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COLLAGENOLYTIC ACTIVITY FROM ISOLATED BONE CELLS

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Summary

A true collagenase (EC 3.4.24.3) which had been discovered previously in bone culture fluids and extracts of whole bone has now been localized to the cellular component of bone. The cellular enzyme bears the same characteristics as that of bone collagenases described earlier. Moreover, it is directly extractable in relatively large quantities. Attempts to demonstrate the presence of a bone cell procollagenase were unsuccessful.

It was also observed that the cells secrete significant amounts of collagenase in vitro. With increasing incubation time the extracellular collagenase levels rise and the intracellular collagenase levels drop.

Introduction

A collagenase (EC 3.4.24.3) which is specific with regard to catalytic activity, activation and inhibition has been demonstrated in the culture fluids of many animal tissues [1,2]. Except for a few tissue types the enzyme has not been directly extractable in detectable quantities. Small amounts of enzyme have been directly extracted from human skin and synovial membrane [3], carcinoma cells [4], polymorphonuclear leukocytes [5], rat [6] and chick long bones [7]. Except for leukocytes no activity has been demonstrated in a well-defined population of cells either cultured or freshly isolated. Recently we have been able to isolate viable, metabolically active bone cells from fetal rat calvaria and directly demonstrate the presence of a comparatively large amount of enzyme that is similar in its activity to collagenase obtained from whole bone cultures.

Materials and Methods

Cell preparation

The cell isolation technique is Procedure III of that described by Dziak and Brand [8]. It is a modification of the procedure of Peck et al. [9] and involves

removing the calvaria of 20—21-day-old fetal rats and digesting the slightly calcified extracellular matrix with bacterial collagenase. The incubation medium consists of a buffered balanced salt solution containing 120 mM NaCl, 3 mM K_2HPO_4, 1.25 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Tris · Cl, 30 mM mannitol, glucose (2 mg/ml), bovine serum albumin (1 mg/ml) and Worthington CLS Type II *Clostridium histolyticum* collagenase (2 mg/ml). After the cells are harvested and washed 5—6 times in enzyme free buffer, cell counts are performed with the aid of a standard hemocytometer. The yields average about $3 \cdot 10^6$ cells/calvarium. The viability and metabolic activity of this bone cell preparation is discussed elsewhere [8,9].

A particulate fraction was prepared by freezing and thawing the freshly isolated cells at least 5 times during which time the cells were completely disrupted and fragmented as judged by microscopic examination. Centrifugation of this suspension at 30 000 × *g* for 30 min yielded a clarified supernatant.

A crude purification of the enzyme was achieved by ammonium sulfate precipitation of the clarified supernatant according to the method of Fullmer and Lazarus [10]. It involved harvesting the proteins precipitating between 20% and 60% ammonium sulfate saturation of the supernatant fraction. As determined by sodium dodecyl sulfate disc gel electrophoresis there were at least 20 protein bands in the crudely purified preparation.

Substrate preparation

The collagenase assay utilizes purified radioactively labeled native rat skin collagen. The substrate is obtained by intraperitoneal injection of young male Spraque-Dawley rats (about 200 g) with 80 μCi of uniformly labeled [^{14}C]glycine, sacrificing the animals after 12 h and purifying neutral salt soluble skin collagen. The skins of the animals are removed, shaved and minced with scissors and are then fragmented in a Waring blender along with chips of solid CO_2 (all procedures from this point on are carried out in the cold, 4° C).

The extraction and purification of the substrate is by a modification of the method of Kaufman et al. [11]. It involves an alternating series of solubilizations and precipitations with 1.0 M NaCl in 0.05 M Tris at pH 7.4 and 15.0% NaCl, respectively. After the last solubilization the solution is acidified to 0.5 M acetic acid and the collagen precipitated with 7.0% NaCl. The pellet is recovered and redissolved in 0.5 M acetic acid and dialyzed for 2—3 days against at least 6 l of 0.02 M NaH_2PO_4, pH 7.4 which precipitates only native collagen. The collagen is then collected, dissolved in 0.5 M acetic acid, dialyzed exhaustively against water and lyophilized.

