MAMMALIAN COLLAGENASE: DIRECT DEMONSTRATION IN HOMOGENATES OF INVOLUTING RAT UTERUS*

Janet N. Ryan** and J. Frederick Woessner, Jr.

Departments of Biochemistry and Medicine and
Program in Cellular and Molecular Biology,
University of Miami School of Medicine,
Miami, Florida 33152

Received May 17, 1971

Summary: *Homogenates of involuting rat uterus contain an enzyme capable of digesting native collagen fibers at pH 7.5 and 37°. Collagenase activity and the uterine collagen are both found in the pellet when the homogenates are centrifuged at 6,000g. Incubation of this pellet at 37° in the presence of calcium leads to the release of soluble degradation products of collagen. It is suggested that the collagenase is firmly bound to its substrate collagen fibers, since it cannot be readily extracted from the pellet.*

Normal growth and remodeling processes and the pathological resorption of tissues require the rapid degradation and removal of collagen, a major structural component of animal tissues. Although native collagen is resistant to attack by most proteases, it is readily digested by specific collagenases. While bacterial collagenases have been well-known for almost half a century, animal collagenases, capable of degrading native collagen at physiological pH, were not known until 1962, when Gross and Lapiere (1) succeeded in demonstrating such an enzyme in the tadpole tail. The key to this demonstration lay in culturing the tissue, suspected of containing collagenase, on reconstituted collagen gels. After 3 to 4 days in culture,

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* This work was supported by United States Public Health Service grant HE 11035 from the National Heart and Lung Institute. Janet N. Ryan received fellowship support from United States Public Health Service training grants HE 05463 from the National Heart and Lung Institute and GM 00649 from the General Medical Sciences. We thank Robert J. Boucek for his helpful criticisms and Mrs. Carolyn Taplin for her excellent technical assistance.

** Present address: Department of Experimental Surgery, University of Arizona, Tucson, Arizona 85721.

sufficient collagenase diffused into the opaque gel to produce a clear zone of lysis around the tissue fragments. By the use of such techniques, collagenases have been demonstrated in a wide variety of tissues by many workers (see review by Eisen et al. [2]). In spite of this success, there are several drawbacks to this method: culturing the tissue is time-consuming and expensive and requires sterile techniques. Furthermore, the activity in the medium may reflect the ability of the tissue to synthesize collagenase under in vitro conditions rather than the actual enzyme activity in the original tissue. With the exception of the granulocyte cell (3) and bone cells (4), attempts to demonstrate collagenase activity directly in homogenates of mammalian tissue have been unsuccessful. This failure to detect the enzyme in tissues suspected of containing collagenase has been attributed to extreme lability of the enzyme, to rapid turnover with only trace amounts present at any time, or to the presence of tissue inhibitors. Recently, Eisen et al. (5) have shown that collagenase can be detected in extracts of skin after separation from inhibitor proteins on Sephadex G-150 columns.

We have succeeded in assaying collagenase directly in tissue homogenates by starting from the premise that the enzyme is not in the soluble fraction of the homogenate, but rather is bound to its substrate, the insoluble collagen fiber. This idea was suggested by the earlier observations of Gallop et al. (6) that bacterial collagenase bound extremely tightly to its fibrous substrate. We selected a tissue with a well-characterized collagenase (7) and an extremely rapid rate of collagen breakdown: the involuting rat uterus.

MATERIALS AND METHODS

The peak of collagen degradation in the involuting rat uterus is known to occur between 48 and 72 hr after parturition (8). Accordingly, uteri were removed from Sprague-Dawley rats 2 days after parturition and homogenized in cold 0.9% NaCl using Ten Broeck homogenizers with motor-driven pestles. The homogenates were centrifuged at 2° at a speed sufficient to bring down

even the small collagen fibrils (6,000g). The pellets were rinsed and resuspended either in buffer or in the 6,000g supernatant. The suspensions were incubated in a metabolic shaker for 20 hr at 37° in the presence of 0.01 M calcium acetate, 0.15 M NaCl, and 0.04 M Tris buffer, pH 7.5. Antibiotics (250 μg streptomycin and 200 units penicillin per ml) were included to prevent bacterial growth. The insoluble collagen of the pellets served as substrate; digestion was detected by the release of soluble hydroxyproline-containing material.

RESULTS AND DISCUSSION

Table I (A) shows that the uterine pellets, washed free of soluble material, released 16% of their total hydroxyproline. No activity could be detected in the supernatant fraction when it was tested on various types of fibrous collagen. This soluble fraction, in fact, consistently inhibits collagenase activity when added to the pellet. Presumably, it contains tissue or serum inhibitors (cf. 5). The enzyme is not released from the pellet by treatment with Triton X-100 nor by repeated washings with low concentrations of salt. It is presumed that the enzyme remains in the pellet because it is bound to collagen fibers.

