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Gelatin: A Poor Substrate for a Mammalian Collagenase

Abstract. *A rabbit tumor collagenase was purified more than 5000-fold. In this form it degrades native collagen in helical conformation at 37°C, pH 7.6, into two fragments, but it had little capacity to cleave gelatin, an indication of the importance of higher-order structure of substrate for this enzyme in pure form. It is likely that, in vivo, enzymes other than collagenase degrade gelatin polypeptides produced by primary collagenolysis.*

Collagen in native conformation is composed of three helical polypeptide (α) chains, each of which is wound around a common axis to give a coiled-coil structure (*1*). It is believed that in vertebrates collagen is degraded by specific enzymes (*2*). These collagenases cleave across the three polypeptide chains at a point three-fourths distant from the amino terminus, yielding two fragments. The data of Sakai and Gross suggest that at physiologic temperatures the two fragments spontaneously denature, and as nonhelical gelatin polypeptides they are susceptible to further degradation by this same collagenase or other proteases (*3*).

It is not known whether, after initial cleavage of the molecule, the collagenase itself has specificity to cleave further the nonhelical gelatin polypeptides it has produced. Two pieces of data suggest that, if this specificity is present, it is not strong. (i) Rheumatoid synovial tissue in culture in vitro synthesizes a neutral protease capable of degrading gelatin to small (<10,000 daltons) fragments at a rate much faster than synovial collagenase (*4*). A similar peptidase is associated with tadpole collagenase (*5*). (ii) Rheumatoid subcutaneous nodule tissue in primary culture synthesizes and releases a collagenase which at 27°C degrades collagen predominantly into two fragments corresponding to TC^A (75 percent of the α chain of collagen) and TC^B (the carboxy terminal 25 percent of the α chain) (*6*). Although 10 percent of the gelatin was broken down to small (<10,000 daltons) fragments, this was explained by the presence of contaminating protease in the enzyme. It was hypothesized that "*in vivo*, after the initial cleavage of collagen into two fragments by collagenase, . . . subsequent breakdown of these primary products of collagenolysis is accomplished by proteases" (*6*). We now present evidence to support this hypothesis. We have demonstrated that a highly purified mammalian collagenase had little specificity for cleavage of intact gelatin or TC^A or TC^B fragments at physiologic temperatures, and that the helical, secondary conformation of collagen provides needed structure for recognition and catalysis by the collagenase.

Collagenase was purified from homogenates of V_2 ascites cell carcinoma that grew after tumor cells were injected intramuscularly into the thigh muscles of adult rabbits (*7*). This rabbit tumor collagenase (RTC) was shown to be preformed in the tumor and was not synthesized in culture in vitro (*7*). When the homogenate was centrifuged, the collagenase sedimented with the nuclear fraction of the cells; it showed maximum activity at *p*H 7 to 8 and was inhibited by the same kinds of compounds (chelating agents, sulfhydryl-containing compounds, and serum) that inhibited the rheumatoid synovial collagenase. It degraded collagen in solution at 24°C into TC^A and TC^B fragments which could be precipitated to form segment-long-spacing aggregates which, when visualized by electron microscopy, confirmed that cleavage occurred at a locus 75 percent from one end of the molecule (*7*).

