PREPARATION OF THREE VERTEBRATE COLLAGENASES IN PURE FORM

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SUMMARY

Human skin, rheumatoid synovial and tadpole collagenases have been obtained in pure form using affinity chromatography with collagen coupled to Sepharose. This simple and reproducible method should have broad applicability in the purification of other animal and human collagenases in sufficient quantities to study in detail their physical and enzymologic properties.

INTRODUCTION

A number of specific animal and human collagenases, important not only in the normal catabolism of connective tissue, but also in amphibian metamorphosis, and in a number of disease states have now been isolated and partially characterized. All these enzymes are similar in that they cleave the native collagen molecule at a small number of specific sites (See recent review by Eisen et al., 1970) (1). Although immunologic differences have been demonstrated between collagenases from different species (2), detailed studies on this interesting class of enzymes have been hampered by difficulties in obtaining highly purified preparations of the individual collagenases.

Gallop et al. (3) were the first to recognize the high affinity of a collagenase for its substrate and to take advantage of this property in the purification of the collagenase from Clostridium histolyticum. The enzyme was allowed to bind to native collagen fibrils and was subsequently recovered after digestion of the substrate and release of the collagenase. Similar use of collagen fibrils for purification of vertebrate collagenases is less than ideal, since the limited cleavage of collagen by these enzymes (1) makes

separation of the collagen degradation products from the enzyme difficult. To overcome this problem, collagen in solution was chosen for coupling to solid matrix (agarose) for use in affinity chromatography. This report describes the preparation of three vertebrate collagenases in pure form using a method that should have broad applicability in the purification of other collagenolytic enzymes.

METHODS

Sources of Collagenase. Human skin collagenase, rheumatoid synovial collagenase and tadpole collagenase were obtained from tissue culture using techniques previously described (4-6). Partial purification of the human enzymes was accomplished by gel filtration of a 0-60 per cent ammonium sulfate precipitate of the collagenase preparations on a column of Sephadex G-150 (2,7). Tadpole collagenase was partially purified by DEAE-cellulose chromatography (6).

Affinity Chromatography. Collagen was coupled to agarose (Sepharose 4B, Pharmacia) according to the method described for other ligands by Cuatrecasas and associates (8,9). Approximately 25 ml of cyanogen bromide-activated Sepharose 4B was suspended in 50 ml 0.2 M $NaHCO_3$ (pH 9.0), and 75 mg of native guinea pig skin collagen, purified by the method of Gross (10), in 25 ml of 0.4 M NaCl, was added immediately. The mixture was stirred gently for 18 hrs at 4°C, filtered, washed with water and equilibrated with 0.05 M Tris-HCl (pH 7.5) containing 0.005 M $CaCl_2$. The efficiency with which native guinea pig skin collagen was coupled to Sepharose was determined by measuring the hydroxyproline content of the collagen-Sepharose beads at the end of the reaction. In every case 70-85 per cent of the collagen was coupled to Sepharose under the described conditions, producing a slurry which contained approximately 1.8 mg collagen per ml of packed Sepharose.

Affinity chromatography was carried out by applying 5-10 mg of enzyme protein to a column (1.2 x 1.5 cm) containing collagen-Sepharose which had been equilibrated with 0.05 M Tris-HCl, 0.005 M $CaCl_2$, pH 7.5. This buffer

was passed through the column until the absorbance at 280 mμ returned to baseline. Elution was accomplished by addition of 1.0 M NaCl to the same buffer. Eluant fractions were dialyzed against water and lyophilized. After reconstitution of the enzyme powder in buffer, samples were assayed for collagenase activity and examined by polyacrylamide gel electrophoresis on 12.5 per cent gels using a Tris-glycine buffer at pH 8.5 (11,12). Unbound material from the first peak was processed in a similar manner and subjected to rechromatography on collagen-Sepharose until no further enzyme activity remained.

Assays. Collagenase activity was measured by the enzymatic release of soluble ^{14}C-glycine-containing peptides from native, reconstituted guinea pig skin collagen fibrils (6). Protein was determined by the method of Lowry et al., (13). Hydroxyproline was measured by the method of Bergmann and Loxley, (14).

