COLLAGENASE AND BONE RESORPTION: ISOLATION OF COLLAGENASE FROM CULTURE MEDIUM CONTAINING SERUM AFTER STIMULATION OF BONE RESORPTION BY ADDITION OF PARATHYROID HORMONE EXTRACT

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SUMMARY: A new method is described for the isolation of tissue collagenase from culture medium containing serum. The method was used to isolate mouse bone collagenase from an in vitro tissue culture system utilizing mouse bone calvaria in which rapid bone resorption was stimulated by the addition of parathyroid hormone extract. The amount of collagenase activity in the tissue culture medium was found to correlate well with the extent of bone resorption observed morphologically The results provide further and more direct evidence that the synthesis and release of bone collagenase is involved in and related to the removal of bone collagen during bone resorption.

The resorption of bone must be accompanied by the degradation and removal of collagen, the major organic, structural component of the bone matrix (1). Native collagen is resistant to attack by most proteases, but is readily digested by specific tissue collagenases (2), including bone collagenases, which have been demonstrated in several species. Mouse bone collagenase has been isolated and partially purified (5,6) and its mode of action and other characteristics demonstrated to be similar to other tissue collagenases, including its inhibition by components present in blood serum (7). In addition to identifying the collagenase in tissue culture media after in vitro culture of bone, bone collagenase has also recently been directly extracted from bone tissue itself (8). Although there has been indirect evidence which has suggested that the amount of collagenase produced by bone cells is directly related to the rate at which bone resorption occurs (1,9), thus linking the synthesis and release of the enzyme to the more general biological phenomenon of bone resorption, there have been

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no previous studies which have directly measured the synthesis and release of the enzyme into the tissue culture media of living bone cultured in vitro, and which have correlated the amount of enzyme activity recovered from the media with the extent of bone resorption which occurred. Thus, in a tissue culture system in which it was morphologically demonstrated that active resorption of living bone occurred (1,9,10), it was not possible to directly isolate and measure the collagenase activity since the tissue culture medium included serum which contains collagenase inhibitor(s). Instead the collagenase activity was measured indirectly by the amount of lysis which occurred in radioactively labelled collagen gels on which the bone fragments were placed (9). In the tissue culture systems which employed media containing no serum, there was no evidence to indicate whether active cellular resorption of living bone occurred under the particular conditions used or whether the bone fragments simply released the enzyme after cell injury or death. This is therefore the first report in which the extent of bone resorption in a living system in tissue culture has been correlated with the amount of collagenase released into the tissue culture media as measured by the direct isolation and subsequent assay of the enzyme.

We have previously shown that heparin increases the production and/or release of mouse bone collagenase in tissue culture (11). It also increases the activity of isolated bone collagenase when it is assayed using collagen in the solid state as the substrate (11). These observations led to a study of the interaction between heparin and mouse bone collagenase using heparin-substituted Sepharose 4B gel, in which it was demonstrated that a strong ionic bond occurs between heparin and collagenase (12,13). This permitted the isolation and purification of the enzyme by this type of affinity chromatography (12,13). By using heparin-substituted affinity chromatography, it was therefore possible to isolate the collagenase from tissue culture media containing serum, and to correlate the amount of collagenase activity present in the media with the extent of bone resorption observed and measured morphologically.

The organ culture methods employed have been reported previously (10).

Briefly, calvaria from 5 day old mice of the Swiss Webster strain were affixed to glass cover-slips and incubated with 2 ml of medium in Leighton-type culture tubes. The tubes were gassed with an atmosphere of oxygen and nitrogen (1:1) and incubated at 37° C in a rotating culture drum apparatus at 1/5 r.p.m. The medium contained heated horse serum and Gey's balanced salt solution (7:3)(14). Parathyroid hormone was added as parathyroid extract (Eli Lilly Co.)(15) and heparin as sodium heparin (Eli Lilly Co.)(15). The medium was changed every two days and the incubation continued for 6 days. The cultures were examined daily under the dissecting microscope to assess bone resorption. The extent of bone resorption which occurred and could be visualized morphologically was recorded as described previously (16). The method assigns a score of 0 (no resorption) to 14 (complete resorption) based on the estimated relative surface area of the bone that is resorbed during the stated time interval.