Using this procedure, purified collagen has been obtained with specific activities as high as 10 000 cpm/mg.

Collagenase assay

Collagenolytic activity was measured by a modification of the method of Nagai et al. [12]. Radioactive substrate is dissolved in a buffered salt solution (125 mM NaCl, 50 mM Tris, 1.4 mM $CaCl_2$, pH 7.4) at a concentration of 4.0 mg/ml by stirring overnight in the cold. Ten μl of test solutions to be assayed are then added to a 200 μl aliquot of the substrate already in a 3 ml centrifuge tube and vortexed to insure an even distribution. Immediately after adding the

test solutions to the substrate the tubes are transferred to a warm room (37°C) and after various periods of incubation the tubes are centrifuged at 27 000 × *g* for 30 min and a 50 μl sample of the supernatant is counted using a scintillation counting solution containing 10% Beckman Bio-Solv BBS-3, a solubilizer for aqueous solutions. The difference between the amount of radioactivity released into the supernatant by the tested substance and a buffer blank is a measure of the amount of collagenolytic activity. Fig. 1 represents the form in which the assay is presented and is a demonstration of the difference in collagen digestion by *Cl. histolyticum* collagenase and trypsin. Note the rapid solubilization by bacterial collegenase and the limited proteolysis by trypsin. The pH of the assay mixtures did not vary from 7.4 in any experiment.

Sodium dodecyl sulfate disc gel electrophoresis

Sodium dodecyl sulfate gel electrophoresis of the collagenase reaction products was performed using a Hoefer SE 500 Vertical Slab electrophoresis apparatus according to the procedure developed by Laemmli [13].

The samples were prepared by running the reaction with partially purified enzyme and purified collagen at 27°C for 4 h in a shaker bath, centrifuging the mixture at 10 000 × *g* for 30 min in a refrigerated centrifuge and exhaustively dialyzing the supernatant against 0.05 M acetic acid, in the cold; 0.5 ml of the acidified collagen solution was then precipitated in the segment-long-spacing form by the addition of 0.2 ml of a 1.0% solution of free ATP. The solution became cloudy in a few seconds and the precipitate was collected after 60 min in the cold. The pellet was then dissolved in sample buffer containing 1.25% β-mercaptoethanol and 1.25% sodium dodecyl sulfate to a final concentration of approximately 1 mg/ml and heated in a boiling water bath for 3 min; 10—20 μl was added to each port on the 5% stacking gel and the electrophoresis was run for approximately 2 h after the dye marker entered the 8% running gel. The gel slab was then removed and fixed in 12.5% trichloroacetic acid for 30 min and stained in 0.05% Coomassie Brilliant Blue R-250 in 10% trichloroacetic acid overnight. Gels were destained in 7% acetic acid.

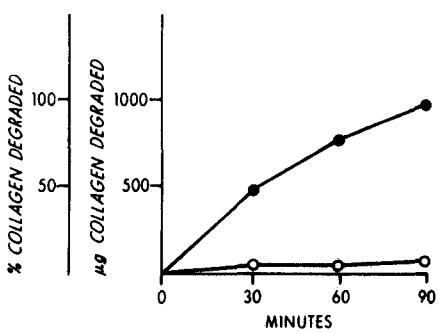


Fig. 1. Demonstration of release of radioactivity by *Cl. histolyticum* collagenase (20 munits/ml) (•) and by trypsin (0.1 mg/ml) (○) expressed as μg collagen degraded and percent of total substrate available for degradation.

Electron Microscopy

Electron micrographs of the collagen fragments in the segment-long-spacing form were taken on a Philips 200 electron microscope. The samples were prepared in a similar fashion to those used for electrophoresis except that the suspension of segment-long-spacing collagen was directly applied in droplets to carbon-coated standard 200 mesh grids, dried, and positively stained with 0.1% phosphotungstic acid for 3 min.

Molecular sieve chromatography

An insulated Bio-Gel A 1.5, 200—400 mesh (8% agarose: Bio Rad Laboratories) column was also used for separation of the collagenous reaction products. Separation was performed by the method described by Piez [14].

