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A COMPONENT OF NORMAL HUMAN SERUM WHICH ENHANCES THE ACTIVITY OF VERTEBRATE COLLAGENASES

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Summary: The activity of vertebrate collagenase is increased by approximately 3-fold in the presence of saturating amounts of a macromolecule found in normal human serum. The activities of collagenases from human skin, rat skin, and tadpole tailfin are all markedly enhanced in the presence of this molecule, but activities of bacterial collagenase, trypsin, chymotrypsin, thermolysin, and a gelatin-specific neutral protease from human skin are unchanged. The enhancer itself has no proteolytic activity and does not change the normal cleavage products of human skin collagenase. The collagenase enhancer is an extremely stable molecule. It is resistant to heat, to extremes of pH at physiological temperature, and appears to be protein in nature. Of particular interest is the requirement that the collagen substrate be in fibrillar form in order for the enhancer to be effective.

Collagenase is an extracellular neutral protease, which is unique in its ability to initiate the enzymatic breakdown of collagen. Because collagenase is a key enzyme in processes of connective tissue remodeling, molecules which modify its activity could be most important in the regulation of collagen turnover at a local level. A number of collagenase inhibitors, derived both from serum (1-4) and from cultured fibroblasts (5) have now been described. However, protein activators, which exist for several enzymes (6-13), have not as yet been demonstrated for any proteases. In this paper, we report the existence of a macromolecule from normal human serum which is a potent enhancer of vertebrate collagenase activity. This molecule is specific to vertebrate collagenases and exerts its effect only when the collagen substrate is in the fibrillar conformation found in tissues. These findings suggest that enhancement of collagenase by this serum component could have important implications for the modulation of enzyme activity in vivo.

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MATERIALS AND METHODS

Materials

Trypsin, chymotrypsin, thermolysin, RNase, DNase, pronase, clostridial collagenase type I, heparin and dextran sulfate were obtained from Sigma Chemical, St. Louis, Missouri.

Preparation of Enhancer

Crude collagenase enhancer was prepared from a 4% solution of human serum in 0.05 M Tris, pH 7.5, 0.15 M NaCl which had been treated in one of two ways to inactivate or remove serum protease inhibitors. In most cases, serum was heated at 80°C for 15 minutes to inactivate the inhibitors. In addition, ammonium sulfate fractionation of unheated serum yielded an inhibitor-free precipitate (0-30% saturation) which contained the crude enhancer.

Preparation of Human Skin Collagenase

Medium obtained from explants of human skin in serum-free organ culture was the source of collagenase, as previously described (14). The crude collagenase was subjected to ammonium sulfate fractionation and then purified by chromatography on CM-cellulose using the method of Stricklin et al. (15). The resulting enzyme preparations are purified about 130 fold. All the collagenase in these preparations was fully active. Crude rat skin and tadpole tailfin collagenases were obtained from organ culture (16)(17), and used without further purification.

Assay Procedures

Collagenase activity was determined by the release of soluble peptides from [^{14}C]glycine-labeled reconstituted native collagen fibrils (17). The collagen was allowed to gel at 37°C for at least 24 h to complete the aggregation process. All collagen preparations employed had specific activities of approximately 30,000 cpm/mg. Incubation of the fibrils with 0.01% trypsin resulted in the release of less than 8% of the total radioactivity, indicating that the collagen fibrils were in helical conformation.

A typical reaction mixture contained 200 µg [^{14}C]glycine-labeled collagen fibrils (approximately 6,000 cpm) in a gel volume of 50 µl, and 175 µl total volumes of 0.05 M Tris, pH 7.5, 0.005 M $CaCl_2$, containing 5 µg purified human skin collagenase with or without enhancer. Enhancer dilutions were always added in a volume of 50 µl, containing 50 µg protein or less. After an appropriate period of incubation, from 1 to 4 h at 37°C, the assay was terminated by centrifugation at 12,000 x g for 10 minutes, and the entire supernatant counted in a liquid scintillation spectrometer.

