Ferguson, S. J., Lloyd, W. J., & Radda, G. K. (1975b) *Eur. J. Biochem. 54*, 127.
Fleischer, N., Rosen, O. M., & Reichlin, M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 54.
Ghosh, P. B., & Whitehouse, M. W. (1968) *Biochem. J. 108*, 155.
Gill, G. N., & Garren, L. D. (1970) *Biochem. Biophys. Res. Commun. 39*, 335.
Glass, D. B., Masaracchia, R. A., Feramisco, J. R., & Kemp, B. E. (1978) *Anal. Biochem. 87*, 566.
Hartl, F. T. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 2094.
Hartl, F. T., & Roskoski, R., Jr. (1979) *Int. Cong. Biochem., XIth, Abstr.*, 259.
Hixson, C. S., & Krebs, E. G. (1979) *J. Biol. Chem. 254*, 7509.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245.
Kupfer, A., Gani, V., Jimenez, J. S., & Shaltiel, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3073.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Meloche, H. P. (1967) *Biochemistry 6*, 2273.
Muneyama, L., Bauer, J. J., Shuman, D. A., Robins, R. K., & Simon, L. N. (1971) *Biochemistry 10*, 2390.
Nitta, K., Brachter, S. C., & Dronman, J. J. (1979) *Biochem. J. 117*, 385.
Pal, P. K., Wechter, W. J., & Colman, R. F. (1975) *J. Biol. Chem. 250*, 8140.
Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691.
Rosen, O. M., & Erlichman, J. (1975) *J. Biol. Chem. 250*, 7788.
Shoji, S., Parmelee, D. C., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Long, G. L., Demaille, J. G., Fischer, E. H., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 848.
Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D. (1976) *Biochem. J. 159*, 409.
Tao, M., Salas, M. L., & Lipmann, F. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 408.
Weber, W., Vogel, C., & Hilz, H. (1979) *FEBS Lett. 99*, 62.
Witt, J. J., & Roskoski, R., Jr. (1975a) *Biochemistry 14*, 4503.
Witt, J. J., & Roskoski, R., Jr. (1975b) *Anal. Biochem. 66*, 253.
Wold, F. (1977) *Methods Enzymol. 46*, 3.
Wyatt, J. L., & Colman, R. F. (1977) *Biochemistry 16*, 1333.
Zoller, M. J., & Taylor, S. S. (1979) *J. Biol. Chem. 254*, 8363.

# Substrate Specificity of the Collagenolytic Serine Protease from *Uca pugilator*: Studies with Collagenous Substrates†

Howard G. Welgus, Gregory A. Grant,* John J. Jeffrey, and Arthur Z. Eisen

ABSTRACT: The collagenolytic protease from *Uca pugilator* was studied with respect to its catalytic properties on collagen types I–V. The crab protease degraded all five collagen types, producing multiple cleavages in the triple helix of each native collagen at 25 °C. The major early cleavage in the $\alpha$1 polypeptide chain of collagen types I–III occurred at a $^3/_4$:$^1/_4$ locus, resulting in fragments electrophoretically similar to the $TC^A$ and $TC^B$ products of mammalian collagenase action. Interestingly, a propensity toward this same cleavage was observed even following thermal denaturation of the substrates. The ability of the crab protease to degrade all native collagen types and to catalyze cleavages at multiple loci in the triple helix distinguishes its action from that of mammalian collagenases. The collagenolytic activity of the crab protease was also examined on fibrillar collagen and compared to that of human skin fibroblast collagenase. Enzyme concentrations of fibroblast collagenase which resulted in the saturation of available substrate sites failed to show such an effect in the case of the crab protease. Binding studies of the crab protease to fibrillar collagen likewise indicated substantially reduced levels of enzyme binding in comparison to fibroblast collagenase. These data suggest that the affinity of the crab protease for native collagen is considerably less than the affinity of mammalian collagenase for this substrate.

The fiddler crab, *Uca pugilator*, is a predacious scavenger that feeds on animal tissues frequently containing collagen as a major constituent protein. A collagenolytic protease isolated from the hepatopancreas of this crustacean has been purified to homogeneity (Eisen et al., 1973) and shown by the determination of the complete covalent structure to be a serine protease (Grant et al., 1980) homologous to the pancreatic serine proteases of vertebrates. This crab protease is a good general protease, displaying a broader peptide bond specificity than either trypsin or chymotrypsin on noncollagenous substrates (Grant & Eisen, 1980). In addition, the enzyme has been shown to cleave the native triple helix of type I collagen in the area of the $TC^A{}_{75}$ locus (Eisen & Jeffrey, 1969; Eisen et al., 1973). Not surprisingly, this protease also effects a rapid conversion of intramolecular cross-linked $\beta$ dimers of collagen to monomeric $\alpha$ chains, indicating an additional site of cleavage at the nonhelical ends of the collagen molecule (Eisen & Jeffrey, 1969). While the crab protease was the first example of a serine protease with significant collagenolytic activity, similar enzymes have now been reported from the dog pancreas (Takahashi & Seifter, 1974), the fungus *Entomophthora coronata* (Hurion et al., 1979), and the insect *Hypoderma*

† From the Division of Dermatology, Department of Medicine and Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110. *Received April 12, 1982.* This work was supported by U.S. Public Health Service Grants AM 31078, GM 27784, AM 12129, and TO-AM 07284.

