A New Method for the Assay of Tissue Collagenase[1] (36297)

SEIZABURO SAKAMOTO, PAUL GOLDHABER, AND MELVIN J. GLIMCHER

Department of Oral Histopathology and Periodontology, Harvard School of Dental Medicine, and the Department of Orthopedic Surgery, Harvard Medical School, Children's Hospital Medical Center, Boston, Massachusetts 02115

Animal collagenases, which cleave undenatured collagen macromolecules in a specific fashion at near neutral pH, have been demonstrated in a wide variety of tissues (1–10). Because the primary action of such enzymes on the collagen macromolecule results in the liberation of two undenatured fragments of the macromolecule approximately one quarter and three quarters of the molecular length, respectively (3, 11), extremely sensitive and specific methods have been required for the detection of such activity. In general, all of the methods utilize purified collagen as the substrate, either in solution or as fibrils in the form of a gel (1, 5, 7, 12). The most widely used technique developed by Lapiere and Gross (13) is based on the release of peptides containing glycine or hydroxyproline into the supernatant fluid after a fixed period of incubation with the enzyme. The amino acids of the peptides released can be measured chemically or the sensitivity of the method increased if the collagen gel has been previously labeled with ^{14}C or ^{3}H glycine or hydroxyproline. However, since the reaction is carried out at 37°, a temperature several degrees higher than the denaturation temperature of the cleaved macromolecules, further degradation and release of peptides can occur by nonspecific proteases and peptidases present in the enzyme preparation tested, or by the nonspecific action of the collagenase itself on the denatured polypeptide chains. To avoid these difficulties and to limit the reaction to the primary action of the enzymes, other techniques have been developed which utilize collagen in solution as the substrate. Under these circumstances the reaction is carried out at a lower temperature so that denaturation does not occur after cleavage of the macromolecule into two undenatured fragments. The reaction is monitored either by following the change in viscosity of the collagen solution with time, or by measuring the change in opacity of the solutions as they form collagen gels after raising the temperature in the presence of EDTA to stop the enzyme reaction (5). Although the methods employing collagen in solution obviate the difficulties due to nonspecific protease or peptidase activity, the sensitivity of the assay is not as great as the technique using radioactively labeled collagen gels.

Since there is difficulty in obtaining sufficient quantities of the enzyme, especially during purification when a large number of fractions eluted from various resin columns containing only small amounts of the enzyme needed to be assayed, it seemed that a combination of the two methods, in which only the specific action of the enzyme was measured, but the sensitivity increased by utilizing radioactively labeled collagen, would be most helpful. Such a method is the subject of the present report. Preliminary data have already been reported (14).

Materials and Methods. Purified, neutral soluble rat skin collagen previously labeled with ^{3}H proline and hydroxyproline (15) (3×10^4 dpm/mg collagen) was used as the substrate. Partially purified mouse bone collagenase was prepared as described (15) and further purified by molecular sieve chromatography through Bio-Gel P-150 resin. Purified bacterial collagenase (Sigma Corporation, Type III) was also used. Protein concentra-

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tions were determined by the method of Lowry (16).

The reaction was carried out at 4° in a small centrifuge tube by mixing 100 μl of a 0.1% solution of the collagen in 0.4 *M* NaCl, with 125 μl of 0.005 *M* Tris–HCl buffer, 0.005 *M* $CaCl_2$, pH 7.6, and 25 μl of the enzyme solution. The solution was then incubated at 25° for various time periods, at the end of which 25 μl of a solution of 0.4 *M* EDTA, pH 7.6, was added to stop the reaction. The tube was then transferred to a 37° water bath and incubated for 2 hr to permit the formation of collagen fibrils from undigested collagen macromolecules, and then centrifuged at 30,000*g* for 20 min to separate the fibrils from the supernatant fluid. Aliquot samples of the supernatant fraction were dissolved in 10 ml of scintillation fluid containing toluene with 5% (v/v) of Bio-Solv-3 (Beckman Instrument Co.) and counted in a liquid scintillation counter. When necessary, quenching corrections were made by the addition of internal standards. A unit of enzyme activity, *U*, is defined as follows:

$$U = (C_{test} - C_{blank})/(C_{total} - C_{blank}) \times 100/hr,$$

where C_{test} are the counts in the supernatant fluid with the enzyme; C_{blank}, the counts in the supernatant fluid using heat-inactivated enzyme; C_{total}, the counts in the original solution prior to incubation at 37°, the volume being made up to that of the test solution with buffer. The value of C_{blank} was also determined by replacing the enzyme solution with buffer. When assaying crude collagenase preparatives, the blank was prepared with 25 μg of crystalline trypsin (Sigma Corporation, 2 × recrystallized) and the reaction terminated by the addition of 25 μl of a 0.4 *M* EDTA solution containing 50 μg of soybean trypsin inhibitor (Sigma Corporation).

