Degradation of Collagen Substrates by a Trypsin-like Serine Protease from the Fiddler Crab *Uca pugilator*†

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ABSTRACT: The collagenolytic properties of a trypsin-like protease from the hepatopancreas of the fiddler crab *Uca pugilator* have been examined. All collagen types, I–V, were attacked by this enzyme. Types III and IV were degraded much more rapidly than types I, II, and V. Crab protease produced multiple cleavages in the triple helix of each collagen at 25 °C; only in the case of type III collagen, however, was a major cleavage observed at a $^3/_4$:$^1/_4$ locus that corresponded to the region of collagen susceptibility to mammalian collagenase action. Additionally, both the affinity and the specific activity of the crab protease for native collagen were lower than those which characterize mammalian collagenase. The results of this study, in conjunction with a previous report on the collagenolytic activity of another serine protease from the fiddler crab [Welgus, H. G., Grant, G. A., Jeffrey, J. J., & Eisen, A. Z. (1982) *Biochemistry 21*, 5183], suggest that the following properties distinguish the action of these invertebrate collagenolytic enzymes from the metalloenzyme collagenases of mammals: (1) broad substrate specificity, including both noncollagenous proteins and collagen types I–V; (2) ability to cleave the native triple helix of collagen at multiple loci; (3) reduced affinity or higher K_m for collagen; and (4) lower specific activity on collagen fibrils.

A new group of invertebrate serine proteases, which have the capacity to cleave the native triple helix of collagen under physiological conditions of pH, temperature, and ionic strength, has recently become recognized. Unlike the metalloenzyme collagenases of mammals these invertebrate collagenases function in a digestive role rather than in a morphogenetic one. Collagenolytic serine proteases have been identified in the fungus *Entomophthora coronata* (Hurion et al., 1979), the insect *Hypoderma lineatum* (Lecroisey et al., 1979), and the fiddler crab *Uca pugilator* (Eisen & Jeffrey, 1969; Eisen et al., 1970, 1973). The first of these to be reported and the most extensively characterized is crab protease I from the fiddler crab. This enzyme has been purified to homogeneity (Eisen et al., 1973) and shown by the determination of its complete covalent structure to be a serine protease (Grant et al., 1980) possessing significant homology to the pancreatic serine proteases of mammals. Furthermore, its specificity with regard to both noncollagenous and collagenous substrates has been documented (Grant & Eisen, 1980; Welgus et al., 1982a). The enzyme is chymotrypsin-like in that it displays a significant degree of preference for phenylalanyl, tryptophanyl, and leucyl residues, although it also cleaves at both positively and negatively charged amino acid residues. With regard to its activity on collagen substrates, crab protease I degrades all five collagen types, producing multiple cleavages in the triple helix of each collagen at 25 °C (Welgus et al., 1982a). The major early cleavage in the α1 polypeptide chain of collagen types I–III occurs at a $^3/_4$:$^1/_4$ locus, resulting in fragments electrophoretically similar to the TC^A and TC^B products of mammalian collagenase action. In addition to cleaving the native triple helix of collagen, this protease also effects a rapid conversion of intramolecular cross-linked β dimers of collagen to monomeric α chains, indicating an additional site of cleavage at the nonhelical ends of the collagen molecule (Eisen & Jeffrey, 1969).

Mammalian collagenases, on the other hand, belong to the class of metalloproteases, are specific for collagen, and do not attack other protein substrates (Harris & Krane, 1974; Seltzer et al., 1977). Most of these collagenases, including those derived from human skin fibroblasts (Stricklin et al., 1977; Welgus et al., 1981) and rabbit synovial cells (McCroskery et al., 1975), can degrade collagen types I (skin, tendon, bone), II (cartilage), and III (skin, blood vessels, gastrointestinal tract) but initiate only a single cleavage in the native molecule at a locus approximately three-fourths of the distance from the amino-terminal end, producing the characteristic $^3/_4$- and $^1/_4$-length products, TC^A and TC^B, respectively (Gross, 1976). At 25 °C, these fragments remain helical and are resistant to any further proteolytic attack. Type IV (basement membrane) and type V (placenta, other tissues) collagens are not susceptible substrates for these enzymes. Liotta et al. (1979) have characterized a mouse bone tumor collagenase which degrades type IV collagen but lacks significant proteolytic activity against the other collagen types. Collagenases from two human sarcomas (Liotta et al., 1981) and from human pulmonary alveolar macrophages (Mainardi et al., 1980) have been reported which are specific for the cleavage of native type V collagen.