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*lineatum* (Lecroisey et al., 1979).

Unlike the crab protease, collagenases which have been identified in mammalian connective tissues belong to the class of metalloproteases, are specific for collagen, and do not appear to attack other protein substrates (Harris & Krane, 1974; Seltzer et al., 1977). Most of these collagenases, which include those derived from human skin fibroblasts (Stricklin et al., 1977; Welgus et al., 1981) and rabbit synovium (McCroskery et al., 1975), can degrade collagen types I (skin, tendon, bone), II (cartilage), and III (skin, blood vessels, gastrointestinal tract) by initiating only a single cleavage in the native collagen 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 $TC^B$ (Gross, 1976). Type IV (basement membrane) and type V (placenta, other tissues) collagens are resistant to proteolytic cleavage by 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 recently been reported which are specific for the cleavage of native type V collagen.

In the present investigation, the collagenolytic protease from *Uca pugilator* has been studied with respect to its catalytic properties on collagen types I–V. Additionally, its action on the three forms of collagenous substrates—fibrillar collagen, collagen in solution, and denatured collagen or gelatin—has also been examined.

## Materials and Methods

*Reagents.* Acrylamide and bis(acrylamide) were purchased from Eastman. Sodium dodecyl sulfate, 99% pure, was obtained from Gallard-Schlesinger. Tris base, bovine pancreatic trypsin, and chymotrypsin were purchased from Sigma. All other chemicals used were reagent grade.

*Purification of Crab Protease.* Crab protease was extracted from the hepatopancreas of live fiddler crabs and purified as previously described (Eisen et al., 1973).

*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, human placenta type V), E. Miller, University of Alabama (bovine cartilage type II, 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 enzyme assays on fibrillar collagen was 0.05 M Tris-HCl, pH 8.0.

The binding of crab protease and human skin fibroblast collagenase to fibrillar collagen was quantitated by analysis of enzyme activity remaining in the buffer of the reaction mixture (Welgus et al., 1980). Briefly, following the incubation of enzyme and $^{14}$C-labeled fibrillar collagen at 25 °C for 20 min, aliquots of unbound collagenase were obtained from the suspending buffer. The aliquots were then incubated with a new [$^{14}$C]collagen substrate gel at 37 °C, and collagenase activity was quantitated following subtraction of the appropriate blanks. Binding studies of the crab protease and human fibroblast collagenase were performed at concentrations of 50 and 100 μg/mL, respectively, to ensure assaying on the linear portion of the activity vs. enzyme concentration curve.

Assays on the different collagen types 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 the crab protease were stopped by the addition of phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 1 mM; those employing human fibroblast collagenase were stopped with 0.04 M ethylenediaminetetraacetic acid (EDTA).

The synthetic substrate *N*-benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide (BzValGlyArgNA) was purchased from Sigma Chemical Co. The hydrolysis of this substrate was followed spectrophotometrically at 385 nm by measuring the rate of liberation of *p*-nitroanilide as previously described (Bundy, 1962, 1963). 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, chymotrypsin, 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 concentration of crab protease was 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).

*Electrophoresis.* Polyacrylamide gel electrophoresis was performed by utilizing the method of King & Laemmli (1971). Following electrophoresis, the protein bands were stained with 1% Coomassie blue.

*$TC^B$ Isolation.* $TC^B$ fragments of chick skin type I collagen were generated by the action of crab protease as described in Figure 1. The reaction solution was made 25% of saturation with ammonium sulfate and centrifuged at 10000*g* for 15 min. The supernatant was retained, dialyzed against water for several hours, and lyophilized. After the sample was redissolved in 0.1 N acetic acid, the inactive crab protease was allowed to precipitate. The supernatant, which contained the $TC^B$ fragments, was used as described in the text.

## Results

The action of the crab protease on native type I collagen in solution is shown in Figure 1A, slot 2. Multiple cleavages are initiated in this substrate, in contrast to the single cleavage which is catalyzed by mammalian collagenase (slot 3). The major early cleavage by the crab protease is at a $^3/_4$ locus, and the resultant $^3/_4$-length fragment derived from the $\alpha$1 polypeptide chain is indistinguishable electrophoretically from $TC^A_{\alpha 1}$ produced by mammalian collagenase. A $^1/_4$-length fragment is also formed with an electrophoretic mobility very similar to $TC^B_{\alpha 1}$. As a consistent observation, there is a protein doublet which migrates in the electrophoretic position of $TC^A_{\alpha 2}$, and it is suspected that this doublet represents $^3/_4$-length cleavages in the $\alpha$2 polypeptide chain at two nearby loci. Although not visible on the gel in Figure 1A, a $^1/_4$-length protein doublet with a similar electrophoretic mobility to $TC^B_{\alpha 2}$ is also produced and is shown in Figure 1B. The $TC^B$ fragments resulting from the action of crab protease on type I collagen in solution were isolated by differential ammonium sulfate precipitation prior to electrophoresis so that a greater amount of protein could be applied to the gel. The top band in Figure 1B corresponds to $TC^B_{\alpha 1}$ while the next two lower