Acrylamide gel electrophoresis (17) was carried out on the supernatant fluid and on the precipitated fibrils after dissolving the precipitates in 3% acetic acid (45° for 30 min) and dialyzing the supernatant fluid against 3% acetic acid at 4°.

Results and Discussion. The number of counts found in the supernatant fluid compared with controls increased as a linear function of the amount of enzyme (Fig. 1). In the case of the partially purified mouse bone collagenase [$(NH_4)_2SO_4$ precipitation (15)], the curve was linear over the range 0–10 μg of protein. It was also linear over the range 0–10 μg for the more highly purified mouse bone collagenase obtained by gel filtration, and over the range 0–0.02 μg of purified bacterial collagenase. The linear relationship was also found to hold for up to approximately 80% of substrate digestion. When 25 μg of trypsin was used as a control, the increase in the amount of radioactivity found in the supernatant fluid over that in the blank was 2–5%.

When testing the activity of column

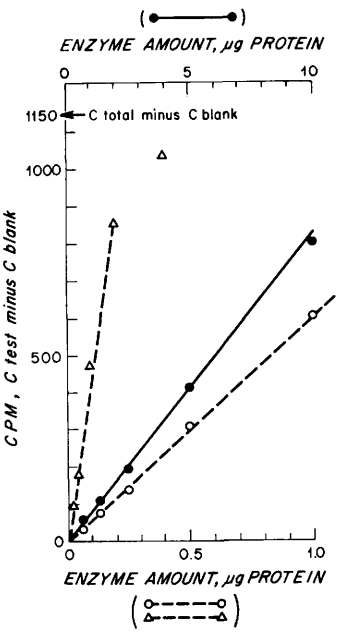

FIG. 1. The calibration curves of the new assay system with three different collagenase samples. Each assay tube contained 1800 cpm. The incubation was carried out at 25° for 4 hr and undigested collagen was allowed to form fibrils at 37° for 2 hr. ●—●: mouse bone collagenase purified by $(NH_4)_2SO_4$ fractionation (the scale at the top); ○- - -○: mouse bone collagenase further purified by gel filtration through Bio-Gel P-150 resin (scale at bottom); △- - -△: purified *Cl. histolyticum* collagenase (Sigma Type III) (scale at bottom).

effluents during the purification of mouse bone collagenase, incubation periods of 30–240 min at 25° were usually sufficient, depending on the concentration of enzyme. A 2 hr period of incubation at 37° was usually sufficient for the formation of collagen gels. In testing cruder extracts, prolonging the incubation period for up to 12 hr prior to fibril formation and prolonging the period for the formation of the collagen gel, were found to be helpful in detecting very small amounts of collagenase activity.

Acrylamide disc gel electrophoresis of the supernatant fluids revealed bands corresponding to the cleaved alpha and beta chains (17) with only trace amounts of alpha and beta components. Similar analyses of the precipitates revealed only the alpha and beta components of denatured collagen similar to those found in control samples.

In comparing the present method with that of Lapiere and Gross (13) using the same radioactively labeled collagen (as gels) as a substrate, we found with crude enzyme extracts that a larger proportion of the collagen was degraded using the method of Lapiere and Gross (13), probably due to the presence of contaminating proteases and peptideases, whereas with the more highly purified mouse bone collagenase fractions, the present method proved to be 40–50% more sensitive.

Since the present method combines both the specificity of the Nagai and Gross (5) assay and the sensitivity of the Lapiere and Gross (13) method, it should prove helpful in the identification, isolation, and purification of animal collagenases.

Summary. A new method to assay the presence and activity of tissue collagenase is presented which combines the specificity of action and the sensitivity of detection of several methods currently in use.

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