In addition to crab protease I, the hepatopancreas of the fiddler crab contains at least one additional distinct collagenolytic serine protease. This enzyme, crab protease II, has recently been purified to homogeneity (Grant et al., 1983) and characterized with respect to its action on noncollagenous substrates. The polypeptide bond specificity of crab protease II and its interaction with specific protease inhibitors are identical with those of bovine trypsin. The amino-terminal sequence of this enzyme displays significant homology with other serine proteases, most strikingly with that of crayfish trypsin. In the present study, the action of crab protease II on collagenous substrates is examined and compared with that of crab protease I and a mammalian collagenase, human skin fibroblast collagenase.

Materials and Methods

Reagents. Acrylamide and bis(acrylamide) were purchased from Eastman. Sodium dodecyl sulfate, 99% pure, was obtained from Gallard-Schlesinger. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris) base and bovine pancreatic trypsin were purchased from Sigma. All other reagents were

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of the highest grade commercially available.

Purification of Crab Protease I and Crab Protease II. Both crab proteases were extracted from the hepatopancreata of live fiddler crabs and purified as described previously (Eisen et al., 1973; Grant et al., 1982, 1983).

Purification of Human Skin Fibroblast Collagenase. Normal human skin fibroblasts were grown in the presence of 10% newborn bovine serum, and the medium was harvested as described by Bauer et al. (1975). Human skin collagenase was purified to homogeneity from serum-containing medium as described by Stricklin et al. (1977).

Sources of Collagen. Collagen preparations were kindly donated by Drs. R. Burgeson, Harbor General Hospital, Torrance, CA (human cartilage type II, human placenta type III), E. Miller, University of Alabama (human placenta type V), and H. Sage, University of Washington (human placenta type IV). Guinea pig skin and chick skin type I collagens were prepared in this laboratory by the method of Gross (1958).

Assay Procedures. Fibrillar collagen assays utilized 50 μL of a 0.4% solution of native, reconstituted [^{14}C]glycine-labeled guinea pig skin type I collagen of specific activity 25 000 cpm/mg. This was allowed to gel at 37 °C overnight to permit completion of the aggregation process. Following incubation of such collagen gels with enzyme, the reaction mixtures were centrifuged at 10000*g* for 10 min, and the supernatant fraction was counted in a liquid scintillation spectrometer. The buffer used for all crab protease I and crab protease II assays was 0.05 M Tris-HCl, pH 8.0. The buffer used for trypsin and human fibroblast collagenase assays was 0.05 M Tris-HCl and 0.01 M $CaCl_2$, pH 7.5.

Assays on the different collagens in solution were performed at 25 °C. NaCl in a final concentration of 0.25 M was utilized in each reaction mixture to prevent any spontaneous gelation at this temperature. Reaction mixtures containing crab protease II were stopped by adding 2.0 μg of leupeptin; the action of crab protease I was stopped with a final phenylmethanesulfonyl fluoride (PMSF) concentration of 1 mM; samples containing human fibroblast collagenase were stopped with 0.04 M ethylenediaminetetraacetic acid (EDTA).

Determination of values for the basic kinetic parameters, K_m and k_{cat}, was performed as described previously (Welgus et al., 1981). Briefly, 4, 8, 12, 16, 20, and 24 μg of human placenta type III collagen in solution were incubated with crab protease II in a final reaction mixture volume of 80 μL. Reaction velocity was quantitated by spectrophotometric scanning of the $^3/_4$-length products on stained polyacrylamide slab gels. In each case, the reaction mixtures were stopped before 25% of the substrate initially present had been degraded.

The synthetic substrate N^{α}-tosyl-L-arginine methyl ester was purchased from Vega Biochemicals. The hydrolysis of this substrate was followed spectrophotometrically at 247 nm. The assay was performed in 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffer, pH 8.0, at room temperature.

Concentrations of trypsin and human fibroblast collagenase were determined spectrophotometrically by the method of Groves et al. (1968). Bovine serum albumin was used to establish the standard curve. The concentrations of crab protease I and crab protease II were determined by amino acid analysis after 24-h acid hydrolysis of aliquots of stock solutions.

The hydroxyproline concentration of the various collagens studied was determined by the method of Bergmann & Loxley (1963).

Polyacrylamide gel electrophoresis was performed by utilizing the method of King & Laemmli (1971). Following electrophoresis, the bands were stained with Coomassie blue.