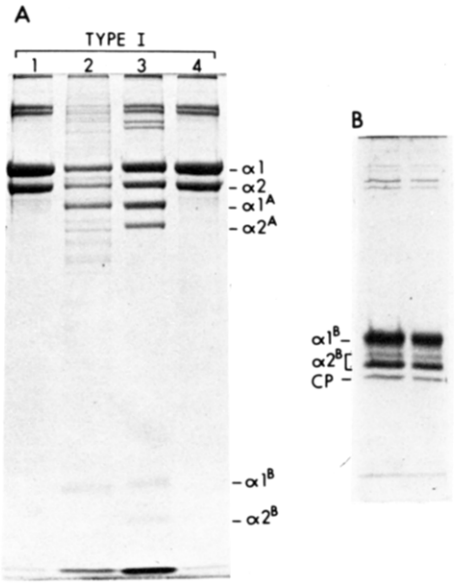


FIGURE 1: Crab protease vs. type I collagen. (A) Chick skin type I collagen (60 μg) was incubated with crab protease (10 μg) at 25 °C for 1 h. The reaction was then stopped by adding PMSF to a final concentration of 1 mM. (Slots 1 and 4) Collagen without added enzyme; (slot 2) collagen incubated with crab protease; (slot 3) collagen incubated with human skin fibroblast collagenase to mark the $TC^A$ and $TC^B$ cleavage products. (B) Chick skin type I collagen (90 mg) was incubated with crab protease (7.2 mg) at 25 °C for 2 h. The reaction was stopped with PMSF, and the $TC^B$ fragments were isolated as described in the text.

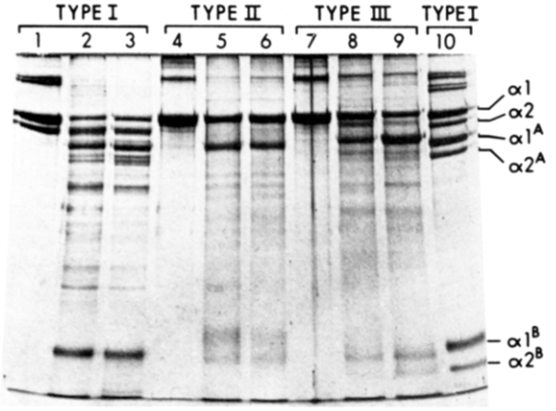


FIGURE 2: Crab protease vs. collagen types I, II, and III. Guinea pig type I, bovine type II, and human type III collagens (40 μg) were incubated with crab protease (12.5 μg) in a total reaction mixture volume of 75 μL. The reactions were stopped by adding PMSF to a final concentration of 1 mM. (Slots 1, 4, and 7) Types I, II, and III collagen, respectively, without added enzyme; (slots 2, 5, and 8) types I, II, and III collagen, respectively, following incubation with crab protease at 25 °C for 30 min; (slots 3, 6, and 9) types I, II, and III collagen, respectively, following incubation for 2 h. Slot 10 shows the $TC^A$ and $TC^B$ cleavage products of type I collagen by human skin fibroblast collagenase.

bands correspond to the $TC^B_{\alpha 2}$ doublet. As would be expected from the characteristic 2:1 ratio of α1 to α2 chains in the collagen substrate, the $TC^B_{\alpha 1}$ band is present in an amount greater than the $TC^B_{\alpha 2}$ bands. The reason for the difference in intensity of the two $TC^B_{\alpha 2}$ bands is not clear, but this may result from the preference of one over the other to subsequent degradation by the crab protease. The lowest band in Figure 1B is due to residual crab protease in the ammonium sulfate fraction, as determined by the migration of a standard solution of the protease. Unfortunately, automated sequence analysis of the isolated $TC^B$ fragments failed to reveal an amino-terminal sequence. On the basis of the amount of protein ana-

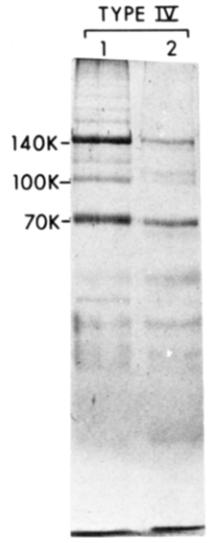


FIGURE 3: Crab protease vs. type IV collagen. Native human type IV collagen (25 μg) was incubated with crab protease (7.2 μg) at 25 °C for 2 h. The reaction was stopped by the addition of PMSF to a final concentration of 1 mM. (Slot 1) Type IV collagen without added enzyme; (slot 2) type IV collagen incubated with crab protease. Note that the 140K, 100K, and 70K components of type IV collagen are all degraded by the crab protease.

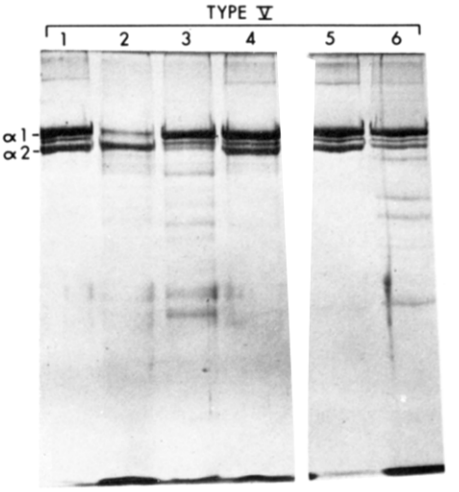


FIGURE 4: Crab protease vs. type V collagen. Native type V collagen (30 μg) was incubated with trypsin (50 μg), crab protease (5 μg), and chymotrypsin (60 μg) at 25 °C for 2 h. The reaction mixtures were stopped by the addition of PMSF to a final concentration of 1 mM. (Slots 1, 4, and 5) Type V collagen without added enzyme; (slot 2) type V collagen plus trypsin; (slot 3) type V collagen plus crab protease; (slot 6) type V collagen plus chymotrypsin.

lyzed and an average expected yield, these fragments acted as if their amino termini were blocked.

