was added to each sample (final concentration 6 M). The tubes were sealed with a Teflon-lined screw cap and hydrolyzed overnight at 110° C.

(3) Hydroxyproline assays: The hydrolyzed samples were dried on a steam bath in a gentle stream of air. The dried samples were resuspended in 50 μl pH 6.0 buffer (0.5 g citric acid/1.2 g sodium acetate/0.34 g NaOH/120 μl glacial acetic acid in a total volume of 10 ml) and 2 μl 0.02% methyl red indicator added. If necessary, the pH of the suspended samples was adjusted to approx. 6.0 with 0.25 M NaOH. The samples were then made up to a final volume of 80 μl with buffer.

Hydroxyproline was measured by colorimetry [8]. The assay was scaled down by using 1/25 of the volume of each reagent added directly to the hydrolysis tubes. After color development, the samples were extracted twice with 300 μl benzene to remove interfering substances. 160 μl of the colored reaction product was transfered to a microcuvette (Pyrocell, 300 μl capacity, 1 cm path length). Absorbance was measured at 557 nm in a Beckman DU spectrophotometer.

Activity is expressed as ng of hydroxyproline solubilized per μg total (pellet + supernatant) hydroxyproline in the overnight incubation at 37° C, after subtraction of the EDTA blank.

*Action of extracted follicular enzyme on synthetic collagenase substrate.* 75 follicles (obtained from rats 54 h after gonadotropin treatment) were homogenized in 900 μl assay buffer, centrifuged at 6000 × *g* for 20 min. Extraction of collagenase from the pellet followed the method of Weeks et al. [7]. The pellet was resuspended in 250 μl 0.05 M Tris buffer (pH 7.6)/0.1 M $CaCl_2$. The suspension was heated, with agitation, at 60° C for 4 min and then cooled in ice; it was centrifuged again and the supernatant used for assay with the synthetic collagenase substrate after activation with 2.5 μg trypsin (2.5 μl of a 1 mg/ml solution) and addition of 9 μg soybean trypsin inhibitor (2.5 μl of a 3.6 mg/ml solution). The peptide, dinitrophenol-Pro-Leu-Gly-Ile-Ala-Gly-D-Arg (Dnp-

peptide, Protein Research Foundation, Osaka, Japan) was dissolved in assay buffer to a final concentration of $4.5 \cdot 10^{-4}$ M. 100-µl aliquots were pipetted into screw-capped tubes, lyophilized and stored dry (4° C). For assay, 100 µl of the follicular extract were added to each of two tubes. To the blank tube, EDTA was added to a final concentration of 0.2 M. Incubation was carried out overnight at 37° C and the reaction stopped by addition of 0.2 ml 1 M HCl. The cleaved peptide fragment containing the dinitrophenol group was extracted in 1 ml ethyl acetate and quantitated by absorbance at 365 nm.

The ethyl acetate layer of the reaction mixture was then dried off at 60° C, the peptide fragment hydrolyzed under nitrogen in 6 M HCl overnight and dried again in the presence of NaOH pellets. Amino acid analysis was done on a Durrum D-500 automatic amino acid analyzer (courtesy of Dr. K. Brew, Department of Biochemistry).

## Results

*(1) Microassay*

The hydroxyproline assay was adapted to detect 25—600 ng of the amino acid in a starting volume of 80 µl (final volume of the reacton mixture after benzene extraction is 0.16 ml). The color production is linear over this range and gives an absorbancy at 557 nm of 1.2 for 600 ng hydroxyproline. The microassay for collagenase was shown to be linear with time when applied to rat uterine tissue (2.5 mg wet weight, 1 µg hydroxyproline). A straight line relationship was found to at least 20 h, at which time 20% of the collagen substrate was solubilized. A single pellet suspension is used to measure collagenase by both the original assay [6] and the microassay; equivalent levels of digestion were obtained in the two assays. Incubation of follicular extracts (150 µg wet weight, 600—700 ng hydroxyproline) also resulted in a linear release of collagen fragments with time. This amount of follicular tissue was the smallest which could be accurately measured in this microassay.