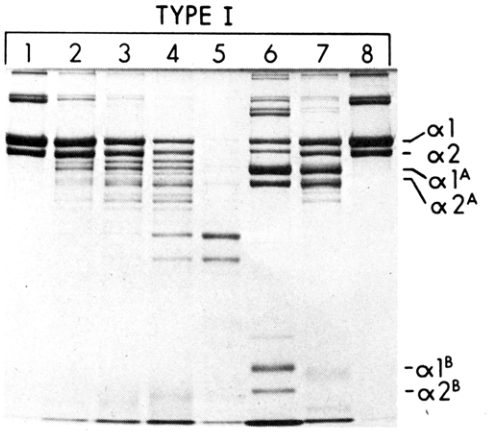

FIGURE 1: Type I collagen cleavage by crab protease II, human fibroblast collagenase, and crab protease I. Type I collagen (30 μg) was incubated at 25 °C with crab protease II, human fibroblast collagenase, and crab protease I. The reaction mixtures were stopped as detailed under Materials and Methods and the samples applied to a 10% polyacrylamide slab gel. Slots 1 and 8, collagen without added enzyme; slots 2, 3, and 4, collagen incubated with 1.6, 4, and 9 μg, respectively, of crab protease II for 1 h; slot 5, collagen incubated with 1.6 μg of crab protease II for 20 h; slot 6, collagen incubated with human fibroblast collagenase (0.12 μg) for 1 h to mark the TC^A and TC^B cleavage products; slot 7, collagen incubated with crab protease I (7 μg) for 1 h.

Results

The cleavages of native type I collagen in solution at 25 °C by crab protease II, human fibroblast collagenase, and crab protease I are compared in Figure 1. Multiple cleavages are catalyzed by crab protease II (slots 2–5), in contrast to the single $^3/_4$:$^1/_4$ cleavage produced by human fibroblast collagenase (slot 6). Crab protease I also catalyzes multiple cleavages in the helix of this collagen, but there is a major initial proteolytic event that results in $^3/_4$-length TC^A products electrophoretically identical with those of the mammalian collagenase (slot 7). Although the products of crab protease II action are in the same general region as those of the crab protease I, no preferred cleavage can be discerned, and none of the products migrates in the exact position of TC^A. Following prolonged digestion (24 h) with crab protease II, these high molecular weight cleavage products are converted to a doublet species of approximately 50 000 daltons (slot 5). Such behavior can most likely be explained by the limited peptide bond specificity of this enzyme (Grant et al., 1983).

Crab protease II also produces multiple cleavages in the native helix of type II collagen (Figure 2). It shares this action with crab protease I, again in contrast to the single proteolytic scission effected by human fibroblast collagenase. Crab protease II preferentially cleaves type II collagen at two distinct loci, resulting in fragments significantly higher and lower in molecular weight than TC^A (slots 2 and 3). This is unlike crab protease I which preferentially cleaves type II collagen at a $^3/_4$:$^1/_4$ locus, giving rise to a major early product (slot 5) which is electrophoretically identical with the TC^A product of mammalian collagenase (slot 4).

In contrast to its degradation of native collagen types I and II, crab protease II catalyzes an early major cleavage in native type III collagen at a locus electrophoretically identical with the $^3/_4$:$^1/_4$ site of human fibroblast collagenase cleavage (Figure 3). Crab protease II action on this substrate is very similar to that of crab protease I; both produce an essentially complete conversion of α chains to $^3/_4$-length TC^A products, prior to any further proteolytic cleavages (slots 2, 3, and 6).

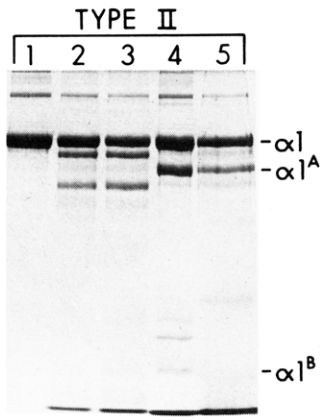

FIGURE 2: Type II collagen cleavage by crab protease II, human fibroblast collagenase, and crab protease I. Type II collagen (20 μg) was incubated at 25 °C with crab protease II, human fibroblast collagenase, and crab protease I. The reaction mixtures were stopped after 1 h as detailed under Materials and Methods, and the samples were applied to a 10% polyacrylamide slab gel. Slot 1, collagen without added enzyme; slots 2 and 3, collagen incubated with 6 and 10 μg, respectively, of crab protease II; slot 4, collagen incubated with human fibroblast collagenase (2.5 μg) to mark the TC^A and TC^B cleavage products; slot 5, collagen incubated with crab protease I (5 μg).