The action of the crab protease on collagen types II–V is shown in Figures 2–4. As observed for the native type I substrate, the crab protease initiates multiple cleavages in the triple helix of collagen types II and III (Figure 2). Furthermore, the major early proteolytic cleavage also occurs at a locus approximately three-fourths the length from the amino-terminal end of the collagen molecule. Thus, as earlier noted for the cleavage of α1(I), a $^3/_4$-length product with the approximate electrophoretic mobility of $TC^A_{\alpha 1}$ is apparent for both α1(II) and α1(III). A single protein band migrating in the electrophoretic position of $TC^B_{\alpha 1}$ is not readily observed, however, due to the susceptibility of this fragment to further cleavage (data not shown). The degradation of native type IV collagen by the crab protease is shown in Figure 3. The constituent 140K, 100K, and 70K protein bands of this sub-

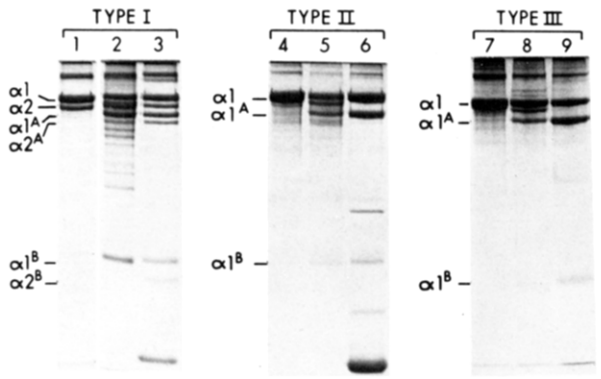


FIGURE 5: Crab protease vs. denatured collagen types I, II, and III. Guinea pig type I, human type II, and human type III collagens (30 μg) were denatured by heating to 70 °C for 15 min. Crab protease (0.03 μg) was then added to the denatured collagens. The reaction mixtures were stopped with PMSF and applied to a 10% polyacrylamide slab gel. Reaction mixtures containing native collagen incubated with human skin fibroblast collagenase are also shown to mark the electrophoretic migration of $TC^A$ and $TC^B$. (Slots 1, 4, and 7) Denatured collagen types I, II, and III, respectively, without added enzyme; (slots 2, 5, and 8) denatured collagen types I, II, and III, respectively, following incubation with crab protease at 37 °C for 30 min; (slots 3, 6, and 9) native collagen types I, II, and III, respectively, incubated with fibroblast collagenase at 25 °C to mark the $^3/_4$- and $^1/_4$-length products of mammalian collagenase action, $TC^A$ and $TC^B$.

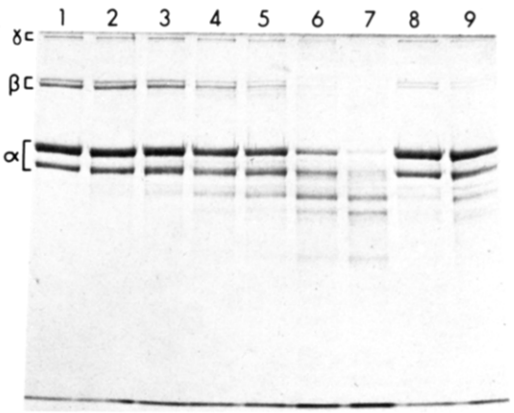


FIGURE 6: Crab protease activity on collagen in solution as a function of time and enzyme concentration. Guinea pig skin type I collagen in solution (25 μg) was incubated with serially doubling quantities of the crab protease (0.2–12.8 μg) at 25 °C for 90 min (slots 1–7). In slots 8 and 9, 0.8 and 1.6 μg of the crab protease were incubated with collagen at 25 °C for 24 h. The reaction mixtures were stopped with a final concentration of 1 mM PMSF and then applied to an 8% polyacrylamide slab gel. (Slot 1) Collagen plus 0.2 μg of crab protease for 90 min; (slot 2) collagen plus 0.4 μg of crab protease for 90 min; (slot 3) collagen plus 0.8 μg of crab protease for 90 min; (slot 4) collagen plus 1.6 μg of crab protease for 90 min; (slot 5) collagen plus 3.2 μg of crab protease for 90 min; (slot 6) collagen plus 6.4 μg of crab protease for 90 min; (slot 7) collagen plus 12.8 μg of crab protease for 90 min; (slot 8) collagen plus 0.8 μg of crab protease for 24 h; (slot 9) collagen plus 1.6 μg of crab protease for 24 h. Note that there is little difference in collagen degradation between slots 3 and 8 and slots 4 and 9, despite a 16-fold increase in the length of incubation. However, a 16-fold increase in enzyme concentration (slots 3 and 7) results in markedly increased collagen degradation.

strate are all cleaved by the enzyme. A comparison of the degradation of native type V collagen by the crab protease, trypsin, and chymotrypsin is shown in Figure 4. The crab protease attacks both the α1 and α2 polypeptide chains of type V collagen, but the α2 chain is cleaved at a much faster rate (slot 3). This preference for the α2 chain is also observed for chymotrypsin (slot 6), whereas trypsin attacks the type V substrate in a different manner, cleaving the α1 polypeptide chain preferentially to α2 (slot 2). Approximately 10-fold quantities of trypsin and chymotrypsin were required to produce cleavage of native type V collagen equivalent to that of the crab protease. Crab protease also degraded the α1 and α2 chains of a second preparation of native type V collagen, but without the α-chain specificity just noted (not shown). Cleavage of this collagen by trypsin also failed to show a preference for either α chain.