Samples were prepared by incubating the whole cell extract with labeled collagen at 27° C for 4 h in a shaker bath, centrifuging the mixture at 10 000 × *g* for 30 min at 4° C, denaturing both substrate and enzymes at 55° C for 20 min and lyophilizing. The radioactivity was then taken up in 800 µl of 1 M $CaCl_2$ in 0.05 M Tris, pH 7.4. Samples were added to the top of the column bed, allowed to run into the bed and covered with eluant buffer. Usually 90—100 fractions of 2.5 ml each were collected. From each fraction 500 µl was added to a liquid scintillation vial containing 6.5 ml of toluene/Omnifluor, 3.0 ml 95% ethyl alcohol and 3.0 ml of BBS-3 solubilizer. This particular counting cocktail was needed to solubilize the high salt buffer.

Analysis of substrate purity

Unambiguous demonstration of collagenase activity from a whole cell extract can best be demonstrated by use of a radioactively labeled collagen substrate which is not contaminated with other labeled proteins. Non-specific proteases are not active against the helical portion of a collagen molecule [1,15] and therefore only a true collagenase will solubilize native purified collagen. For these reasons the purity of our substrate was confirmed in a number of different ways including molecular sieve chromatography, analytical ultracentrifugation, sodium dodecyl sulfate electrophoresis, and attempted digestion by non-specific proteases. There was no indication of any contamination by either labeled or non-labeled non-collagen protein.

Results

Bone cell collagenase

Fig. 2 is a demonstration of collagenolytic activity from a bone cell particulate fraction and a 30 000 × *g* clarified supernatant of the particulate fraction. A 10 µl aliquot, representing the extract from 10^6 bone cells was added to the substrate and incubated at 37° C. After 1, 2 and 4 h the collagen gels were pelleted by centrifugation and the amount of collagen degraded was calculated. The solubility of the enzyme is demonstrated by the presence of activity in the clear supernatant.

Collagenolytic activity has been reported from granulocytes [5] and the possibility exists that some collagenase was derived from contaminating blood cells. A particulate fraction of rat blood with the same number of granulocytes

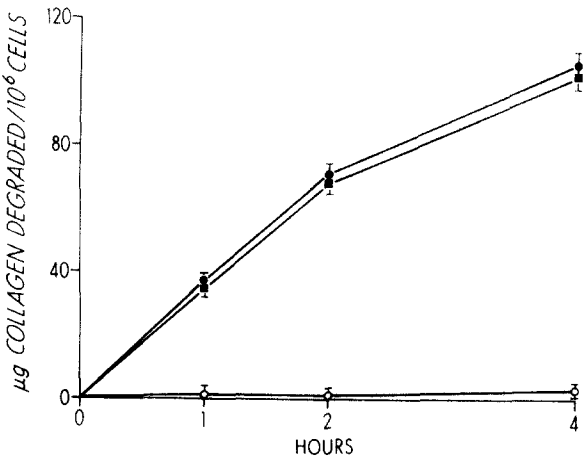


Fig. 2. The demonstration of collagenolysis by bone cell extract (●) and the contribution due to rat granulocyte (○) in the extract. Solubility ofthe enzyme is demonstrated by activity in a clarified 30 000 × *g* supernatant fraction of the extract (■). Each point in this figure as well as all the subsequent figures is representative of at least 6 determinations and the vertical bars represent ± S.E. of the mean.

as in the bone cell fraction was prepared and separately assayed. The granulocyte curve in Fig. 2 shows the contribution of granulocytes in the bone cell preparation. Relatively little activity could be attributed to this cell population.