Gelatin specific protease was assayed by the method of Harris and Krane (18) using [^{14}C]labeled heat-denatured collagen as the substrate. Activities of trypsin, chymotrypsin, and thermolysin were assayed by measuring trichloroacetic acid soluble peptides resulting from proteolysis of casein(19).

RESULTS

When protease inhibitors were removed from human serum either by salt fractionation or as a result of heat inactivation, a non-dialyzable molecule

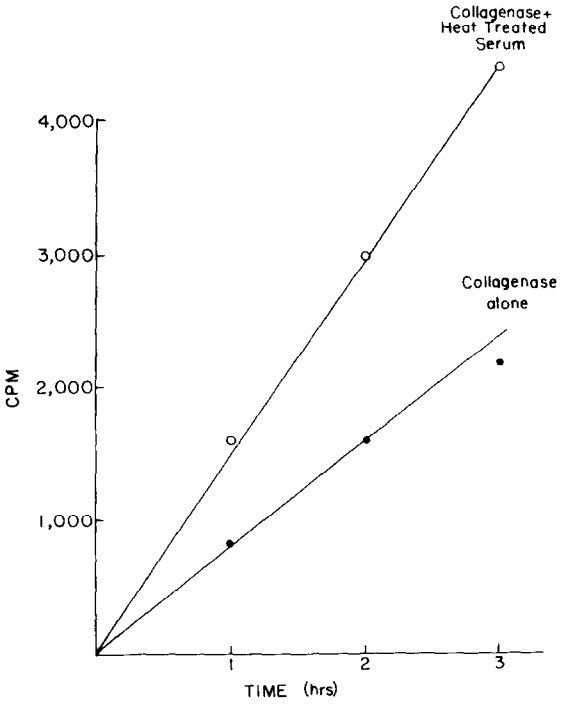


Figure 1. The effect of serum enhancer on the collagenase catalyzed digestion of collagen. Purified collagenase (5 µg protein) was incubated with [^{14}C]glycine-labeled collagen with and without 50 µl of 4% human serum which had been previously heated at 80°C for 15 min. Parallel reactions were terminated by centrifugation at the times indicated, and the solubilized collagen counted in a scintillation spectrophotometer.

remained which, when added to human skin collagenase, significantly increased the amount of collagen solubilized. Figures 1 and 2 show the effect of heat treated serum on the rate of the collagenase-catalyzed hydrolysis of labeled collagen and the effect of concentration on the enhancement. At saturating concentrations of serum there was a maximum activity increase of 2-4 fold. Figure 3 demonstrates that this enhancement was constant over a 3-fold concentration of collagenase. Both crude and highly purified human skin collagenase were equally affected by interaction with this molecule.

Of particular interest was the observation that enhancement occurred only when the collagen substrate was in the native fibrillar form. When collagen

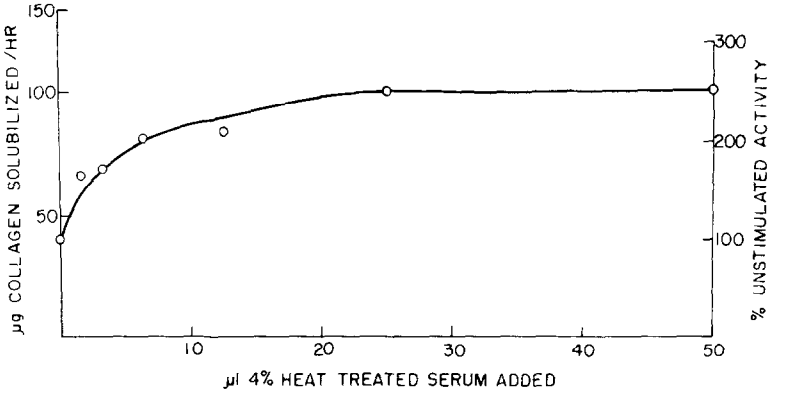


Figure 2. Effect of concentration of heat-treated serum on the enhancement of collagenase activity. Increasing amounts of 4% heated serum were added to human skin collagenase (5 µg protein) and the reaction incubated for 90 min. at 37°C.