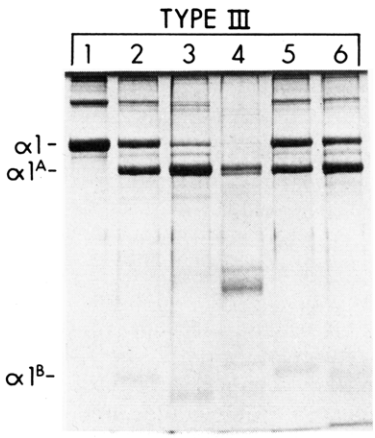

FIGURE 3: Type III collagen cleavage by crab protease II, human fibroblast collagenase, and crab protease I. Type III collagen (20 μg) was incubated at 25 °C with crab protease II, human fibroblast collagenase, and crab protease I. The reaction mixtures were stopped as detailed under Materials and Methods and the samples applied to a 10% polyacrylamide slab gel. Slot 1, collagen without added enzyme; slots 2 and 3, collagen incubated with 0.018 and 0.14 μg, respectively, of crab protease II for 1 h; slot 4, collagen incubated with 0.4 μg of crab protease II for 20 h; slot 5, collagen incubated with human fibroblast collagenase (0.003 μg) for 1 h to mark the TC^A and TC^B cleavage products; slot 6, collagen incubated with crab protease I (5 μg) for 1 h.

With continued incubation, however, this primary cleavage product is further degraded into smaller fragments (slot 4), unlike mammalian collagenase which cannot degrade the TC^A product of its type III digestion (slot 5).

Native collagen types IV and V are also successfully attacked by crab protease II (Figure 4). These substrates are resistant to the action of mammalian collagenase but are susceptible to degradation by crab protease I (Welgus et al., 1982a).

All five types of native collagen in solution were attacked by crab protease II in a manner that is dependent upon both time and enzyme concentration (not shown). In contrast, crab protease I degradation of this form of collagen substrate is peculiar in that it varies only with enzyme concentration, not with length of incubation (Welgus et al., 1982a). Because the

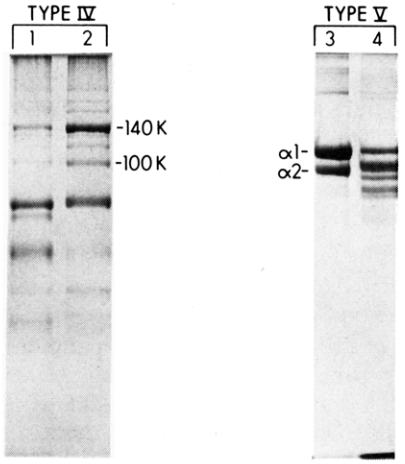

FIGURE 4: Cleavage of collagen types IV and V by crab protease II. Type IV collagen (20 μg) and type V collagen (20 μg) were incubated at 25 °C with crab protease II for 1 h. The reaction mixtures were stopped as detailed under Materials and Methods and the samples applied to a 10% polyacrylamide slab gel. For the type IV collagen substrate, the 140K and 100K bands which are shown represent the major pepsin-derived fragments of this molecule. Slot 1, type IV collagen incubated with 0.1 μg of crab protease II; slot 2, type IV collagen without added enzyme; slot 3, type V collagen without added enzyme; slot 4, type V collagen incubated with 1.2 μg of crab protease II.

Table I: Comparative Rates of Degradation of Native Collagen Types I–V by Crab Protease II and Trypsin[a]

collagen type	[crab protease II] (μg/mL)	[trypsin] (μg/mL)
I	150	*b*
II	300	*b*
III	1.6	270
IV	6	800
V	80	5000

[a] Fifteen micrograms of native collagen types I–V was incubated at 25 °C for 60 min with 25 μL of crab protease II or trypsin. The enzyme concentration required to produce 50% degradation of substrate α chains is recorded. [b] Denotes resistance of this substrate to significant enzymatic cleavage.

time and concentration dependence of crab protease II action are more enzymologically conventional, the relative rates of cleavage of the five collagen types could be measured and the basic kinetic parameters, K_m and k_{cat}, examined. In Table I, the comparative rates of cleavage of collagen types I–V by crab protease II and trypsin are shown. Collagen types III and IV are rapidly degraded by the crab protease; types I, II, and V are cleaved more slowly. Type III collagen is known to be susceptible to certain general proteases such as trypsin (Miller et al., 1976) and thermolysin (Wang et al., 1978), presumably because of a looser helix in the region of the $^3/_4$:$^1/_4$ collagenase cleavage site. In the case of trypsin, an Arg_{780}–Gly_{781} bond is cleaved which is only one triplet carboxy terminal to the Gly_{775}–Leu_{776} bond attacked by mammalian collagenase (Miller et al., 1976; Highberger et al., 1978). As seen in Table I, crab protease II is approximately 170 times as efficient as trypsin in catalyzing this or a similar cleavage. Native type IV collagen contains several areas in its primary structure where glycine is not found in every third amino acid position (Schuppan et al., 1980); these regions are presumably nonhelical. Crab protease II is more than 100 times as effective as trypsin in degrading type IV collagen. Type V collagen could be regarded as essentially resistant to tryptic digestion. The concentration of trypsin required, 5 mg/mL, is sufficient to produce some cleavages even in native type I collagen. It

