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IMMUNOLOGIC RELATIONSHIP OF A PURIFIED HUMAN SKIN COLLAGENASE TO OTHER HUMAN AND ANIMAL COLLAGENASES

EUGENE A. BAUER, ARTHUR Z. EISEN AND JOHN J. JEFFREY

Division of Dermatology, Department of Medicine, Washington University School of Medicine, St. Louis, Mo. 63110 (U.S.A.)

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SUMMARY

Antibodies prepared to a number of animal and human collagenases have been used to investigate the relationships between these enzymes. The human skin collagenase used as an immunogen was one of two chromatographically separable peaks of collagenolytic activity obtained from culture media of normal human skin. On the basis of their electrophoretic mobility on polyacrylamide gel, they have been designated as fast-moving or slow-moving human skin collagenase. The fast moving enzyme was further purified and used to produce an anti-human skin collagenase antibody. Anti-fast-moving human skin collagenase antibody gives a reaction of complete identity on immunodiffusion with slow-moving skin collagenase as well as with preparations of human gingival and rheumatoid synovial collagenases. In addition, anti-fast-moving human skin collagenase antibody produces complete inhibition of these other human collagenases, suggesting that these enzymes are closely related proteins.

Antibodies to tadpole and rat uterine collagenases are immunologically not identical when compared with one another or to the human skin enzyme. Crustacean hepatopancreas and bacterial collagenases also fail to react with anti-fast-moving human skin collagenase antibody, further indicating species specificity among collagenases.

INTRODUCTION

Collagenases have now been isolated from a number of animal[1–3] and human tissues[4–9]. Most of these enzymes are not directly extractable from tissues, but can only be isolated from culture media in which the tissues have been grown. The collagenases from human granulocytes[9] and crustacean hepatopancreas[3] differ in this respect and are extractable from tissue.

All human collagenases, so far isolated and characterized, are capable of degrading collagen in native fibrillar form at physiologic pH and temperature. In solution these enzymes are characterized by their ability to cleave the native collagen molecule without disrupting its helical structure[7–9]. In addition, the specificity of these col-

lagenases on the collagen molecule is remarkably similar in that they all cleave the native molecule across its three constituent polypeptide chains, producing two fragments, one three-quarters and the other one-quarter the length of the molecule. This mode of action of human collagenase is very similar, if not identical, to that of the collagenase isolated from tadpole tailfin[10,11]. Although these and certain other biochemical similarities exist between human and tadpole enzymes[7–9], a more precise comparison between collagenases as proteins had not been possible since none of the human enzymes has, so far, been obtained in pure form and the amphibian collagenase has only been partially purified[12].

Antibodies produced to a number of different enzymes have proven useful in studying the relationships between these enzymes and analogous enzymes from other species or from individual tissues of an organism[13–17]. Since both animal and human collagenases exhibit a number of biochemical similarities, it seemed possible that specific antibodies to various collagenases would be of value in elucidating the degree of organ heterogeneity among these enzymes and in determining whether or not they are antigenically species specific.

This paper deals with the use of anti-collagenase antibodies produced against a purified human skin collagenase to assess the immunologic relationships of this enzyme to a variety of other human and animal collagenases.

METHODS

Normal human skin was cultured in Dulbecco's Modified Eagle's medium at 37° in an atmosphere of O_2–CO_2 (95:5, v/v) as previously described[7]. Culture medium was changed daily for 10 days and media having collagenolytic activity were pooled, dialyzed at 4° against several changes of distilled water, lyophilized and stored at −20°. Specimens of human gingival tissue, rheumatoid synovium and *post-partum* rat uterus were cultured in a similar fashion. Tadpole tailfin (*Rana catesbeiana*) was cultured as described by NAGAI *et al.*[12].