The degradation of denatured collagen types I, II, and III by the crab protease was next examined. The respective native collagens were thermally denatured into random gelatin chains by subjecting them to a temperature of 70 °C for 15 min. As expected, all three denatured substrates were degraded by the crab protease (Figure 5), but the quantity of enzyme needed to extensively cleave the denatured gelatin chains was less than 1% of that required to similarly cleave native collagen. Interestingly, for each denatured collagen type, a major band can be identified which migrates in the approximate position of $TC^A_{\alpha 1}$ of the respective collagen. Thus, it appears that even in the absence of the triple helix, and with the potential for many proteolytic cleavages, there is a propensity for catalysis at a $^3/_4$:$^1/_4$ locus in the denatured chains of α1(I), α1(II), and α1(III).

A puzzling observation in collagenase assays which utilized collagen in solution at 25 °C was that quantities of the crab protease in excess of equimolar concentrations to collagen were required to achieve and maintain significant collagen degradation as a function of time. This was not the case with assays of either fibrillar collagen or denatured collagen, which were performed at 37 °C. Figure 6, slots 1–7, illustrates the effect of serially doubling concentrations of crab protease incubated with guinea pig skin type I collagen in solution at 25 °C for 90 min. Each slot contains 25 μg of collagen; crab protease is present in quantities which range from 0.2 (slot 1) to 12.8 μg (slot 7). Slots 8 and 9 contain reaction mixtures identical with slots 3 and 4, respectively, but were incubated for 24 h. Comparison of these slots clearly shows that, despite increasing the time of incubation 16-fold, the extent of the degradation of collagen α chains was virtually unchanged; the only evidence of any continued catalysis, in fact, was a small amount of conversion of intramolecularly cross-linked β and γ components to the monomeric α form. On the other hand, when slot 7, which represents the incubation of collagen with 16 times as much enzyme as slot 3 but for an identical duration, is now compared with slot 8, which contains the same amount of enzyme as slot 3 but has been incubated 16 times longer (24 h), it is clear that increasing the enzyme concentration is far more effective in promoting catalysis than increasing the time of incubation an equivalent amount. To further examine this, it was necessary to determine whether the crab protease was stable over the entire 24 h of incubation. This was assessed by measuring enzyme activity with the synthetic substrate *N*-benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide (Grant & Eisen, 1980) at time = 0 min, 90 min, and 24 h from aliquots of the same reaction mixtures as are shown in Figure 6. Crab protease activity at all enzyme concentrations was unchanged at each time point examined, indicating that the enzyme was fully stable throughout the assay.

The action of crab protease was next investigated on type I fibrillar collagen at 37 °C. The degradation of collagen fibrils was always linear as a function of time for up to 24 h, even at concentrations of the crab protease equal to the lowest concentrations employed for the assays on collagen in solution just discussed. In Figure 7, the dependence of reaction velocity on crab protease concentration (A) is compared to a similar plot for human skin fibroblast collagenase (B). Concentrations

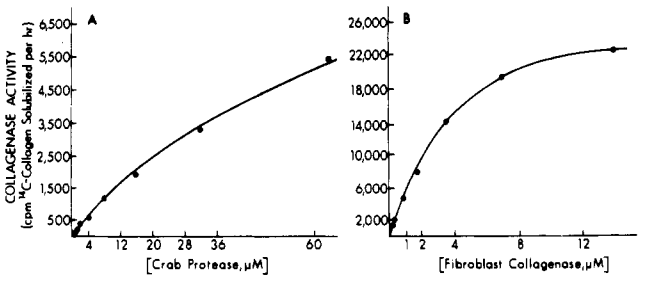


FIGURE 7: Crab protease and fibroblast collagenase activity on fibrillar collagen vs. enzyme concentration. (A) Crab protease activity on fibrillar collagen vs. enzyme concentration. Aliquots (40 μL) of increasing concentrations of the crab protease were incubated with 200 μg (50 μL) of fibrillar collagen at 37 °C. All reaction mixtures were stopped after approximately one-third gel lysis, and the resultant activity was normalized to time = 60 min. Concentrations of the crab protease ranged from 10 to 1600 μg/mL. (B) Human fibroblast collagenase activity on fibrillar collagen vs. enzyme concentration. Aliquots (40 μL) of increasing concentrations of fibroblast collagenase were incubated with 200 μg of fibrillar collagen at 37 °C. All reaction mixtures were stopped after approximately one-third gel lysis, and the resultant activity was normalized to time = 60 min. Concentrations of fibroblast collagenase ranged from 10 to 800 μg/mL. Note that substrate saturation is clearly evident with the fibroblast collagenase, but there is little indication of this effect with the crab protease, even at comparable or higher enzyme concentrations.