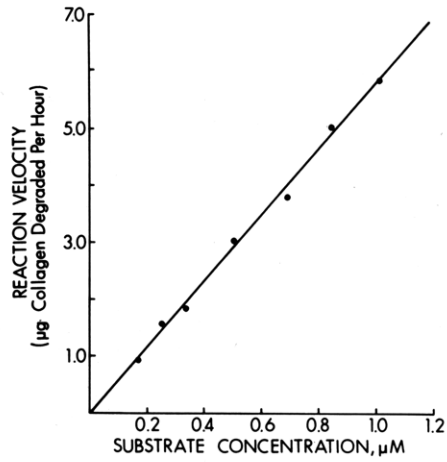

FIGURE 5: Reaction velocity vs. substrate concentration. Crab protease II (0.01 μg) was incubated at 25 °C with 4, 6, 8, 12, 16, 20, and 24 μg of type III collagen for 1 h in a total reaction mixture volume of 80 μL. The samples were then applied to an 8% polyacrylamide slab gel, and reaction velocity was quantitated as described previously by spectrophotometric scanning of the stained $^3/_4$-length TC^A products (Welgus et al., 1981). Note the lack of any discernible substrate saturation.

would seem apparent, then, that collagen types III and IV, which are known to have some defect in triple-helical structure, with regard to the degree of either helix tightness or regions of frank nonhelicity, are attacked more readily by crab protease II than the other collagen types.

Since crab protease II initiates only a single cleavage in native type III collagen until nearly all the α chains are degraded, this substrate seemed well suited for the determination of values for the kinetic parameters K_m and k_{cat}. Shown in Figure 5 is the resultant velocity vs. substrate concentration plot, using increasing amounts of type III collagen, ranging from 0.05 to 0.3 mg/mL. The constituent data points are linear, indicating a lack of any discernible substrate saturation and implying that these substrate concentrations, in the micromolar range, are still well below the K_m of the crab protease II for collagen. Unfortunately, the viscosity of collagen increases rapidly at concentrations ≥0.5 mg/mL (von Hippel, 1967), thus prohibiting the use of higher substrate concentrations in this assay system.

Certain proteolytic enzymes such as chymotrypsin and pepsin are capable of releasing peptides from native collagen without altering its helical structure (Rubin et al., 1965). Bornstein et al. (1966) have clearly demonstrated that such proteases convert intramolecular cross-linked β components of collagen to monomeric α chains. This action is the result of the cleavage of susecptible peptide bonds located in the short nonhelical region at the N-terminal end of the collagen molecule near the site of the intramolecular cross-links. Figure 6 shows the incubation of type I collagen with crab protease II at approximately 10% of the enzyme concentration required to produce substantial cleavage of helical collagen. Clearly, there is significant loss in the staining of β-collagen protein bands, with a concomitant increase in the staining of α-chain bands. It is noteworthy that the trypsin-like crab protease II can effect this proteolytic scission, while trypsin lacks such a capability (Eisen & Jeffrey, 1969).

The time course of the degradation of fibrillar collagen at 37 °C by crab protease II is shown in Figure 7. Interestingly, cleavage of this substrate, measured by the release of [^{14}C]-glycine, is not linear as a function of time. A consistent observation was that the rate of degradation in the first hour exceeds that of subsequent cleavage. After 1 h, however, the

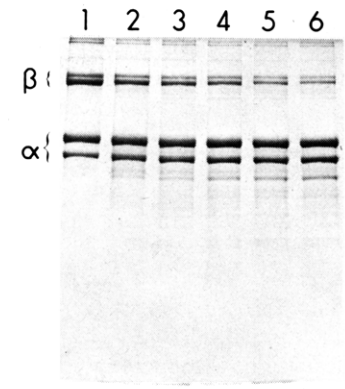

FIGURE 6: Cleavage of intramolecular cross-linked β dimers of collagen into monomeric α chains. Chick skin type I collagen (12 μg) was incubated at 25 °C with crab protease II (1.2 μg) for increasing lengths of time. The reaction mixtures were stopped with leupeptin and then applied to an 8% polyacrylamide slab gel. Slot 1, collagen without added enzyme; slots 2, 3, 4, 5, and 6, collagen incubated with crab protease II for 30 min, 1 h, 2 h, 3 h, and 4 h, respectively. Note the decrease in β components of collagen and the concomitant increase in α components.