Enzyme purification

Crude human skin collagenase powder was dissolved in 0.05 M Tris–HCl (pH 7.5) with 0.005 M $CaCl_2$ to a concentration of approx. 20.0 mg protein per ml. $(NH_4)_2SO_4$ fractionation was carried out at pH 7 and 0° to a final saturation of 50%. The 50% precipitate was dissolved in 0.05 M Tris–HCl (pH 7.5) with 0.005 M $CaCl_2$ and dialyzed against large volumes of the same solution.

Gel filtration was performed using reverse flow at 4° on a column (1.2 cm × 100 cm) of Sephadex G-150 equilibrated with 0.05 M Tris–HCl (pH 7.5) containing 0.005 M $CaCl_2$ and 0.2 M NaCl. Fractions having collagenolytic activity were pooled, dialyzed against 0.05 M Tris–HCl (pH 7.5) with 0.005 M $CaCl_2$ and concentrated by pressure dialysis at 4°. Further purification was obtained on a column (0.9 cm × 60 cm) of Sephadex G-75 equilibrated with the same buffer.

Human gingival and rheumatoid synovial collagenases were obtained from crude enzyme solutions following precipitation with $(NH_4)_2SO_4$ to 50% saturation. Tadpole collagenase was partially purified as described by NAGAI *et al.*[12]. Further purification of this enzyme was achieved by preparative electrophoresis on polyacrylamide gel[18]. Highly purified preparations of rat uterus and crustacean hepato-

pancreas collagenases were obtained by chromatographic techniques to be described in detail elsewhere. Partially purified bacterial collagenase (*Clostridium histolyticum*) was obtained commercially (Worthington Biochemical Corp.).

Preparation of anti-enzyme antisera

Adult male, white rabbits weighing 5–6 kg were injected initially with 0.6 mg of purified human skin collagenase in complete Freund's adjuvant. Rabbits whose sera showed precipitating antibodies were given a booster injection at 3 weeks and bled again 1 week later. Sera were pooled and taken to 33% saturation at 0° with $(NH_4)_2SO_4$ (pH 7.0). This method was found to give a good yield of γ-globulin while excluding those serum proteins[19] which have an inhibitory effect on collagenase[20]. The $(NH_4)_2SO_4$ precipitate was dissolved in 0.05 M Tris–HCl (pH 7.5) containing 0.1 M NaCl, dialyzed against the same buffer and adjusted to a concentration of 25 mg protein per ml before use.

The rat uterus collagenase used for immunization contained only a single protein band as determined by disc electrophoresis. Tadpole collagenase, however, was not homogeneous and contained four protein components. γ-Globulin preparations against these two enzymes were prepared in a fashion similar to that used for human skin collagenase.

Immunodiffusion and immunoelectrophoresis

Gel diffusion was done according to OUCHTERLONY[21]. Micro-immunoelectrophoresis[22] was performed on 0.5% Ionagar (Colab) buffered with 0.05 M Tris–HCl (pH 7.5) containing 0.1 M NaCl. Enzyme preparations were subjected to electrophoresis at 4° for 45 min at 300 V in Tris–HCl buffer (pH 7.4). Following electrophoresis the antibody preparations were allowed to react at 25° for 48 h.

Enzyme inhibition

Increasing amounts of anti-human skin collagenase antibody were added as γ-globulin fraction to a constant volume of enzyme and preincubated for 1–2 h at 25°, for 1 h at 37° and overnight at 4°. The resulting precipitates were removed by centrifugation and residual collagenolytic activity in the supernatant determined by lysis of [^{14}C]collagen gels at 37° for 12 h.

Assays

Collagenase activity was determined by a method that depends on the enzymatic release of soluble [^{14}C]glycine containing peptides from native reconstituted, guinea pig skin collagen fibrils[7,12]. Noncollagenolytic protease activity was determined at neutral pH using casein as a substrate[23]. Protein was determined by the method of LOWRY *et al.*[24].