RESULTS AND DISCUSSION

Figure 1 demonstrates a typical affinity chromatogram obtained by

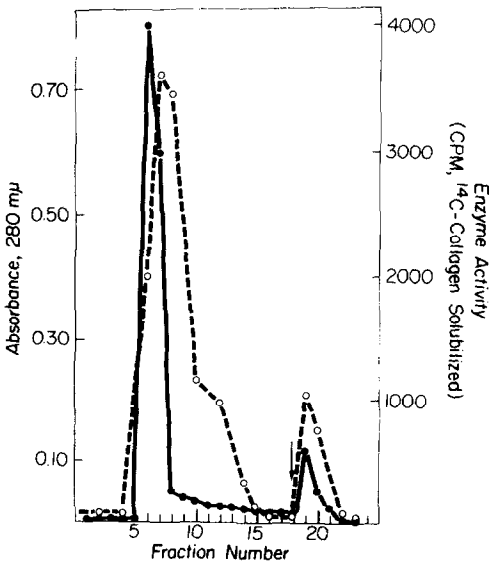

Figure 1. Affinity chromatography of human skin collagenase on collagen-Sepharose. A sample of 10.0 mg of partially purified enzyme protein was applied to a column (1.2 x 1.5 cm) and effluent fractions of 5.0 ml were collected at a rate of 15.0 ml/hr. Elution was accomplished by the addition of 1.0 M NaCl to the eluant buffer (arrow). Collagenase activity was measured by incubating 100 μl of the eluant fractions on ^{14}C-collagen fibrils for 6 hrs at 37°C. (See text.) ●——● absorbance at 280 mu; o---o, enzyme activity.

passing human skin collagenase, partially purified by gel filtration on Sephadex G-150, through collagen-Sepharose. Similar chromatograms are obtained with rheumatoid synovial and tadpole collagenases. Although only partial adsorption of collagenase activity occurs, that fraction which binds can be eluted with a buffered solution of 1.0 M NaCl. Harsher methods of elution, such as lowering the pH, have been used with other enzymes (8), but the lability of collagenases at acid pH makes such methods impossible (4-6).

The enzymatically active eluant fraction of human skin collagenase appears as a single band on polyacrylamide gel electrophoresis (Fig. 2A). Occasionally minor contaminating protein bands may be present, which usually can be removed by rechromatography on fresh collagen-Sepharose. Starting with 10.0 mg of partially purified collagenase, a single chromatographic procedure rarely yields more than 0.5 mg of the purified enzyme.

Table 1 shows the purification scheme for human skin collagenase. By a single passage of partially purified enzyme through collagen-Sepharose, an approximate 20-fold increase in the specific activity of this enzyme is achieved with 15-20 per cent recovery of initial activity. If contaminating proteins remain in the eluate, rechromatography results in more highly purified prepar-

Table 1. Purification of Human Skin Collagenase

Stage	Specific Activity*	Total Protein (mg)	Total Activity	Recovery (%)
Crude	0.66	404.0	266.6	---
Ammonium sulfate 0-60%	1.82	149.5	272.1	102.1
Sephadex G-150	3.41	67.2	229.2	86.0
Collagen-sepharose	12.10	3.5	42.4	15.9

* Specific Activity= µg collagen solubilized/min/mg protein.

ations. However, the extremely low protein concentrations and the attendant difficulty in their accurate determination make precise measurements of specific activity impossible.

The appearance on polyacrylamide gel electrophoresis of synovial and tadpole collagenases is shown in Figure 2B and 2C respectively. In addition to the electrophoretic criterion for purity of the rheumatoid synovial and tadpole collagenases, the enzymes purified by collagen-Sepharose chromatography elicit monospecific anti-collagenase antisera in rabbits (7,15), a further indication of the purity of these preparations.