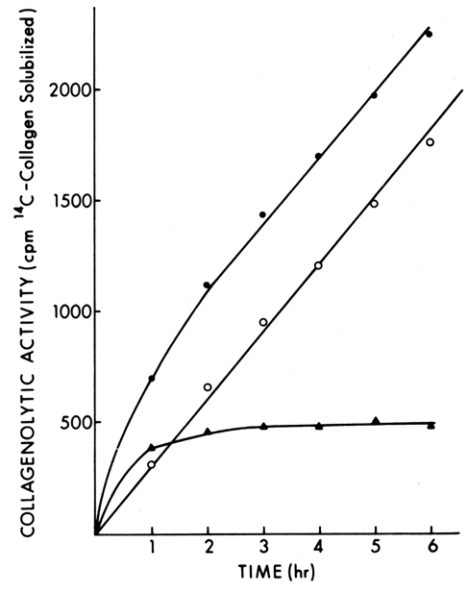

FIGURE 7: Crab protease II activity on collagen fibrils. Crab protease II (50 μL; 37 μg/mL) and trypsin (50 μL; 37 μg/mL) were incubated at 37 °C with fibrillar collagen. (●) Crab protease; (▲) trypsin; (○) crab protease following subtraction of the trypsin blank.

rate is essentially constant. Such an initial burst in collagenolysis does not occur in fibrillar collagen degradation by either crab protease I or human skin collagenase (Welgus et al., 1982a, 1980). For further examination of this phenomenon, the stability of crab protease II was assessed by measuring its activity against the synthetic substrate N^{α}-tosyl-L-arginine methyl ester following increasing lengths of incubation at 37 °C. No loss in enzyme activity was observed, even after 4 h at this temperature. It has long been known that when trypsin is incubated with ^{14}C-labeled collagen fibrils, approximately 5–10% of the total counts present in the substrate gel are rapidly solubilized with little further release of radioactivity after the first few minutes of the reaction (Lapiere & Gross, 1963). Although the exact nature of this process is not understood, proteolytic scission in the helical region of the native molecule is not believed to occur. When crab protease II digestion of fibrillar collagen is corrected for a trypsin blank performed simultaneously, the resultant enzyme activity is now linear throughout the entire time of the reaction (Figure 7).

This most likely represents the true collagenolytic action of the crab protease. The specific activity of the crab protease on native type I fibrillar collagen, calculated accordingly, is 102 μg of collagen solubilized (mg of enzyme)$^{-1}$ min^{-1}.

Discussion

In this investigation, the collagenolytic properties of crab protease II, a trypsin-like serine protease isolated from the hepatopancreas of the fiddler crab, have been delineated. This enzyme and the previously reported crab protease I both cleave the triple helix of all native collagen types, I–V, under physiological nondenaturing conditions. This broad collagen type specificity appears to be a common property of these invertebrate collagenolytic serine proteases, in clear distinction to the more limited specificity inherent to mammalian metalloenzyme collagenases. Another common denominator of the collagenolytic action of such invertebrate enzymes is their ability to catalyze multiple cleavages in the triple helix of each native collagen type, whereas mammalian collagenases can initiate only a single proteolytic scission.

The single cleavage of collagen types I–III produced by mammalian collagenase occurs at a $^3/_4$:$^1/_4$ locus in the native molecule. The predisposition for catalysis at this site has been suggested to result from an intrinsically looser helix in this region, since other potentially susceptible peptide bonds located throughout the molecule are not cleaved as long as the triple-helical structure is maintained (Welgus et al., 1982b). In the case of type III collagen, the helix is sufficiently loose in this area to allow cleavage of the native molecule by general proteases such as trypsin (Miller et al., 1976) and thermolysin (Wang et al., 1978). Although cleavage by general proteases does not occur in type I collagen, helix coil renaturation studies have shown that a peptide fragment which contains the collagenase cleavage site has far less capacity to re-form its triple-helical structure as compared to similar peptides obtained from other regions of the molecule (Highberger et al., 1978). Crab protease I preferentially attacks collagen types I–III at loci very close to the mammalian collagenase cleavage site, and in each case, the products formed are electrophoretically very similar to, if not identical with, TC^A and TC^B. In contrast, crab protease II preferentially cleaves only type III collagen at such a locus. Type I collagen is attacked at many loci simultaneously, and type II is preferentially cleaved at two sites, neither of which result in products that correspond to TC^A. It would seem that in the case of crab protease II degradation of type I and II collagens, primary structure may play an important role in addition to secondary helical conformation in determining the susceptibility of these substrates to proteolytic cleavage. Nevertheless, when type I collagen is thermally denatured and then exposed to the crab protease, the resultant pattern of cleavages is different from those produced in the native substrate (data not shown), presumably reflecting a release of constraints on proteolytic action imposed by the triple-helical structure.

The relative rates of crab protease II cleavage of native collagen types I–V are shown in Table I. Collagen types III and IV are much more susceptible substrates than types I, II, and V, perhaps reflecting easier access to sites of proteolysis due to a comparatively looser helix or regions of complete nonhelicity as previously discussed (vide supra). The biochemical similarities of crab protease II to trypsin have been reported (Grant et al., 1983), including an identical polypeptide bond specificity. The ability of the crab protease to cleave native types III and IV 100–200-fold more readily than trypsin and to degrade the trypsin-resistant collagens, types I, II, and V, underscores the special capacity of these invertebrate collagenolytic enzymes to effectively deal with the triple-helical structure of native collagen as compared to most serine proteases.