RESULTS

Enzyme preparations

Human skin collagenase separated into two peaks of active material following gel filtration on Sephadex G-150 (Fig. 1). When each area of activity was subsequently chromatographed on G-75, Peak I emerged with the void volume, whereas Peak II

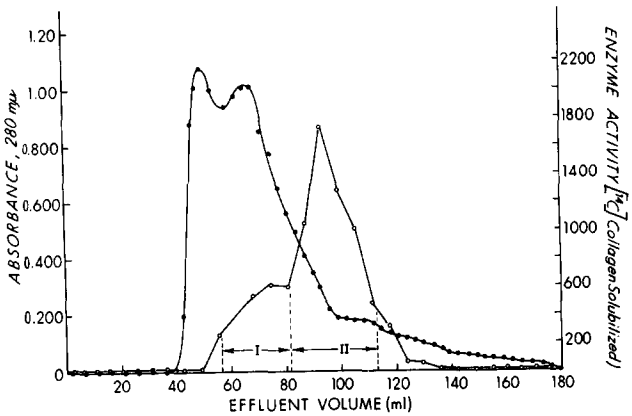


Fig. 1. Gel filtration of human skin collagenase separated on Sephadex G-150 with 0.05 M Tris–HCl (pH 7.5) containing 0.2 M NaCl and 0.005 M $CaCl_2$, as eluant. A sample of 50 mg was applied to the column. Effluent fractions of 3.5 ml were collected at a rate of 10.5 ml/h. ●—●, absorbance at 280 mμ; ○—○, enzyme activity.

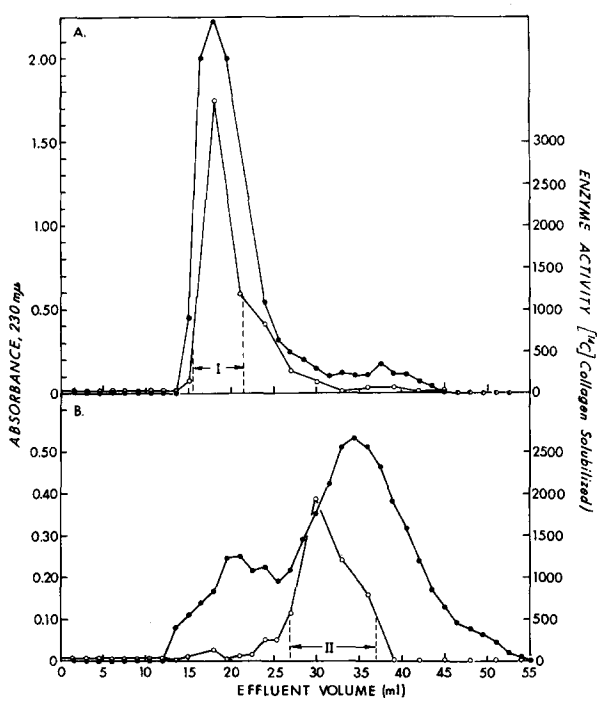


Fig. 2. Rechromatography on Sephadex G-75 of two peaks (I and II) of human skin collagenase previously separated by Sephadex G-150. The column was 0.9 cm × 60 cm and effluent fractions of 1.5 ml were collected. ●—●, absorbance at 230 mμ; ○—○, enzyme activity. A. Peak I from Sephadex G-150. A sample of 30 mg was applied in a volume of 1.5 ml. B. Peak II from Sephadex G-150. A sample of 1.5 mg was applied in a volume of 1.5 ml.

was retarded by the G-75 (Fig. 2). On polyacrylamide gel electrophoresis Peak I was found to be a heterogeneous mixture migrating slower than Peak II, which appeared as a single band near the buffer front (Fig. 3). The faster migrating human skin collagenase preparation(HSC_f) cleaved the collagen molecule in a fashion identical to that originally described for crude human skin collagenase and was found to be free of noncollagenase proteolytic activity. This electrophoretically pure protein was used for immunization.