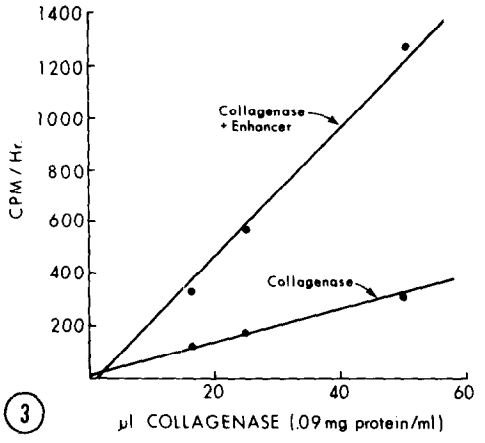
TABLE I

ENHANCEMENT OF COLLAGENASE ACTIVITY ON COLLAGEN FIBRILS AT 27°C AND 37°C

Incubation Temperature	Collagenase Activity, cpm		Fold Enhancement
	Collagenase Alone	Collagenase + Enhancer	
27°C	306	1002	3.3
37°C	558	1698	3.0

Fibrils were formed at 37°C, incubated at 27°C. The same preparation of collagenase and enhancer were used at both temperatures. The assay at 27°C was incubated for 18 hours, while that at 37°C was incubated for 2 hours. The reaction at 27°C was terminated with 20 mM EDTA and the gel incubated at 37°C for 5 min. before centrifugation.

in solution (27°C) was employed, no enhancement of collagenase activity could be demonstrated. However, when collagen fibrils were used at the same temperature (27°C), the degree of enhancement produced was identical to that observed at 37°C (Table I). Furthermore, in the presence of serum enhancer, the products of collagenase activity on the fibrillar substrate at 27°C were the normal



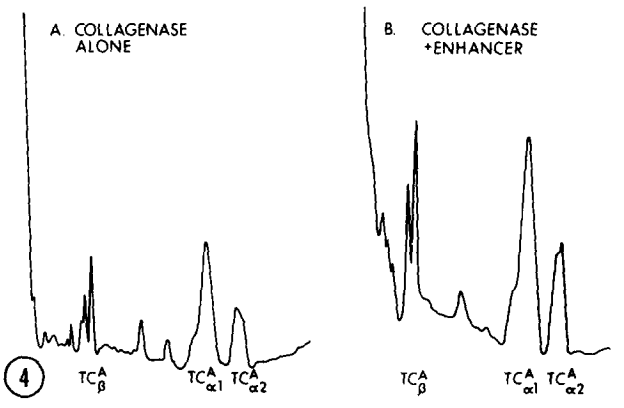


Figure 3. Enhancement of collagenase activity as a function of enzyme concentration. 50 µl of 4% serum heated to 80°C for 15 minutes was added to the concentrations of collagenase indicated and the reaction incubated for 90 minutes at 37°C.

Figure 4. Densitometric scans of acrylamide gels showing soluble cleavage products from collagenase incubated at 27°C with fibrillar collagen. The increase in product detected by the scans was equivalent to the observed increase in activity as shown in Table I. a) Collagenase alone. b) Collagenase and enhancer.

3/4-1/4 cleavage fragments previously reported for human skin collagenase (14) (Fig. 4 a,b). Thus, collagenase produced no additional cleavages in the collagen molecule in the presence of the enhancer. Enhancer preparations themselves displayed no proteolytic activity toward either collagen or gelatin.

The enhancement of the activity of purified collagenase could not be explained simply as a protein protective effect. Bovine serum albumin, when added in concentrations up to 0.5 mg per assay mixture, failed to increase collagenase activity, whereas partially purified enhancer preparations (approximately 4 µg of protein) produced maximum enhancement of collagenase activity. Furthermore, unlike their effect on mouse bone collagenase (20), heparin and dextran sulfate failed to affect appreciably the activity of human skin collagenase.