Modifiers of bone cell collagenase activity

The collagenolytic enzymes harvested from whole bone cultures have been shown to be similar to other mammalian collagenases with regard to inhibitors and metal ion requirements [15]. The enzyme obtained from our isolated cell preparations also possesses these properties. Fig. 3 is a compilation of these characteristics. Heating the particulate fraction at 55°C for 20 min caused destruction of all protease activity. Rat serum at a concentration of 5% caused a 92% inhibition at 8 h. The inhibitory factor in serum is believed to be in the α_2 Macro globulin (α_2 M) fraction which is known to have other anti-protease activity as well [16]. EDTA at a concentration of 15 mM significantly decreases col-

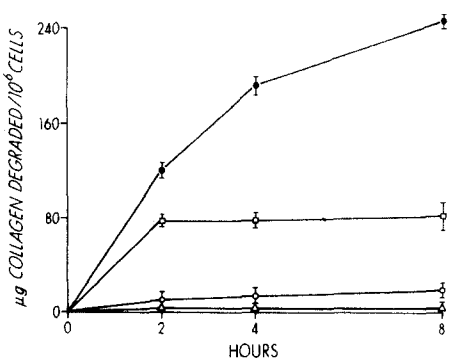
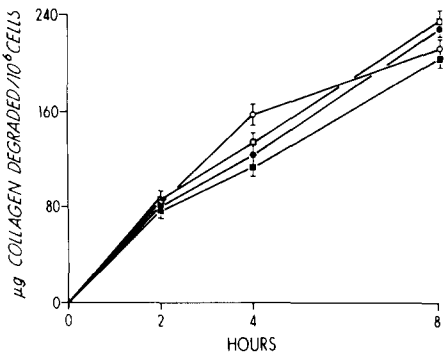


Fig. 3. This figure is a summary of the effects of EDTA (□), serum (○), and heat (△) on the extractable bone cell collagenase (●).



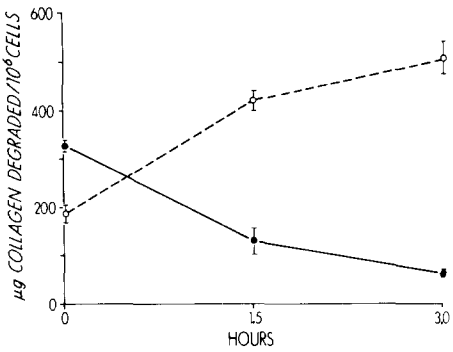


Fig. 4. Attempts to demonstrate the presence of a collagenase zymogen according to Vaes [17]. Bone cell extract (●), bone cell extract + trypsin (4 µg/ml for 15 min) + soya-bean trypsin inhibitor (8 µg/ml for 15 min) (○), bone cell extract + soya-bean trypsin inhibitor (8 µg/ml for 15 min) (■), bone cell extract + heat-denatured albumin (10 mg/ml) (□).

Fig. 5. The time course of secretion of collagenase from bone cells (●) and the appearance of collagenase in the medium (○). The medium values (○) are corrected for a dilution of the secreted collagenase by the medium. The values along the abscissa are times of cell incubation after isolation and the values along the ordinate are a measure of the amount of collagen degraded at one assay time, 6.5 h. The cells were pre-incubated for 15 min before the zero hour time point was taken.

lagenolysis by the bone cell fraction, indicative of a divalent cation dependence, most probably Ca^{2+}. The EDTA treatment does not inhibit collagenolysis as completely as serum because there are cellular proteases which are Ca^{2+} independent but still susceptible to α_2 M inactivation [16]. The time course of the digestion with EDTA also implies that only a small portion of the total radioactivity can be solubilized and is consistent with the model of less specific protease digestion of the non-helical regions of a collagen molecule.

Attempts to demonstrate a procollagenase

Fig. 4 is a summary of attempts to demonstrate a zymogen of the enzyme according to the method of Vaes et al. [17]. In their work considerable amounts of collagenase activity were released from mouse bone with a trypsin pretreatment. No such phenomenon could be shown with this bone cell collagenase. The possibility of a procollagenase being completely activated by cell proteases was also explored. By providing large amounts of competing protein (10 mg/ml heat denatured bovine serum albumin) cell protease activity would have been slowed and zymogen activation may have been inhibited. However the bovine serum albumin treated fractions did not show a depression. Rather there was a slight but insignificant increase in collagen digestion. Heat denatured albumin alone at 10 mg/ml had no effect on degradation of the collagen substrate.