Immunologic properties

On immunodiffusion, anti-fast-moving human skin collagenase antibody gave a single precipitin band with its antigen, fast-moving human skin collagenase (Fig. 4A). Anti-rat uterus antibody similarly yielded a single precipitin band when reacted with crude enzyme solution. Anti-tadpole antibody reacted against crude enzyme solutions

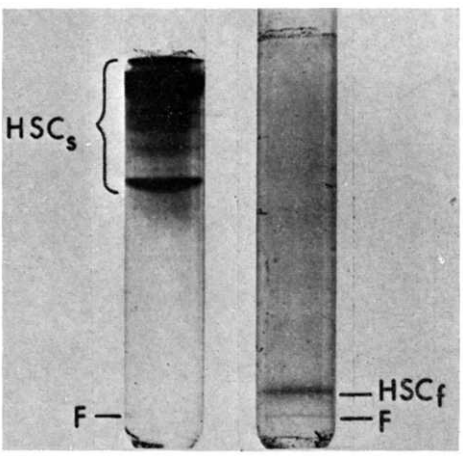


Fig. 3. Polyacrylamide gel electrophoresis of human skin collagenase following rechromatography of Peaks I and II on Sephadex G-75 (see text for details). 75 μg protein was applied to the upper gel. Electrophoresis was carried out in 12.5% acrylamide at a constant current of 5 mA per tube. Gel on the left shows the heterogeneous, slow-moving enzyme (HSC_s). On the right is the fast-moving enzyme (HSC_f). F = buffer front.

displayed several precipitin bands, probably reflecting the relative impurity of the immunogen. When fast-moving human skin collagenase and the human skin collagenase preparation that migrates more slowly on polyacrylamide electrophoresis (slow-moving humin skin collagenase) (HSC_s) were allowed to react with anti-fast-moving human skin collagenase antibody a reaction of complete identity occurred (Fig. 4A). Anti-fast-moving human skin collagenase antibody also demonstrated identity with both human gingival and rheumatoid synovial collagenase preparations (Fig. 4B) indicating that major antigenic determinants are shared by human skin collagenase and these other enzymes. In contrast, when precipitin reactions between human skin collagenase (fast-moving) and its antibody were compared to reactions between tadpole collagenase and its respective antibody, the relationship was one of nonidentity (Fig. 4C). Similar results were obtained with rat uterine collagenase and its antibody (Fig. 4D). Furthermore, no reactions were seen on immunodiffusion when anti-fast-