The enzyme was purified by extraction of frozen and thawed tumor ho-

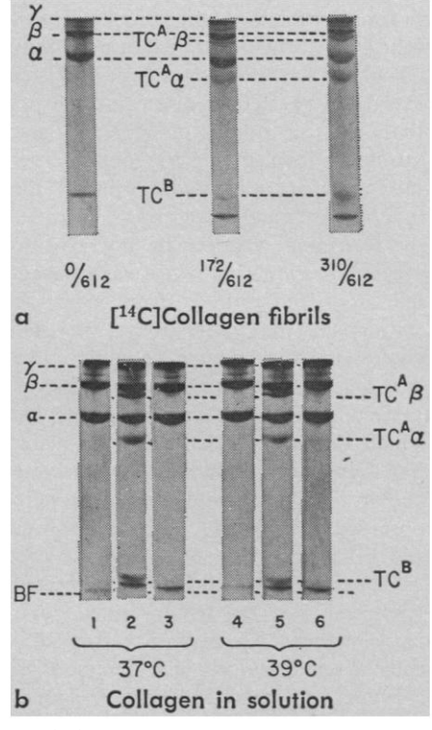

Fig. 1. (a) Polyacrylamide disc gel electrophoretic patterns of reaction mixtures of RTC and [^{14}C]collagen fibrils incubated at 37°C. Each reaction contained 50 μl of 0.4 percent [^{14}C]collagen in 0.2*M* NaCl (*p*H 7.6) (allowed to form fibrils by incubation at 37°C for 16 hours), to which was added 8 μg of RTC diluted to 200 μl in 0.1*M* tris · HCl, *p*H 7.6, 0.005*M* $CaCl_2$ (TC). At three intervals (0, 2, and 4 hours) two tubes were removed from incubation. One tube was centrifuged at 15,000 rev/min for 4 minutes at room temperature; the radioactivity in the supernatant was determined, the number of counts per minute in the supernatant of control gels was subtracted from this, and the results—0/612, 172/612, and 310/612—indicate the radioactivity in the sample supernatant expressed as a fraction of total radioactivity. The reaction in the other tube was halted by addition of ethylenediaminetetraacetate (EDTA), and remaining fibrils were solubilized by cooling to 4°C and subsequent addition of a small amount of 1.0*M* acetic acid. Portions of the solubilized reaction mixture were used for disc gel electrophoresis. (b) Results of incubation of collagen and gelatin in solution at 37°C with and without collagenase. All reactions were carried out in semimicro viscometers. Each reaction mixture contained 200 μl of gelatin (produced by 10 minutes of incubation at 45°C) or native collagen, 19 μg of RTC in 20 μl of TC or an equivalent amount of TC buffer without enzyme, 580 μl of 0.1*M* tris · HCl (*p*H 7.6), 0.2*M* NaCl, 0.005*M* $CaCl_2$, and 100 μl of 0.5*M* D-arginine (*p*H 7.6) to a final volume of 1.0 ml. After 20 minutes of incubation at 37°C or 39°C each reaction was stopped by additions of EDTA. Portions (75 μl) were subjected to electrophoresis on polyacrylamide gel and stained. Numbers correspond to samples plotted as a function of specific viscosity (η_{sp}) in Fig. 2: 1, control collagen, 37°C; 2, collagen plus RTC, 37°C; 3, gelatin plus RTC, 37°C; 4, control collagen, 39°C; 5, collagen plus RTC, 39°C; and 6, gelatin plus RTC, 39°C; *BF*, buffer front.

mogenate with 0.1*M* tris·HCl, *p*H 7.6, 0.005*M* $CaCl_2$, ammonium sulfate precipitation, elution from DEAE–Sephadex A-50, and a subsequent elution from a column (1.5 by 60 cm) of Bio-Gel A-1.5. The enzyme had a specific activity [measured as ^{14}C-labeled collagen solubilized (counts per minute) per milligram of protein] more than 5000-fold that found in the original homogenates.

Collagen was obtained by injecting guinea pigs with ^{14}C-labeled glycine and proline 6 hours before the animals were killed. The skin was removed and collagen was extracted and purified (*8*, *9*).

Viscometric studies were carried out in Cannon-Manning semimicro viscometers in a water bath with temperature controlled at ± 0.1°C. L-Arginine (0.05*M* final concentration) was added to each reaction mixture to prevent fibril formation of collagen at higher temperatures (*10*). We determined previously that L-arginine does not alter the rate of cleavage of collagen in solution by collagenase. Reaction products were monitored by polyacrylamide disc gel electrophoresis (*11*).

Figure 1a shows acrylamide gels of reaction products of RTC and reconstituted [^{14}C]collagen fibrils at 37°C. The gels showed that: (i) the collagen molecules in fibrils at 37°C were cleaved to fragments consistent with TC^A and TC^B and that no smaller fragments were produced; and that (ii) the radioactivity in supernatants of matched specimens roughly paralleled the percent degradation of α and β chains to TC^A and TC^B seen on gels, suggesting that once cleaved, fragments of collagen are incapable of remaining attached to fibrils.