Secretion of bone cell collagenase

In many instances where the isolated cells were incubated after isolation it was noted that the activity in the cell particulate fraction decreased with incubation time. The levels of collagenase in the medium were then monitored during a 3-h incubation period and the results are displayed in Fig. 5. It appears

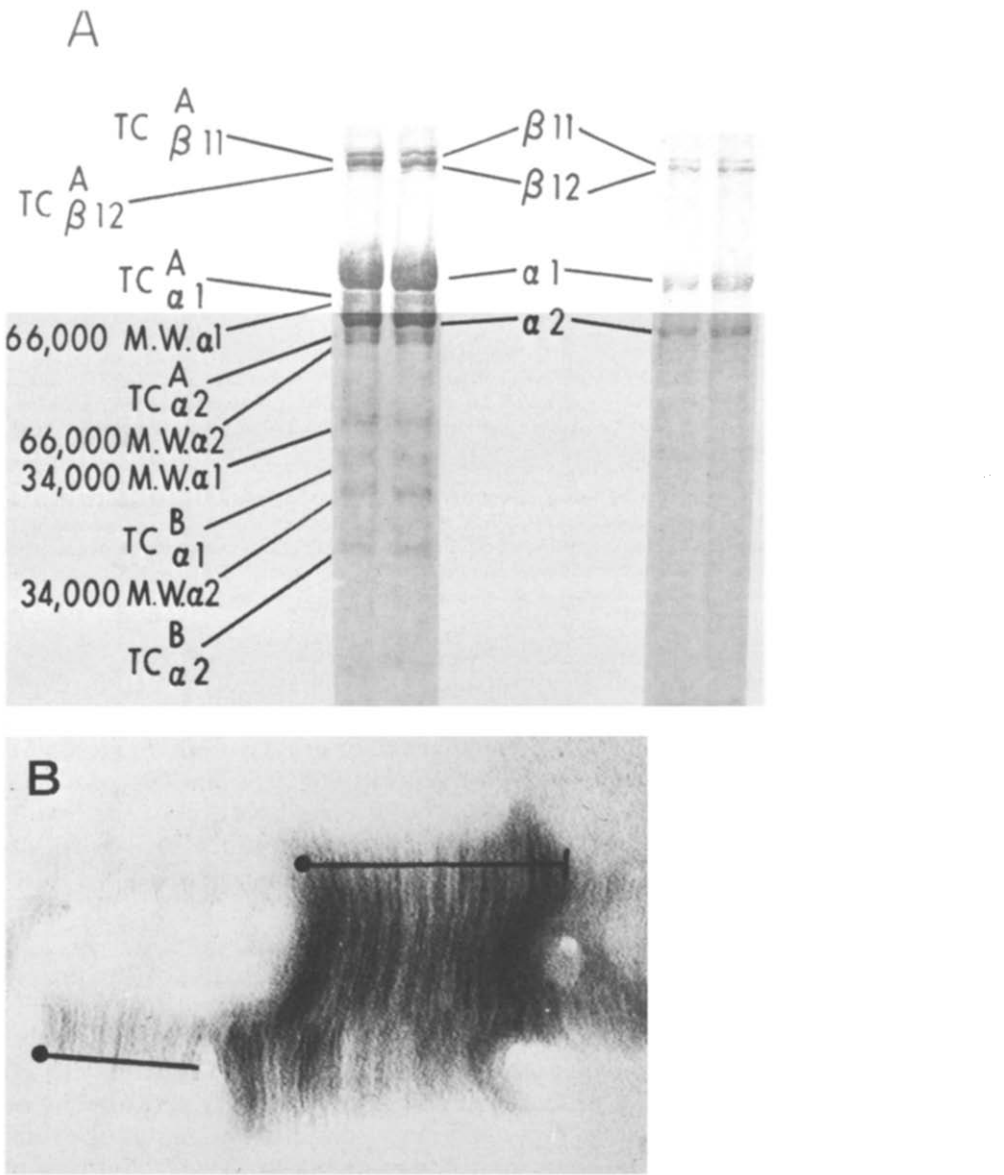


Fig. 6. This figure is a demonstration of the characteristic reaction products of bone cell collagenase with purified collagen. In Part A, the purified collagenase and a buffer control were reacted with native collagen. The reaction products are displayed using sodium dodecyl sulfate-polyacrylamide electrophoresis. ($TC^{A}_{\beta 11}$ migrated to a position which overlapped β_{12} and could not be resolved). Part B is an electron micograph both native (●———) and partially degraded (●———) collagen precipitated in the segment-long-spacing form.