Before engaging in further studies of follicular enzyme, it was important to show that the microassay would respond quantitatively to different enzyme concentrations. Since it is impossible to vary the levels of bound enzyme (relative to substrate), collagenase had to be extracted from its substrate and different concentrations of the soluble enzyme added back to collagen substrate. Rat uterine collagenase was used for these studies since it was available in relatively large amounts. Follicular pellets containing collagen were prepared as described in Methods. The addition of varying amounts of uterine collagenase to follicular collagen resulted in a linear release of solubilized collagen up to at least 20% digestion of the substrate. Therefore, the endogenous follicular collagen is a suitable substrate for enzyme levels digesting up to 20% of the total collagen.

*(2) Properties of the follicular enzyme*

Collagenase activity could not be detected directly in homogenates of Graafian follicles (Table I). Brief exposure (3 min) to trypsin followed by addition of soybean trypsin inhibitor to block trypsin, revealed collagenolytic activity (Table I). Doubling the trypsin concentration had no further effect. Addi-

TABLE I

ACTIVATION OF THE FOLLICULAR ENZYME

Treatment with trypsin and aminophenylmercuric acetate was described under Methods. Each treatment used at least two different batches of follicles; the total number of determinations is shown.

| Tissue fraction | Treatment | Number of determinations | Activity (ng hydroxyproline solubilized/μg total hydroxyproline) ** |
|---|---|---|---|
| Whole homogenate | None | 5 | n.d. |
| | Trypsin * | 13 | 67.2 ± 6.7 } $P > 0.5$ *** |
| | Aminophenylmercuric acetate (1 mM) | 7 | 75 ± 12 } |
| Pellets | None | 5 | 32 ± 8 |
| | Aminophenylmercuric acetate (1 mM) | 4 | 65 ± 17 |

n.d., not detectable.
* 0.2 μg per follicle.
** Mean ± S.E.
*** Student's *t*-test

tion of soybean trypsin inhibitor to the follicular enzyme (activated by aminophenylmercuric acetate) did not diminish the collagenolytic activity.

Aminophenylmercuric acetate (1 mM) also unmasked latent collagenase (Table I). The difference in activity resulting from enzymic (trypsin) or chemical activation is not significant ($P > 0.5$), nor did the two reagents together give greater activity.

Approximately half of the total activity seen in activated whole homogenates is apparent in pellets without the necessity of activation (Table I). This may represent active enzyme which is obscured in whole homogenates by soluble inhibitors. The pellet activity is further enhanced by aminophenylmercuric acetate treatment indicating that most of the activity of the whole homogenates is recovered in the insoluble fraction. It is important to note that this insoluble fraction probably does not contain most proteins associated with membranous or sub-cellular fractions since the homogenates were prepared in 0.25% Triton X-100. The follicular enzyme may be bound to the insoluble collagen.

Table II shows the activation of the enzyme by $Ca^{2+}$. Addition of 10 mM $CaCl_2$ to the follicle pellets results in approx. 2-fold increase in enzyme activity, bringing it to the full activity level seen in Table I. The difference between the untreated and calcium-treated samples is significant ($P = 0.02$). Further increase in the $CaCl_2$ does not enhance the activity. Also shown in the table is the fact that 10 mM $CaCl_2$ added to the EDTA-treated enzyme restores part of the lost activity.