The collagen substrate specificity of one collagenase, human skin fibroblast collagenase, has been defined in terms of the kinetic parameters K_m and k_{cat}, with respect to both type and animal species of substrate origin (Welgus et al., 1981). Despite very large differences in values for k_{cat}, the K_m for all susceptible collagens (types I–III) is approximately 1–2 μM. Similar determinations were attempted in this study for the cleavage of type III collagen by crab protease II. From the data shown in Figure 5, the K_m of the crab protease for its most readily degraded collagen is unmeasurable but must be considerably greater than 1 μM. Unfortunately, due to the inherent viscosity of collagen, the substrate concentration could not be raised sufficiently to attain any detectable evidence of saturation. These data, and the results of binding studies to fibrillar collagen previously reported for crab protease I (Welgus et al., 1982a), would seem to indicate that the affinity of such invertebrate collagenolytic enzymes for collagen is significantly less than that which characterizes the more specific mammalian collagenases.

The capacity to cleave intramolecularly cross-linked β dimers of type I collagen into monomeric α chains has been shown to depend upon proteolytic cleavage of the short nonhelical N-terminal end of the molecule that contains the cross-linking site (Bornstein et al., 1966). This nonhelical fragment consists of 16 amino acid residues which precede the repeating helical (Gly-X-Y) sequences (Bornstein & Traub, 1979). The ninth amino acid position in this fragment is occupied by the lysine residue that serves as the site of cross-link formation. Cleavage must occur at this lysine or C terminal to this lysine in order to effect conversion of β dimers into monomeric α components. In the case of chymotrypsin and cyanogen bromide, a Met–Gly bond is cleaved that is located in the first triplet of the helical segment of the collagen molecule (Bornstein et al., 1966). Trypsin can cleave Lys-9 only when it contains a free amino group; following conversion of this amino group to an aldehyde and the subsequent formation of the intramolecular cross-link, proteolytic cleavage by trypsin at this residue can no longer occur. Thus, trypsin is unable to catalyze the conversion of cross-linked β dimers into α chains. Since crab protease II has essentially the same residue specificity as trypsin, but yet can effect this catalysis, the crab enzyme must either cleave this Lys-9 bond following cross-link formation, which is unlikely, or else cleave the next C-terminal susceptible bond, which is an Arg–Gly bond located only three triplets into the helical portion of the molecule.

The activity of crab protease II on reconstituted collagen fibrils at 37 °C initially appeared to be unusual in that collagenolysis did not proceed in a linear fashion as a function of time (Figure 7). This observation was explained by the apparent trypsin-like ability of the crab protease to nonspecifically release peptides, possibly from nonhelical regions of the molecule, during the first few minutes of incubation with the fibrillar collagen substrate, which is then superimposed on its true collagenolytic action. Thus, when a "trypsin blank" is subtracted from the overall rate of collagenolysis, degradation is linear from time zero, and this resultant activity is taken to reflect the true collagenolytic capacity of the enzyme. The specific activity of crab protease II, calculated by correcting for this tryptic activity, is 102 μg mg^{-1} min^{-1}. This is similar to that of crab protease I, 86 μg mg^{-1} min^{-1}, but both values are significantly less than the specific activity of human

skin fibroblast collagenase on fibrillar collagen, 850 $\mu g\ mg^{-1}\ min^{-1}$ (Welgus et al., 1982a).

Studies of both crab protease I and crab protease II suggest that certain collagenolytic properties of these invertebrate serine proteases distinguish them from the metalloenzyme collagenases found in mammals. The collagenolytic enzymes of invertebrates possess a far broader collagen specificity than mammalian collagenases, successfully attacking all five collagen types which are known to exist. Unlike the mammalian enzymes, the invertebrate proteases can cleave the collagen triple helix at multiple loci, rather than only at a single $^3/_4$:$^1/_4$ site. The affinity and catalytic rates of the invertebrate enzymes for native collagen substrates are considerably lower than those for the mammalian collagenases, perhaps reflecting an accommodation for their biological role as digestive enzymes capable of attacking other protein substrates as well. Continued study of collagenolytic invertebrate enzymes will be necessary to determine whether these properties are shared with yet other memmbers of this growing subgroup of serine proteases and if they will provide insights into the mechanism of collagenolysis.

Acknowledgments

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Registry No. Collagenase, 9001-12-1; trypsin, 9002-07-7.