that collagenase is rapidly secreted from the cells and accumulates in the surrounding medium. Both the constant cell number and the rise in total collagen degradation at each time point suggest that cell death and lysis are not the reason for appearance of collagenase in the medium.

Characterization of bone cell collagenase products

It is now commonly accepted that demonstration of collagenolytic activity from animal sources requires an elucidation of the macromolecular degradation products. In order to preserve the helical nature of these products and prevent further proteolysis by less specific proteases in the crudely purified bone cell collagenase it was necessary to run the reaction at 27° C. Lowering the temperature slowed collagenolysis but it inhibited the helix-coil transition of the amino-terminal tropocollagen fragment (TC^A) and the carboxy-terminal tropocollagen fragment (TC^B).

When the crudely purified enzyme and a buffer control were reacted with native collagen and prepared for sodium dodecyl sulfate electrophoresis as described in the Materials and Methods the patterns in Fig. 6 were consistently observed. The control always displayed only four bands corresponding to α_1, α_2, β_{11} and β_{12}. The electrophoretic pattern of the collagen which was reacted with crudely purified enzyme contained a number of new protein bands. In addition to the intact chains there was also noted a fragment of approx. 150 000

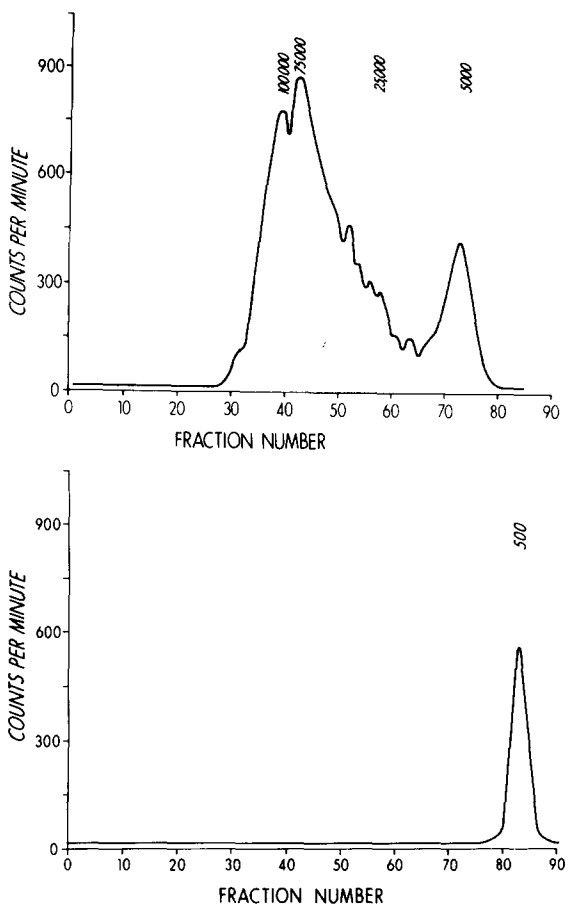


Fig. 7. Gel filtration chromatogram of a denatured mixture from the reaction of bone cell extract (top) and *Cl. histolyticum* collagenase (bottom) with native labeled collagen substrate at 27° C.