Rat uterus collagenase can be extracted from endogenous collagen by $CaCl_2$ in high concentration (0.1 M) at elevated temperature (60° C) [7]. By the same method, it was possible to extract an enzyme from follicular homogenates which hydrolyzed the synthetic collagenase substrate dinitrophenol-Pro-Leu-Gly-Ile-Ala-Gly-D-Arg of Masui et al. [9]. The amino acid sequence of this peptide is the one recognized by collagenase in the α1-chain of the collagen

TABLE II

EFFECT OF EDTA AND $CaCl_2$

Batches of 30 follicles were rinsed with 500 μl Tris buffer, pH 7.6, containing 0.9% NaCl, and then homogenized in this buffer. Aminophenylmercuric acetate was added to a final concentration of 1 mM. An aliquot was allowed to stand for 1 h (4°C) in the presence of 1 mM EDTA; a portion of this aliquot was assayed without further additions (Blank; this reading was subtracted from all measurements) and $CaCl_2$ (10 mM) was added to another fraction before incubation. The rest of the homogenate was assayed without addition or in the presence of varying concentrations of $CaCl_2$ as indicated. Two batches of follicles were tested; total number of determinations is shown.

| Addition | Number of determinations | Activity (ng hydroxyproline solubilized/μg total hydroxyproline) * |
|---|---|---|
| None | 6 | 34.8 ± 7.2 } $P = 0.02$ ** |
| 10 mM $CaCl_2$ | 7 | 75 ± 12 } |
| 20 mM $CaCl_2$ | 2 | 60 (50, 70) |
| 50 mM $CaCl_2$ | 2 | 65 (60, 70) |
| 1 mM EDTA, then 10 mM $CaCl_2$ | 6 | 56 ± 12.2 |

* Mean ± S.E.
** Student's *t*-test.

molecule and the collagenase-susceptible bond is glycine-isoleucine [9]. The follicular extract split more than 90% of the peptide substrate in an overnight incubation and this hydrolysis was blocked by EDTA. The peptide fragment released by enzymic action and soluble in ethyl acetate was shown by quantitative amino acid analysis to have the composition Pro (13 nmol), Leu (15 nmol) and Gly (13 nmol), confirming the point of cleavage at the Gly-Ile bond.

## Discussion

Our studies have shown for the first time that mature Graafian follicles produce a collagenolytic activity which has the properties of a true collagenase. In addition, we have developed a microassay which can be used to quantitate collagenase in minute tissue samples (0.15—2.5 mg).

All known mammalian collagenases are inhibited by EDTA and activated by $Ca^{2+}$. A number of these enzymes are irreversibly inhibited by EDTA, but the human corneal and rat skin collagenase are partially reactivated upon addition of excess $CaCl_2$ [10,11]. The follicular enzyme seems to resemble these latter collagenases.

Latent collagenases which can be activated by either enzymic or chemical treatment have been found in several tissues [12,13]; it is believed that they represent enzyme · inhibitor complexes. Trypsin activation may be the result of selective destruction or binding of the inhibitor, while chemical activation by aminophenylmercuric acetate may result from the disruption of the enzyme · inhibitor complex. Trypsin activation of latent enzyme forms is not unique to collagenases, but activation of an occult enzyme by aminophenylmercuric acetate has been reported only for true collagenase [12].

The peptide substrate, dinitrophenol-Pro-Leu-Gly-Ile-Ala-Gly-D-Arg was hydrolyzed by follicular homogenates with the specificity of collagenase, since the Gly-Ile bond was the only bond split. The Dnp-peptide should not be con-

fused with the peptide substrate (carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala) used by Espey and Rondell [16] to assay follicular extracts. This latter peptide is suitable for the assay of *Clostridium histolyticum* collagenase. However, it is not cleaved by animal collagenase [17] nor do the enzymes that cleave this peptide have any action on collagen [18].

In conclusion, we believe that the properties of the follicular enzyme identify it as a true collagenase: ability to hydrolyze collagen substrate; calcium activation; inhibition by EDTA; association with the insoluble tissue fraction, activation by trypsin or aminophenylmercuric acetate, and the specific hydrolysis of the Dnp-peptide.

The demonstration of the production of this collagenase in ovarian follicles in vivo supports the concept that collagenase is involved in the process of ovulation. The further substantiation of this concept awaits the quantitation of changes of this enzyme preceding ovulation.

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