References

Bauer, E. A., Stricklin, G. P., Jeffrey, J. J., & Eisen, A. Z. (1975) *Biochem. Biophys. Res. Commun. 64*, 232.

Bergmann, I., & Loxley, R. (1963) *Anal. Chem. 35*, 1961.

Bornstein, P., & Traub, W. (1979) *Proteins (3rd Ed.) 8*, 411–632.

Bornstein, P., Kang, A. H., & Piez, K. A. (1966) *Biochemistry 5*, 3803.

Eisen, A. Z., & Jeffrey, J. J. (1969) *Biochim. Biophys. Acta 191*, 517.

Eisen, A. Z., Bauer, E. A., & Jeffrey, J. J. (1970) *J. Invest. Dermatol. 55*, 359.

Eisen, A. Z., Henderson, K. O., Jeffrey, J. J., & Bradshaw, R. A. (1973) *Biochemistry 12*, 1814.

Grant, G. A., & Eisen, A. Z. (1980) *Biochemistry 19*, 6089.

Grant, G. A., Henderson, K. O., Eisen, A. Z., & Bradshaw, R. A. (1980) *Biochemistry 19*, 4653.

Grant, G. A., Eisen, A. Z., & Bradshaw, R. A. (1982) *Methods Enzymol. 80*, 722.

Grant, G. A., Sacchettini, J. C., & Welgus, H. G. (1983) *Biochemistry 22*, 354.

Gross, J. (1958) *J. Exp. Med. 108*, 215.

Gross, J. (1976) in *Biochemistry of Collagen* (Ramachandran, G. N., & Reddi, A. H., Eds.) pp 275–317, Plenum Press, New York.

Groves, W. E., Davis, F. C., Jr., & Sells, B. (1968) *Anal. Biochem. 22*, 195.

Harris, E. D., Jr., & Krane, S. M. (1974) *N. Engl. J. Med. 291*, 557, 605, 652.

Highberger, J. H., Corbett, C., Kang, A. H., & Gross, J. (1978) *Biochem. Biophys. Res. Commun. 83*, 43.

Hurion, N., Fromentin, H., & Keil, B. (1979) *Arch. Biochem. Biophys. 192*, 438.

King, J., & Laemmli, U. K. (1971) *J. Mol. Biol. 62*, 465.

Lapiere, C. M., & Gross, J. (1963) in *Mechanisms of Hard Tissue Destruction*, pp 663–694, Publication No. 75 of the American Association for the Advancement of Science, Washington, DC.

Lecroisey, A., Boulard, C., & Keil, B. (1979) *Eur. J. Biochem. 101*, 385.

Liotta, L. A., Abe, S., Robey, P. G., & Martin, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2268.

Liotta, L. A., Lanzer, W. L., & Garbis, S. (1981) *Biochem. Biophys. Res. Commun. 98*, 184.

Mainardi, C. L., Seyer, J. M., & Kang, A. H. (1980) *Biochem. Biophys. Res. Commun. 97*, 1108.

McCroskery, P. A., Richards, J. F., & Harris, E. D., Jr. (1975) *Biochem. J. 152*, 131.

Miller, E. J., Finch, J. E., Jr., Chung, E., & Butler, W. T. (1976) *Arch. Biochem. Biophys. 173*, 631.

Rubin, A. L., Drake, M. P., Davison, P. F., Pfahl, D., Speakman, P. T., & Schmitt, F. O. (1965) *Biochemistry 4*, 181.

Schuppan, D., Timpl, R., & Glanville, R. W. (1980) *FEBS Lett. 115*, 297.

Seltzer, J. L., Jeffrey, J. J., & Eisen, A. Z. (1977) *Biochim. Biophys. Acta 485*, 179.

Stricklin, G. P., Bauer, E. A., Jeffrey, J. J., & Eisen, A. Z. (1977) *Biochemistry 16*, 1607.

von Hippel, P. H. (1967) in *Treatise on Collagen* (Ramachandran, G. N., Ed.) pp 253–338, Academic Press, New York.

Wang, H. M., Chan, J., Pettigrew, D. W., & Sodek, J. (1978) *Biochim. Biophys. Acta 533*, 270.

Welgus, H. G., Jeffrey, J. J., Stricklin, G. P., Roswit, W. T., & Eisen, A. Z. (1980) *J. Biol. Chem. 255*, 6806.

Welgus, H. G., Jeffrey, J. J., & Eisen, A. Z. (1981) *J. Biol. Chem. 256*, 9511.

Welgus, H. G., Grant, G. A., Jeffrey, J. J., & Eisen, A. Z. (1982a) *Biochemistry 21*, 5183.

Welgus, H. G., Jeffrey, J. J., Stricklin, G. P., & Eisen, A. Z. (1982b) *J. Biol. Chem. 257*, 11534.