The culture media from each 2 day change were pooled (24 ml) and dialyzed against 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$ for 16 h at 4° C. The dialyzed samples were passed through a column of heparin-substituted Sepharose 4B gel which was prepared after the method of Iverius (17). After charging the sample, the column was washed with 50 ml of the same buffer solution. The bound proteins were then eluted by a linear gradient of Ca-acetate and NaCl obtained by a two-chamber, constant level gradient device, the mixing chamber containing 100 ml of the starting buffer (50 mM Tris-HCl, pH 7.6) and the reservoir an equal amount of limiting buffer (0.5 M Ca-acetate and 0.5 M NaCl in 50 mM Tris-HCl buffer).

Figure 1 shows a typical elution profile of the affinity chromatography. The bulk of the serum proteins emerge as a non-binding, breakthrough peak. As the ionic strength of the elution buffer is increased, a considerable amount of protein is eluted as a second peak. At higher ionic strengths, a small broad peak emerges. This third protein peak was found to contain the collagenase activity. The results are similar to those obtained in a study using the collagenase isolated from serum-free culture medium of mouse bone (12). Only trace amounts or no collagenase activity at all was detected in the media obtained

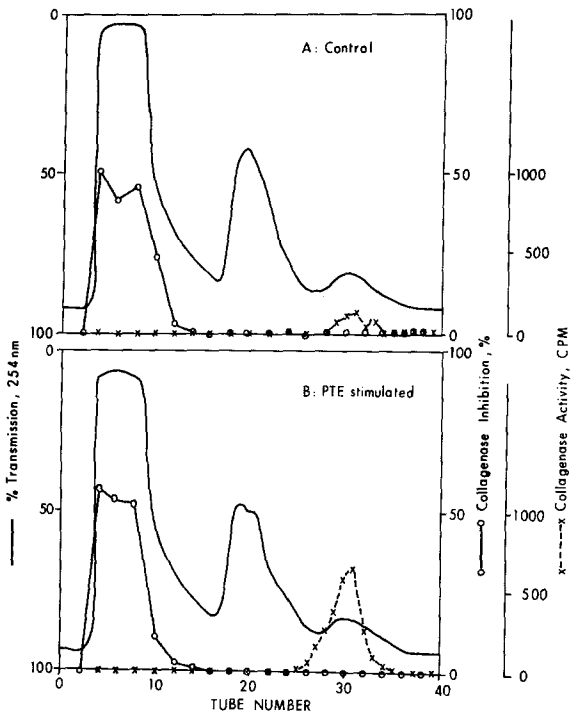

Fig. 1 Affinity chromatography of culture medium of bone (24 ml) using a column (1.6 x 5 cm) of heparin-substituted Sepharose 4B gel. Elution of protein from the column was monitored at 254 nm by a Uvicord I (LKB). Flow rate was 30 ml/h and 6 ml fractions were collected. Collagenase activity was measured in each fraction using 50 µl of the sample by a method employing radioactively labelled reconstituted collagen fibrils (10) incubated for 16 h. Sodium azide was included in each reaction mixture at a concentration of 1 mg/ml to prevent bacterial growth. Collagenase inhibitor activity in each fraction was determined by measuring the extent (%) of inhibition which occurred in the appropriate collagenase assay system using isolated mouse bone collagenase (11) to which 50 µl of each fraction was added.

from control groups in which very little bone resorption was noted morphologically (Table 1, Figure 1A). In contrast, significant amounts of collagenase were isolated from the culture medium where there was morphological evidence that extensive bone resorption had occurred as a result of the addition of parathyroid extract and heparin (Table I, Figure 1B). Serum collagenase inhibitor emerged in the breakthrough peak and was not retarded by the heparin-substituted Sepharose 4B gel.