M_r, amino terminus of β_{12} ($TC^A_{\beta 12}$) (the amino terminus of β_{11}, $TC^A_{\beta 11}$, migrated to a point which overlapped β_{12} and could not be resolved) fragments of approximately 75 000 M_r, amino terminus of α_1 and α_2 ($TC^A_{\alpha 1}$, $TC^A_{\alpha 2}$) fragments of approx. 25 000 M_r, carboxy terminus of α_1 and α_2 ($TC^B_{\alpha 1}$, $TC^B_{\alpha 2}$) and fragments of approx. 66 000 and 34 000 M_r. The origin of these last two products is discussed in a later section. When samples of either collagen digested by *Cl. histolyticum* collagenase, or the crudely purified bone cell enzyme alone were taken through the same procedure and electrophoretically analyzed, no bands were apparent.

The electron micrograph of Fig. 6 also provides evidence of the specific degradation of native collagen by bone cell collagenase.

The digestion products of the total cell extract were also studied using molecular sieve chromatography. The reaction was carried out in a similar fashion to that for electrophoresis and electron microscopy except that the reaction mixture was immediately denatured at 55° C for 20 min. Using this technique it was possible to show a radioactively labeled intact α chain of 100 000 M_r, a fragment of about 75 000 M_r and a series of macromolecular fragments around 25 000—40 000 M_r (Fig. 7). The inability to show a single peak at 25 000 M_r may be due to non-specific protease degradation of the fragments or to the presence of other cleavage products by the bone cell collagenase itself. When the collagenase reaction was run at 37° C nearly all of the radioactivity appeared around the 5000 M_r peak. The products of the reaction of bacterial collagenase with labeled substrate under the same conditions eluted at a point corresponding to approx. 500 M_r.

Discussion

An enzyme with the capabilities of degrading native collagen in a fibrillar form has been identified in whole bone cultures by many investigators and in extracts of whole bone by Aer [6]. The data presented here show that this enzyme as well as other proteases can be found in isolated bone cells. The amount of activity in the isolated bone cells is very high compared to that of other isolated cell types. Lazarus et al. [5] required 100 million cells to demonstrate activity with human granulocytes, whereas less than one million fetal bone cells could produce collagen degradation.

The characteristics of bone cell collagenase are similar to those of collagenases from whole bone and other tissues. It is heat and serum inactivated, Ca^{2+} requiring, soluble in a physiological buffer and cleaves the collagen molecule into macromolecular fragments at neutral pH. In addition, it appears that the mechanism for the in vivo secretion of collagenase is retained by an isolated population of cells devoid of a collagen matrix.

The possibility that some collagen degradation was due to *Cl. histolyticum* collagenase contamination was considered, however, Fig. 3, 6 and 7 provide evidence that the bacterial enzyme was not responsible for the digestion of substrate. Fig. 7 displays the products of *Cl. histolyticum* collagenase as compared to bone cell collagenase. There was no exopeptidase activity in the bone cell extract. Fig. 3 demonstrates that bone cell collagenase is inhibited by 92% with rat serum. *Cl. histolyticum* collagenase is not significantly inhibited by serum

[2,18] and Fig. 6 directly characterizes the products using sodium dodecyl sulfate-disc gel electrophoresis and electron microscopy.

Unlike the mouse bone collagenase of Vaes et al. [17] this bone cell collagenase appears to be stored intracellularly in an already activated form. Attempts to artificially activate a zymogen or to prevent activation by cellular proteases according to their procedure yielded no change in the collagenolytic activity of the bone cell enzyme.

In addition to the demonstration of a 75 000 M_r and a 25 000 M_r fragment it was noted in the gel electrophoresis pattern the appearance of other fragments of about 34 000 and 64 000 M_r. These fragments could be from further degradation by non-specific proteases which with time can degrade the initial collagenase reaction products even at 27°C [19], or they may be actual cleavage products of the bone cell collagenase as has been previously reported with enzymes from other tissues [20,21].

The preparation of a collagenase from whole bone culture and now from bone cells has been documented. However, as is commonly known there are different bone cell types in whole tissue any of which could be producing collagenase. In subsequent reports utilizing bone cell collagenase content and other criteria we will demonstrate the separation of a heterogeneous population of cells into fractions which show certain differentiated cell characteristics. In addition a cell culturebioassay system for collagenase has been developed and can be used to test the effect of hormones and other factors on the synthesis and release of collagenase in both mixed and separated bone cell populations.

Acknowledgements

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