of fibroblast collagenase of approximately 7 μM result in the saturation of substrate sites, whereas there is little evidence of substrate saturation with the crab protease at comparable or higher enzyme concentrations. Furthermore, assay of the supernatant of these reaction mixtures for collagenase activity following enzyme binding at 25 °C to 200 μg of fibrillar collagen revealed that less than 5% of the crab protease (initial concentration = 50 μg/mL) had bound as compared to 20% of an equimolar solution (100 μg/mL) of human fibroblast collagenase. Thus, the lack of substrate saturation exhibited by the crab protease appears to correlate with a comparatively reduced affinity of this enzyme for the fibrillar collagen substrate. From the plots of enzyme activity vs. concentration (Figure 7A,B), the specific activities of the crab protease and human fibroblast collagenase on fibrillar collagen are 86 μg of collagen solubilized $mg^{-1}$ $min^{-1}$ and 850 μg $mg^{-1}$ $min^{-1}$, respectively.

## Discussion

In this study, the collagenolytic properties of the serine protease from the hepatopancreas of the fiddler crab, *Uca pugilator*, have been examined. The crab protease successfully attacks all native collagen types which are presently known to exist—types I–V. It thus has a broader collagen specificity than any of the mammalian collagenases which have been so characterized. In further distinction to such mammalian enzymes, the crab protease catalyzes multiple cleavages in the triple helix of each native collagen type at 25 °C. It shares this ability to attack the native collagen helix at multiple loci with the collagenase from the bacterium *Clostridium histolyticum*—a metalloenzyme with a molecular weight of approximately 100 000 (Siefter et al., 1959).

The mode of attack of the crab protease on native collagen types I–III in solution is of considerable interest. The major early cleavage product of each respective α1 polypeptide chain is a $^3/_4$-length fragment with the approximate electrophoretic mobility of $TC^A_{\alpha 1}$, the $^3/_4$-length peptide which results from the single cleavage by mammalian collagenase of collagen types I–III. Unlike mammalian collagenase, however, multiple catalytic events either follow or occur simultaneously with this $^3/_4$:$^1/_4$ cleavage. In the case of type I collagen, a protein band migrating in a similar position to $TC^B_{\alpha 1}$ is readily apparent. In collagen types II and III, a single such $^1/_4$-length fragment could not always be identified, due to its susceptibility to further degradation. The α2 chain of type I collagen is also attacked in a characteristic manner—with the formation of a protein doublet which migrates at the approximate electrophoretic position of $TC^A_{\alpha 2}$. Purification of $TC^B$ following type I collagen digestion demonstrated $^1/_4$-length fragments corresponding to $TC^B_{\alpha 2}$. When subjected to automated sequence analysis, the $TC^B$ fragments acted as if their amino termini were blocked. While the exact reason for this behavior has not yet been determined, it is possible that if cleavage of the α chains occurred on the amino side of glutamine residues, the acidic conditions used for isolating these fragments would have promoted cyclization and thus rendered the peptides resistant to sequence analysis. Although this is only speculation, it is interesting to note that there is a glutamine residue present in both α1 and α2 chains immediately preceding the glycine residues at the point where mammalian collagenase cleaves the α chains.

There has been much speculation as to why mammalian collagenases attack native collagen at only a single locus ($Gly_{775}$–$Ile_{776}$ in α1; $Gly_{775}$–$Leu_{776}$ in α2; Highberger et al., 1978), while numerous other potentially susceptible residues on both α chains are not cleaved. Studies of helix coil renaturation of a peptide of α1(I) containing the mammalian collagenase cleavage site (Highberger et al., 1979) and the known susceptibility of native type III collagen to cleavage by general proteases such as trypsin and thermolysin (Miller et al., 1976; Wang et al., 1978; Gadek et al., 1980) have led to considerable conjecture that mammalian collagenase specificity is due to a lower degree of helicity or "looser helix" around the collagenase cleavage site. The degradation of native collagen types I–III by the crab protease would seem to support this notion, since in each case the major early products reflect cleavage at a $^3/_4$:$^1/_4$ locus, even though this enzyme clearly has the capacity to attack the triple helix at multiple other sites. It is then very surprising that the α1 polypeptide chain of denatured collagen types I–III is preferentially cleaved by the crab protease at a similar, if not identical, locus to the native substrate. This propensity toward the formation of $^3/_4$-length products even on radomly coiled gelatin α chains would seem to imply that, in addition to the degree of helicity, some aspect of primary structure also exists which makes this a preferred site for the cleavage of collagenous substrates by the crab protease. In this respect, it is most interesting that the major initial cleavage in denatured collagen types I–III by human skin fibroblast collagenase also results in $^3/_4$- and $^1/_4$-length fragments electrophoretically identical with the $TC^A$ and $TC^B$ products of its action on native collagen (Welgus et al., 1982). Even though the crab protease and fibroblast collagenase are unrelated biochemically and have a different peptide bond specificity, it would appear that amino acid sequences favorable to both are located in this region of the collagen molecule.