The specificity of the enhancer was examined using a variety of proteases. As shown in Table II, concentrations of enhancer which fully activate human skin

TABLE II

EFFECT OF COLLAGENASE ENHANCER FROM HUMAN SERUM ON COLLAGENASES FROM VARIOUS SOURCES AND ON SELECTED PROTEOLYTIC ENZYMES

Enzyme Source	Enzyme Alone	Enzyme + Enhancer	Fold Increase
	cpm - Blank		
Human Skin Collagenase	1279	3096	2.4
Rat Skin Collagenase	398	758	1.9
Tadpole Collagenase	508	953	1.8
Bacterial Collagenase	2531	3114	1.2
Human Skin Gelatin - Specific Protease	974	744	0.8
	O.D.		
Trypsin	0.405	0.381	0.9
Chymotrypsin	0.561	0.555	1.0
Thermolysin	0.864	0.864	1.0

In the assays on [^{14}C]collagen and [^{14}C]denatured collagen (gelatin-specific protease), enzyme was incubated for 90 minutes at 37°C with 50 µl of 4% heat-treated serum. In the trypsin, chymotrypsin and thermolysin assays, 5 µg trypsin and chymotrypsin and 0.25 µg thermolysin were incubated with 2 ml of 0.5% casein and 300 µl of crude enhancer for 30 minutes. This amount of enhancer is 5-fold in excess of that necessary to fully activate human skin collagenase.

Blanks are complete reaction mixtures incubated with substrate in the absence of enzyme.

collagenase were also capable of increasing the activity of crude rat skin and tadpole tailfin collagenases. Bacterial collagenase from Clostridium histolyticum, however, showed no significant activation in the presence of heat-treated serum. In contrast to human skin collagenase, a gelatin-specific neutral protease found in organ cultures of human skin (unpublished) was unaffected by the serum enhancer (Table II). Finally neither trypsin, chymotrypsin, nor thermolysin was affected

by concentrations of heat-treated serum capable of fully stimulating human skin collagenase.

The collagenase enhancer is extremely stable, retaining 100% activity at 80°C for 20 minutes. However, 90% of the activity was lost during the same interval at 100°C. Exposure to extremes of pH, from pH 2-12, for 1 h at either 0°C or 37°C, had no effect. The enhancer was resistant to digestion by DNase, RNase, trypsin, chymotrypsin and thermolysin but was destroyed by incubation with pronase.

DISCUSSION

Protein activators have now been described for a variety of enzymes of disparate catalytic activity. The best described are those for cyclic AMP phosphodiesterases (6), RNA polymerases (7), phenylalanine hydroxylase (8,9) and galactosyl transferase (10). To date, however, none has been described for a proteolytic enzyme, and the molecule described here appears to be the first such entity. The mechanism of action of the enhancer is unknown at this time, but it is unlikely that it affects the specificity of the collagenase, since the products of the activated collagenase are identical to those produced by the enzyme in the absence of the enhancer. Furthermore, the enhancer appears to possess no proteolytic activity of its own. Thus, it is most likely that this molecule acts by changing the kinetic properties of collagenase. In view of the fact that enhancement requires the fibrillar form of the substrate, it is tempting to speculate that the enhancer acts to promote proper alignment of the enzyme with the specific locus in the collagen molecule at which collagenolytic cleavage occurs. Such a mechanism has been proposed for the activators of a number of DNA-dependent RNA polymerases (21-23).

It should be emphasized that the increase in collagenase activity in the presence of serum enhancer is not due to conversion of a zymogen to its active form. The human skin collagenase used in these studies contains no proenzyme (15)

Furthermore, repeated attempts to activate purified collagenase proenzyme with enhancer preparations gave negative results (data not shown).

The collagenase enhancer appears to share some properties with other protein activators described to date, such as its stability to heat and to acid. The inactivation by pronase has led us to the tentative conclusion that this molecule is at least partly protein in nature. Additionally, preliminary evidence suggests that the enhancer is of relatively low molecular weight-approximately 20,000 daltons (unpublished).

The physiological role and the cells responsible for the production of the collagenase enhancer is presently unknown. It seems, especially in view of the requirement for fibrillar collagen, that the enhancer could play an important role in the control of tissue remodeling processes at a local level, both in normal and in pathological states.

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