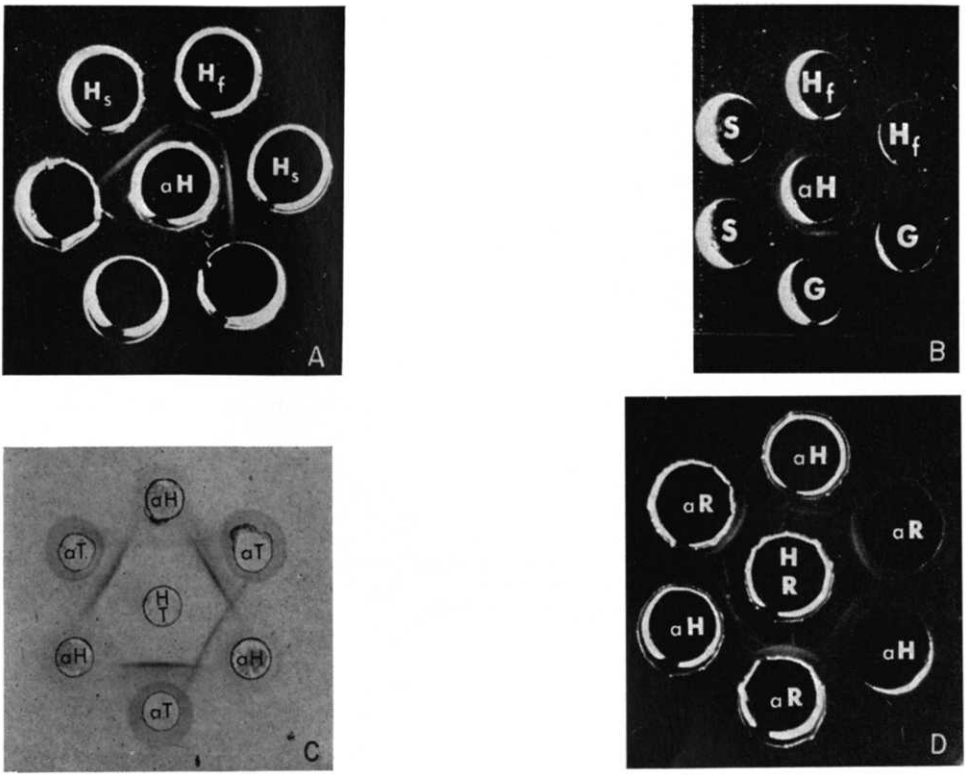


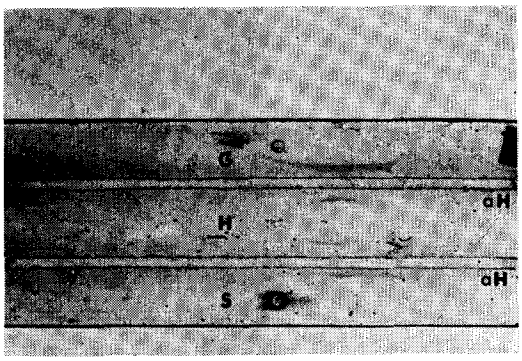
Fig. 4. Gel diffusion analysis of antisera against human skin, rat uterus and tadpole collagenases. A. H_f, fast-moving human skin collagenase; H_s, slow-moving human skin collagenase ;aH, fast-moving human skin collagenase antibody. B. H_f, fast-moving human skin collagenase; G, gingival collagenase; S, rheumatoid synovial collagenase. C. H, fast-moving human skin collagenase; T, tadpole collagenase; aH, anti-fast-moving human skin collagenase antiserum; aT, anti-tadpole collagenase antiserum. D. H, fast-moving human skin collagenase; R, rat uterus collagenase; aH, anti-fast-moving human skin collagenase antiserum; aR, anti-rat uterus collagenase antiserum.

moving human skin collagenase, anti-tadpole or anti-rat uterine antibodies were reacted with crustacean hepatopancreas or bacterial collagenases.

Further evidence for cross-reactivity between human collagenases was obtained when human skin (fast-moving), synovial and gingival collagenases were subjected to immunoelectrophoresis. Under these conditions the enzyme preparations all migrated toward the cathode and gave closely corresponding precipitin arcs with anti-fast-moving human skin collagenase antibody (Fig. 5). The double precipitin arc seen opposite the synovial collagenase may indicate the presence of two enzymes which, although electrophoretically separable, gave a pattern of identity with fasts moving human skin collagenase by immunodiffusion (Fig. 4B).

Enzyme inhibition

Anti-fast-moving human skin collagenase antibody produced almost complete inhibition of human skin, rheumatoid synovial and gingival collagenases (Fig. 6). The neutralization curves obtained with skin enzyme were similar whether a crude



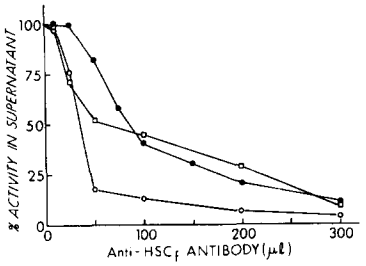


Fig. 5. Immunoelectrophoresis of human skin (H), gingival (G) and rheumatoid synovial (S) collagenases and their subsequent reaction with anti-fast-moving human skin collagenase antiserum (aH). Cathode is at the right.