Unlike mammalian collagenases, which generally cleave only collagen types I, II, and III, the crab protease also degrades native collagen types IV and V. The 140K, 100K, and 70K components of type IV collagen were all cleaved by this enzyme. The α1 and α2 chains of two different native type V preparations were likewise attacked. In one preparation, the α2 polypeptide chain was cleaved preferentially to the α1 chain. Concentrations of trypsin and chymotrypsin 10-fold greater than those of the crab protease also cleaved type V collagen. Like the crab protease, chymotrypsin attacked the

$\alpha 2$ chain preferentially to $\alpha 1$. Trypsin, on the other hand, manifested the opposite selectivity, attacking $\alpha 1$ at a faster rate than $\alpha 2$. In this regard, it is interesting that studies of the catalytic properties of the crab protease on noncollagenous substrates have shown that this enzyme is closer in its peptide bond specificity to chymotrypsin than to trypsin (Grant & Eisen, 1980). The action of these proteases on native type V collagen supports this observation. It should also be noted that Sage and co-workers (Sage et al., 1981) have found preferential degradation of the $\alpha 2$ chain of type V collagen by elastase. In a different type V preparation, also from human placenta, $\alpha$-chain selectivity was not observed for either the crab protease or trypsin. These data would seem to be consistent with the known existence of at least some heterogeneity [homopolymers of $\alpha 1$(V), heteropolymers of $\alpha 1$(V) and $\alpha 2$-(V)] in the chain composition of type V collagen, even within the same tissue of the same animal species (Rhodes & Miller, 1978; Sage et al., 1981).

The observation that at least equimolar concentrations of the crab protease to native type I collagen in solution are required to produce even moderate substrate degradation is very puzzling. Clearly, increasing enzyme concentration was far more effective in promoting catalysis than increasing the time of incubation by an equivalent amount (Figure 6). This occurred despite the retention of normal enzyme activity against a synthetic substrate, *N*-benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide, for at least 24 h at 25 °C. Such behavior was not observed for either denatured collagen or the fibrillar substrate. Explanations for this phenomenon could include (1) the requirement for more than one molecule of the crab protease to bind to collagen in order to cleave the triple helix, (2) competitive inhibition by the helical products of protease action at 25 °C, and (3) nonspecific binding. Unfortunately, the experimental data do not allow us to distinguish between these hypotheses.

In contrast to the findings with collagen in solution, fibrillar collagen degradation by the crab protease at 37 °C was linear as a function of both time and enzyme concentration (Figure 7A). Interestingly, a comparison of the plots of reaction velocity vs. enzyme concentration for the crab protease and human skin fibroblast collagenase at equimolar concentrations revealed marked substrate saturation with the latter enzyme but little evidence of this effect with the former (Figure 7A,B). Assessment of enzyme binding to fibrillar collagen showed that less than 5% of the crab protease in a typical reaction mixture had bound whereas approximately 20% of the fibroblast enzyme had done so. The $K_m$ of fibroblast collagenase on native collagen types I, II, and III is approximately 1 $\mu$M (Welgus et al., 1981). We strongly suspect that the $K_m$ of the crab protease on type I collagen is much greater. This would account for the lack of substrate saturation at enzyme concentrations where fibroblast collagenase appears to be saturating available binding sites, as well as for the markedly reduced enzyme binding at an equimolar concentration of both enzymes. The $K_m$ of the crab protease has been measured on small molecular weight noncollagenous peptides (Grant & Eisen, 1980) and was found to be approximately 0.1 mM on these substrates. A $K_m$ for collagen equal to or greater than this value would be consistent with the experimental observations.

In this study, we have investigated the collagenolytic activity of crab protease with regards to collagen type and different levels of substrate organization (denatured $\alpha$ chains, collagen in solution, and fibrillar collagen). Crab collagenase, a serine protease, possesses the unique ability among this class of enzymes to cleave the collagen triple helix in a location similar to mammalian collagenases, which are metalloproteases. Although their physiologic roles appear to be quite different, the fact that a serine protease and a metallogenzyme have in common the ability to cleave native collagen suggests that there may be certain structural features, especially in their ability to bind collagen, which are common to both. Thus, investigations into the nature of the interaction of crab protease with collagen may lead to a better understanding of the structural properties of this substrate that relate to its degradation.

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References

Bergmann, I., & Loxley, R. (1963) *Anal. Chem. 35*, 1961.
Bundy, H. F. (1962) *Anal. Biochem. 3*, 431.
Bundy, H. F. (1963) *Arch. Biochem. Biophys. 102*, 416.
Eisen, A. Z., & Jeffrey, J. J. (1969) *Biochim. Biophys. Acta 191*, 517.
Eisen, A. Z., Henderson, K. O., Jeffrey, J. J., & Bradshaw, R. A. (1973) *Biochemistry 12*, 1814.
Gadek, J. E., Fells, G. A., Wright, D. G., & Crystal, K. G. (1980) *Biochem. Biophys. Res. Commun. 95*, 1815.
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.
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.
Highberger, J. H., Corbett, C., & Gross, J. (1979) *Biochem. Biophys. Res. Commun. 89*, 202.
Hurion, N., Fromentin, H., & Keil, B. (1979) *Arch. Biochem. Biophys. 192*, 438.
King, J., & Laemmli, V. K. (1971) *J. Mol. Biol. 62*, 465.
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.
Rhodes, R. K., & Miller, E. J. (1978) *Biochemistry 17*, 3442.
Sage, H., Pritzl, P., & Bornstein, P. (1981) *Biochemistry 20*, 3778.
Seltzer, J. L., Jeffrey, J. J., & Eisen, A. Z. (1977) *Biochim. Biophys. Acta 485*, 179.
Siefter, S., Gallop, P. M., Klein, L., & Meilman, E. (1959) *J. Biol. Chem. 234*, 285.
Stricklin, G. P., Bauer, E. A., Jeffrey, J. J., & Eisen, A. Z. (1977) *Biochemistry 16*, 1607.
Takahashi, S., & Seifter, S. (1974) *Isr. J. Chem. 12*, 557.