The active collagenase fractions (tube numbers 27-33) were collected and concentrated almost to dryness in a Diaflo filter cell with a PM-10 membrane

TABLE I

The Amount of Collagenase Isolated by Affinity Chromatography From the Tissue Culture Medium of Bone in Control Samples and in Samples in Which Bone Resorption Was Stimulated by the Addition of Parathyroid Hormone Extract (PTE)

Experimental Group	Bone Resorption[a] (Morphological Score)	Collagenase Activity[b] (units/ml)
Control:		
Heparin, 10 units/ml	1.9±0.21	0.05
PTE treated:		
Heparin, 10 units/ml plus PTE, 0.1 units/ml	12.7±0.85	2.30

[a] The extent of bone resorption morphologically is expressed as the mean ± one standard deviation for four bone cultures.

[b] Collagenase activity values represent the activity units per ml of the pooled culture medium of each experiment. One collagenase unit is defined as the amount of enzyme activity necessary to digest 1 μg of collagen in 1 h under the assay conditions described.

(Amicon). Subsequently, starting buffer was added to the Diaflo filter and the procedure repeated several times. The final volume of the sample was 1.5 ml. The concentrated sample then was centrifuged and aliquot portions of the supernatants assayed for collagenase activity. Table I shows that the collagenase activity in the culture medium of resorbing bone is significantly higher than that in the culture medium from the control sample. Two other similar experiments have also revealed similar results: only a trace of collagenase activity in the control media and comparable levels of collagenase activity in the culture media of resorbing bone (1.91 and 2.49 units/ml).

The sample of collagenase isolated by affinity chromatography was mixed with rat skin collagen solutions, incubated at 25° C, and the changes in viscosity followed as previously described (11). Control experiments were carried out in which the solution containing the enzyme was replaced with buffer. Figure 2 shows that the enzyme sample reduced the specific viscosity of the collagen solution by 65% in 20 h. The reaction products of the collagen used as the sub-

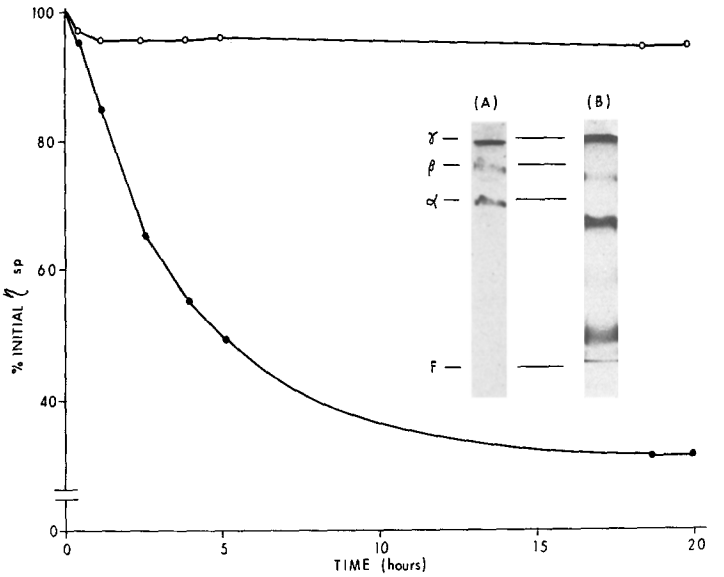

Fig. 2 The effect of collagenase isolated from the culture medium of bone on the viscosity of rat skin collagen solution at 25° C. The percentage change in specific viscosity is shown on the ordinate. o——o control, ●——● with enzyme sample (27.0 units in 0.4 ml). Insert is an acrylamide gel disc electrophoresis of the reaction products of rat skin collagen from the viscometry experiments at the end of 20 hours. (A) Control, (B) with enzyme added.

strate were analyzed by disc gel electrophoresis. The patterns showed that the collagenase attacked monomeric collagen at a single site in a manner similar to that of mouse bone collagenase isolated from serum-free culture medium of mouse tibiae (5) and other tissue collagenases (2)(Fig. 2).

A direct correlation was demonstrated between the extent of bone resorption occurring in living bone in tissue culture as measured morphologically and the amount of collagenase activity in the tissue culture media by a method which directly isolates the enzyme prior to assay. Preliminary studies have also shown a direct dose response relationship between the amount of Parathyroid Hormone Extract added and the collagenase recovered in the tissue culture medium.

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