Fig. 6. Inhibition of collagenase by anti-human skin collagenase antibody. Reactions were carried out using 40 μl of purified fast-moving human skin collagenase at a protein concentration of 1.0 mg/ml. Neutralization curves for rheumatoid synovial and gingival collagenases were obtained using 40 μl of a 50% $(NH_4)_2SO_4$ sulfate preparation containing 5.0 mg protein per ml. Varying amounts of anti-fast-moving human skin collagenase (HSC_f) γ-globulin, at a protein concentration of 25 mg/ml, were added to a final volume of 340 μl. ●—●, human skin collagenase; □—□, gingival collagenase; ○—○, synovial collagenase.

preparation of human skin collagenase or fast-moving human skin collagenase was used. In addition, virtually identical inhibition curves were obtained with rheumatoid synovial and gingival collagenases. In contrast, anti-tadpole collagenase antibody was incapable of inhibiting human collagenases.

Although whole rabbit serum itself can inhibit these enzymes, γ-globulin preparations from nonimmunized animals produced less than 10% inhibition at the highest concentration of γ-globulin used in the experiments. This low level of inhibition was probably due to the contamination of the γ-globulins with small amounts of α-globulins, which have been found to inhibit human skin collagenase[20].

DISCUSSION

Human skin collagenase shares a number of biological and biochemical properties with other human collagenases as well as with the amphibian enzyme. The enzymes appear to be synthesized *de novo* only from viable cells and are not detectable in culture media until approx. 24–48 h of incubation[5,7,8,10,12]. In addition, enzyme production is inhibited by freeze-thawing and, in the case of skin and tadpole tailfin, by puromycin[25,26]. All of these enzymes have similar pH optima and are inhibited by EDTA[7,8,12] and by human serum[7,8,20]. From results obtained by gel electrophoresis and from electron microscope studies of segment long spacings, it has also been shown that human[7], synovial[8], gingival (unpublished observations) and tadpole[10,11] collagenases cleave the collagen molecule in a susceptible region, one-quarter the distance from the "B" (C-terminal) end. In addition, studies of tadpole enzyme–collagen reaction mixtures have shown that cleavage of the collagen molecule by this enzyme results in the release of N-terminal leucine and isoleucine and C-terminal glycine[10,27]. Our recent observations (unpublished) indicate that human skin collagenase also

liberates N-terminal leucine and isoleucine after cleavage and, thus may attack the same peptide bond as does the tadpole enzyme. The collagenase from rat uterus[2] and crustacean hepatopancreas[3] also produce these two fragments initially but differ from the human and amphibian enzymes in that they continue to digest the cut end of the three-quarter length fragment resulting in a number of smaller fragments.

In spite of the many similarities between human skin and tadpole collagenases, on an immunologic basis, these enzymes are not identical. The fact that the two enzymatic activities are so alike, despite immunologic differences, suggests that the active sites are probably similar and have been preserved phylogenetically[13]. The presence of species specificity among collagenases is demonstrated further by the fact that fast-moving human skin collagenase and rat uterine collagenase antisera give a reaction of nonidentity and anti-fast-moving human skin collagenase shows no reaction either to crustacean hepatopancreas or to bacterial collagenase. In addition, the immunologic evidence presented demonstrates clearly that human collagenases from skin, synovium and gingiva, share significant antigenic determinants and are closely related. It remains to be determined, however, whether the human enzymes are identical in all respects.

Of interest is the presence in the media from human skin cultures of two species of skin collagenase. Whether this represents two distinct enzymes or whether fast-moving human skin collagenase is a subunit of the electrophoretically slow-moving collagenase is not, as yet, known. It is clear, however, that not only are fast-moving and slow-moving human skin collagenases immunologically identical but are also closely related to other human collagenases. Antibodies to purified fast-moving human skin collagenase should, therefore, serve as a valuable tool for further investigating the biological properties of human collagenases.

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