It is not sufficient for the demonstration of collagenase activity merely to show the solubilization of hydroxyproline-containing material at neutral pH. It must also be shown that fragments smaller than an α-chain (molecular weight 100,000) are produced and that the substrate is native collagen, not susceptible to attack by nonspecific proteases such as trypsin. The first point was established by membrane experiments. Ninety per cent of the solubilized, hydroxyproline-containing material passed through a membrane which has an exclusion limit of 100,000 daltons (Diaflo XM-100, Amicon Corp., Lexington, Mass.); 30% of the material was able to pass through cellulose dialysis membranes. This material is smaller than α-chains since controls of heat-denatured soluble collagen from guinea pig skin passed through these two membranes to the extent of only 40% and 5% respectively.

TABLE I. Determination of Collagenase Activity in Uterine Homogenates

A. Localization of Collagenase

Fraction Incubated	Per Cent Collagen Digested
Entire Homogenate + Calcium	7
Pellet Resuspended in Buffer + Calcium	16
Pellet Resuspended in Buffer + EDTA	1
Pellet Resuspended in Supernatant + Calcium	9
Supernatant Incubated with Rat Tail Tendon	< 1

B. Ability of Collagenase to Attack Native Collagen

Enzyme Tested	Per Cent Collagen Digested
Tissue Collagenase	13
0.01% Trypsin	8
Sum of the Above	21
Collagenase + Trypsin Combined, 20 Hr	21
Collagenase, 20 Hr, Followed by	
Trypsin + EDTA (0.01 M), 24 Hr	22

The assay is described under Methods. Each digest contained approximately 800 μg hydroxyproline (5.6 mg collagen) in a volume of 5 ml. Blank corrections were based on similar tubes shaken at 4°; corrections did not exceed 1-2%. The digestion of collagen by 0.01% trypsin (200 units/mg, Worthington Biochemical Corp., Freehold, N.J.) was determined in the presence of 0.001 M EDTA; and digestion by both trypsin and the tissue collagenase, in the presence of 0.01 M calcium acetate but no EDTA. After incubation, the suspensions were chilled and centrifuged for 30 min at 30,000g; pellets and supernatants were separately hydrolyzed and assayed for hydroxyproline (9).

The second point was established by determining the amount of trypsin-susceptible denatured collagen that was present and comparing it to the amount of collagen digested by the tissue collagenase. No matter how carefully the uterine tissue is homogenized, significant amounts of trypsin-susceptible collagen are always present. However, collagenase releases significantly more hydroxyproline over a 20-hr period than does 0.01% trypsin; in nine experiments, the percentage of collagen digested by collagenase was 16 $\pm$ 3.5 (mean $\pm$ S.D.) and by trypsin was 10 $\pm$ 2.0 ($P < 0.005$). Higher concentrations of trypsin released no further hydroxyproline. The digestion of collagen by trypsin is almost complete within 4 hr, whereas the collagenase activity continues to release collagen products at a constant rate for 48 hr (Fig. 1) and degrades more than 25% of the tissue collagen in this period. The pellet contains no trypsin-like enzyme as indicated by the complete inhibition of activity by EDTA.

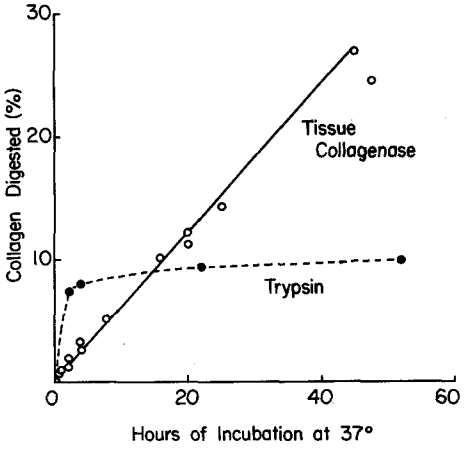

Fig. 1. Kinetics of collagenase action. Digestion of collagen by the tissue collagenase and by 0.01% trypsin. Conditions for the two incubations are as described in Table I.

It is concluded that collagenase attacks a fraction of collagen which is completely inaccessible to trypsin action. Moreover, it is possible that the collagenase and trypsin act on two separate fractions of the collagen since their effects are additive, whether the two enzymes act in concert or consecu-

tively; these data are presented in Table I (B). The enzyme activity of the pellet is further shown to be due to collagenase in that it agrees in all properties with the collagenase isolated from cultures of rat uterus by Jeffrey and Gross (7): it requires calcium, is inhibited by EDTA, is unaffected by cysteine (1 mM), has a pH optimum of 7.5, and causes a limited cleavage of collagen at 30°.

These findings indicate that any assessment of the amount of collagenase in a tissue should include not only the soluble forms (5) but also the fraction of enzyme found in the tissue pellet and presumably bound to its substrate.

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