Having demonstrated that RTC was incapable of cleaving further the gelatin fragments produced by its action upon intact collagen, we examined the effect of RTC on intact gelatin chains at 37°C and at 39°C. This latter temperature is above the denaturation temperature of collagen in solution and was chosen to prevent any significant folding of gelatin to collagen, a process that occurs to a limited degree in solutions of gelatin at temperatures below the denaturation temperature (37°C) of collagen in solution. No change in the very low specific viscosity of gelatin and collagenase was noted (Fig. 2). If allowance is made for the fall in viscosity of the control collagen at 39°C compared with 37°C, it can be seen that the rate of cleavage of collagen by the enzyme at 39°C was less than at 37°C. There was a faint suggestion of TC^A in the reaction mixture of gelatin and RTC (No. 6) at 39°C, but it is clear that gelatin is a poor substrate for collagenase at either 37°C or 39°C (Fig. 1b).

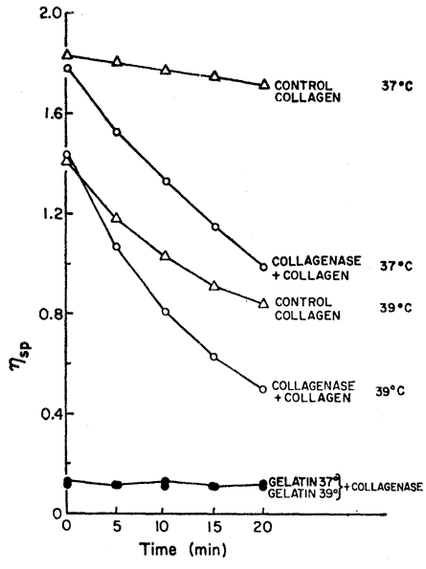

Fig. 2. Specific viscosity (η_{sp}) is plotted against time for six reaction mixtures of collagen or gelatin, with and without RTC, at 37°C and 39°C. The extent of degradation in each mixture is shown by disc gel electrophoresis in Fig. 1b.

At temperatures less than 37°C, increasing amounts of gelatin substrate were cleaved to TC^A and TC^B; at 27°C, after a 2-hour reaction between enzyme and collagen or gelatin, both substrates were degraded at a similar rate (*12*). Beier and Engel found that 26°C was close to optimal for forming "collagen-type" renatured material from thermally denatured substrate (*13*). We have hypothesized that at 27°C during a 2-hour incubation the gelatin was sufficiently renatured to permit some cleavage at the susceptible locus.

There are significant implications in these data. The high degree of specificity of the RTC for native collagen indicates that, in addition to the primary sequence of amino acids, the secondary helical structure of the triple-stranded molecule is important for enzyme recognition and catalysis. The coiled-coil conformation of collagen protects the molecule from degradation by most tissue proteases but it obviously facilitates cleavage by a specific collagenase. It is unlikely that synthetic substrates for collagenase used by some laboratories in lieu of native collagen (*14*) have structure sufficient for recognition by mammalian collagenases such as this one. We have attempted to assay the rate of cleavage of partially renatured chains (provided by Klaus Kühn and his colleagues, Munich, Germany) in the form of $(\alpha 1)_3$ and $(\alpha 2)_3$. Native collagens from which these α chains were prepared have $(\alpha 1)_2\alpha 2$ form. When the purified RTC was used it was found that the rate of cleavage of these renatured molecules was proportional to the amount of collagen fold (estimated by specific viscosity). The $(\alpha 1)_3$ and $(\alpha 2)_3$ had a specific viscosity one-fourth to one-third that of native collagen at the same concentration, and were cleaved by RTC at only one-tenth the rate of native $(\alpha 1)_2\alpha 2$ collagen.

Careful attempt at renaturation of pure α chains or of peptides produced by cyanogen bromide cleavage of collagen should yield structures with sufficient helical conformation so that RTC could utilize them as substrate. This would facilitate preparation of homogeneous fragments amenable to sequence determination of amino acid sequences about the common cleavage site of many diverse collagens.

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