Wang, H. M., 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., Jeffrey, J. J., Stricklin, G. P., & Eisen, A. Z. (1982) *J. Biol. Chem.* (in press).

# Small-Angle X-ray Scattering Study of Halophilic Malate Dehydrogenase†

M. H. Reich, Z. Kam, and H. Eisenberg*

ABSTRACT: Malate dehydrogenase from the organism *Halobacterium marismortui* was studied in solutions of varying salt concentration by using a small-angle X-ray system employing a linear position sensitive detector. Considerations pertaining to the study of absorbing multicomponent solutions are presented. The radius of gyration of halophilic malate dehydrogenase was found to be 31.8 ± 0.6 Å and the shape of the molecule spheroidal. The scattering from prolate ellipsoids of eccentricity between 1 and 2 best fitted the data while for oblate ellipsoids the scattering was best fitted for eccentricities between 1 and 0.5. No significant change in the radius of gyration or anisotropy of halophilic malate dehydrogenase was found in the range of NaCl concentrations studied (1–4 M). The contrast matching electron density was found to be 0.407 ± 0.002 e/Å$^3$. A parallel study of bovine serum albumin yielded within experimental error a similar contrast matching electron density of 0.404 ± 0.006 e/Å$^3$. This information combined with the diffusion coefficient and the amount of water and salt associated with halophilic malate dehydrogenase renders the existence of an outer hydration shell unlikely. The data are rather consistent at low resolution with a spheroidal particle of uniform electron density.

Halophilic organisms provide an interesting example of biological adaptation to extreme conditions. Much interest has centered around the mechanism by which halophilic enzymes function in the extreme salinity of the intracellular medium of halobacteria (the intracellular salt concentration exceeds 4 M KCl and 2 M NaCl). Most nonhalophilic enzymes cannot function under such conditions while the halophilic enzymes are inactivated at ionic strengths typical of normal intracellular environments. The unusual behavior of halophilic enzymes leads to the expectation that their structure has characteristic and possibly unique features. The amino acid composition of halophilic malate dehydrogenase reveals a greater than usual excess of acidic amino acid residues (16–18 mol % compared with 7–9 mol % for nonhalophilic proteins), a higher content of borderline hydrophobic amino acids (alanine and threonine), and an unusually high ratio of lysine to arginine (Mevarech et al., 1977).

Malate dehydrogenase is an enzyme that catalyzes the reaction of L-malate with NAD to form oxaloacetate and NADH. Nonhalophilic malate dehydrogenase is a dimer consisting of identical subunits and has a molar mass ($M_2$) of between 60 000 and 72 000 g/mol for various species. Malate dehydrogenase from *Halobacterium marismortui* is also a dimer, of molar mass 85 000 g/mol. Thermodynamic analysis of contrast variation studies by analytical ultracentrifugation and quasi-elastic light scattering (Eisenberg, 1976) have shown an unusual degree of water and salt interaction with halophilic malate dehydrogenase (Pundak & Eisenberg, 1981; Pundak et al., 1981). Large amounts of water and salt were found to be associated to halophilic malate dehydrogenase (for water, 0.8 g/g of protein; for salt, 0.3 g/g of protein). For comparison, a similar study of water and sodium chloride association to bovine serum albumin found only 0.24 g of water/g of protein and 0.01 g of salt/g of protein associated (Eisenberg et al., 1978).

It has recently been reemphasized (Eisenberg, 1981) that information derived from the analysis of the intensity of zero-angle (forward) scattering, independent of the type of the incident radiation employed, is equivalent to the information derived from the ultracentrifuge. However, from the angular dependence of the scattering, additional information about macromolecular structure in solution may be obtained. Small-angle X-ray scattering in particular can be used to determine a hierarchy of basic structural information about proteins in solution. From the change in zero-angle intensity as the solvent electron density is changed, the dependence of the electron density increment of the protein on the above parameter may be derived. This is entirely analogous to the study of the mass density increment by ultracentrifugation and may be similarly analyzed. The next most accessible quantity that can be measured from the scattering at low angles is the electronic radius of gyration, which may also depend upon the electron density contrast. Further information about the shape of the molecule can be obtained at higher angles by fitting the scattering intensity to the theoretical scattering curves from simple models as ellipsoids of rotation of varying eccentricity. Procedures (Glatter, 1979) relying on the analysis of the scattering curves over the whole angular range, and the fitting of more detailed models (Pilz et al., 1975; Serdyuk et al., 1979), may be applied. In this work, we have confined ourselves to the lower resolution structural features.

X-ray scattering measurements were made of halophilic malate dehydrogenase in buffers of varying NaCl concentrations (1–4 M) and at different protein concentrations. The range of protein concentrations depended upon the salt concentration of the buffer as it proved difficult to measure the scattering from the lowest protein concentrations in the higher NaCl concentration range.

Measurements of bovine serum albumin were also made under the same conditions as the above measurements, so that a comparison of the scattering from halophilic malate dehydrogenase with a nonhalophilic protein could be made. For construction of a low-resolution model of the structure of

† From the Polymer Research Department, Weizmann Institute of Science, Rehovot, Israel. *Received March 5, 1982.*

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