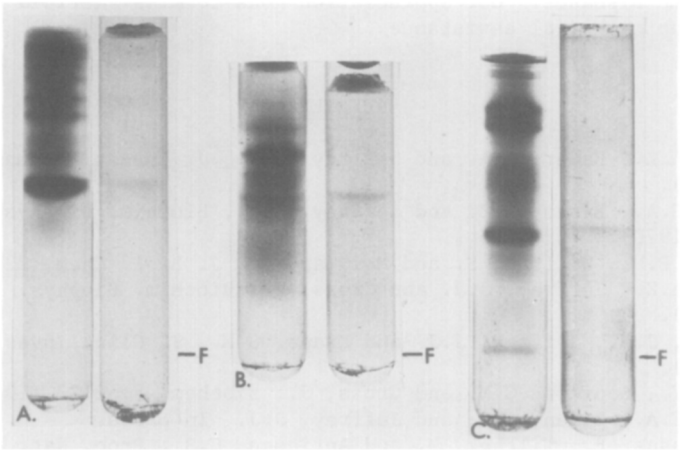

Figure 2. Polyacrylamide gel electrophoresis of vertebrate collagenases purified on collagen-Sepharose. 12.5% gels were used at 5mA per tube. In each case, the gel on the left shows the pattern of the partially purified enzymes. Samples contained 100-150 µg of protein. The gels on the right show the purified collagenases eluted from collagen-Sepharose. These samples contained 50-70 µg of protein. (A) Human skin collagenase. (B) Rheumatoid synovial collagenase. (C) Amphibian collagenase. F = buffer front.

Although good yields of collagenase activity can be obtained by passing crude enzyme preparations through collagen-Sepharose, a number of contaminating proteins also bind to the matrix. Thus, the use of affinity chromatography with collagen-Sepharose after initial partial purification of these enzymes reduces the likelihood that non-specific adsorption of components present in the crude mixtures will occur.

The present study is the first to describe the complete purification of these vertebrate collagenases. Both electrophoretic and immunologic (7,15)

criteria of enzyme purity indicate the feasibility of using affinity chromatography as a simple and reproducible method for obtaining pure preparations of each of these collagenases. Using the pure enzymes thus obtained, it has been possible to study some of the immunologic properties of rheumatoid synovial and tadpole collagenases (7,15). It is now possible to obtain pure vertebrate collagenases in amounts sufficient to examine in detail their physical and enzymologic properties. Such studies are currently in progress.

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REFERENCES

1. Eisen, A.Z., Bauer, E.A. and Jeffrey, J.J., J. Invest. Dermatol. 55, 359 (1970).
2. Bauer, E.A., Eisen, A.Z. and Jeffrey, J.J., Biochim. Biophys. Acta 206, 152 (1970).
3. Gallop, P.M., Seifter, S. and Meilman, E., J. Biol. Chem. 227, 891 (1957).
4. Eisen, A.Z., Jeffrey, J.J. and Gross, J., Biochim. Biophys. Acta 151, 637 (1968).
5. Evanson, J.M., Jeffrey, J.J. and Krane, S.M., J. Clin. Invest. 47, 2639 (1968).
6. Nagai, Y., Lapiere, C.M. and Gross, J., Biochem. 5, 3123 (1966).
7. Bauer, E.A., Eisen, A.Z. and Jeffrey, J.J. In press.
8. Cuatrecasas, P., Wilchek, M. and Anfinsen, C.B., Proc. Nat. Acad. Sci. 61, 636 (1968).
9. Cuatrecasas, P., J. Biol. Chem. 245, 3059 (1970).
10. Gross, J., J. Exp. Med. 107, 247 (1958).
11. Davis, B.J., Ann. N.Y. Acad. Sci. 121, 404 (1964).
12. Jovin, T., Chrambach, A. and Naughton, M.A., Anal. Biochem. 9, 315 (1964).
13. Lowry, O., Rosebrough, N., Farr, A. and Randall, R., J. Biol. Chem. 193, 265 (1951).
14. Bergmann, I. and Loxley, R., Anal. Chem. 35, 1961 (1963).
15. Eisen, A.Z., Bauer, E.A. and Jeffrey, J.J., Proc. Nat. Acad. Sci. 68, 248 (1971).